BBRC

Bioscience Biotechnology Research Communications

VOLUME-12 NUMBER-2 (April-June 2019)
Print ISSN: 0974-6455
Online ISSN: 2321-4007
CODEN: BBRCBA
www.bbrc.in
University Grants Commission (UGC)
New Delhi, India Approved Journal

An International Peer Reviewed Open Access Journal For Rapid Publication

Published By:
Society for Science & Nature (SSN)
Bhopal India
Indexed by Thomson Reuters, Now Clarivate Analytics USA
ISI ESCI SJIF=4.186
Online Content Available: Every 3 Months at www.bbrc.in

SOCIETY
FOR SCIENCE AND NATURE
SCIENCE FOR LIFE

Registered with the Registrar of Newspapers for India under Reg. No. 498/2007
Bioscience Biotechnology Research Communications
VOLUME-12 NUMBER-2 (April-June 2019)

Continued Inside Cover

Printed By:
Society For Science & Nature Bhopal India
C-52, HB Colony, Koh-e-Fiza
Bhopal - 462001, INDIA

ISSN 0974-6455

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Continued From Back Cover

Bioscience Biotechnology Research Communications
(Abbreviation: Biosc. Biotech. Res. Comm.)
Web of Science (WoS ESCI Thomson Reuters, Clarivate Analytics USA & UGC India) Indexed Journal

About the Journal

Bioscience Biotechnology Research Communications, BBRC is a broad based internationally indexed official publication of Society for Science & Nature (SSN) since 2008. The international journal publishes peer reviewed original research papers, exciting reviews and short communications in basic and applied areas of life sciences and the upcoming state of the art technologies, including Biology and Medicine on a fast track. The young editorial team of *Biosc. Biotech. Res. Comm.* tries hard to provide a high quality flawless format of scientific communication for the popularization and advancement of science, worldwide. During these years hundreds of peer reviewed research papers of very high quality have been published in *Biosc. Biotech. Res. Comm.* and authors like Kiran Shaw Majumdar of Biocon, Bangalore have contributed to *Biosc. Biotech. Res. Comm.* helping it achieve high readership in a short span of time. Reviewing the published research articles, it becomes evident that on an average, about 7 papers out of 10 are subjected to healthy revisions in *Biosc. Biotech. Res. Comm.* making quality reading. We owe this achievement to our reverend reviewers! We hope the standards set by *Biosc. Biotech. Res. Comm.* will improve further making this international journal unique and easily accessible to the scientific fraternity across the globe. In its tenth year of successful existence as a scholarly publication, *Biosc. Biotech. Res. Comm.* has now become an open access Thomson Reuters ISI ESC Web of Science/Clarivate Analytics USA Indexed journal also approved by University Grants Commission (UGC) Ministry of Human Resource Development, Government of India, New Delhi and has a NAAS-2019, Government of India, Indian Council of Agricultural Research (ICAR) New Delhi rating of 4.38 and SJIF 4.196.

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Bhopal, India.

Bioscience Biotechnology Research Communications
International Open Access Peer Reviewed Journal For Rapid Publication
(Indexed in Leading National and International Scientific Citation Agencies
Approved by University Grants Commission (UGC) New Delhi)
(NAAS 2019 Journal Score – 4.31 SJIF 2018 4.196)
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Biosci. Biotech. Res. Comm. 12(2): 203-210 (2019)

BBRC
Bioscience Biotechnology
Research Communications

Floral variety of *Fabaceae* Lindl. family in gully ecosystems in the south-west of the Central Russian Upland

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ABSTRACT

The multi-year geobotanical studies have shown that in the south-west of the Central Russian Upland the floristic composition of gully ecosystems includes 274 genera, which are combined into 65 families. The species from 3 families: *Asteraceae* Dumort., *Fabaceae* Lindl. and *Poaceae* Barnh. take the lead in the taxon hierarchy. The value of the generic coefficient, which is calculated by the number of species per genus, is quite significant and is equal to 1.81. It has been established that *Fabaceae* Lindl. species have extensive presentation (it comes second place in the first triad of families). Among *Fabaceae* species, a particular importance is given to wild related cultural species, which have high biological, and resource value by a mix of morphological and qualitative characteristics and which are potential selection sources for improvement of various economically useful features. The most striking example include species of the *Medicago: Medicago sativa L.* genus and the *Medicago var* Mart. hybrid genus. The *M. sativa L.* and *M. varia* Mart. coenopopulations in gully mouths are the most complete and they often have continuity of species distribution by age groups. It has been found that any forms, which act as carriers of multidimensionality recessive mutation, i.e. mf-mutation, have a

ARTICLE INFORMATION:

Corresponding Author: liset@bsu.edu.ru
Received 20th March, 2019
Accepted after revision 31st May, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/1

high degree of occurrence in local populations. With seed renewal, the proportion of species with mf-mutation is from 5 to 55% in families (the offspring of the first plant). The *M. sativa L.* and *M. varia* Mart. forms, which have been identified by us in the natural habitats of gully geocomplexes, have a number of valuable selection crop-related characteristics: high seed production, good leaf coverage and high protein content.

KEY WORDS: FLORISTIC COMPOSITION OF PHYTOCENOSES, MEDICAGO SATIVA L., MEDICAGO VARIA MART., MF-MUTATION, MULTIPLICIT

INTRODUCTION

Due to some, ecological and climatic changes which have been observed in the last decades we have to make a new assessment of flora changes in the regions with ecotones, for example, a forest-steppe zone. Some plant species can actively adapt to any environmental changes thanks to the fact that they are ready to be divided into separate populations, which are tolerant to the conditions of new ecological niches. Even small changes in natural heterogeneous populations cause new systematic taxon's and ecotypes. Their use is of great importance for the creation of a new source material for breeding and the formation of new biological resources (Givnish, 2002).

The form-building process is most active on the territories with complex geological history and a variety of ecological-climatic and soil conditions, in mountainous or foothill areas and in ancient agricultural centres (Takhtajan, 1986, Vavilov, 1992, Goncharov, 2007). The south-west part of the Central Russian Upland largely corresponds to the above-mentioned historical, geographical, ecological and anthropogenic conditions. It is important that the Dneprovskoe and Valdaiskoe glaciation did not affect a significant part of the south-west of the Central Russian Upland. The unique features of the Central Russian Upland landscapes are based on a wide spread of typical karst and cretaceous landscape complexes with involvement of exposed and shallow chalk rocks, which make the region similar to foothills. Therefore, the study region has all the conditions, which accompany primary microevolution processes at the level of microgeocenter (Zhukovsky, 1971, Diamond, 2002, Dumacheva et al., 2015). The recent decline in the share of arable land due to more stringent environmental requirements has created space for the formation of autogenous successions (Lisetskii et al., 2016a) which include both new relationships among plants in communities (Degtyar' and Chernyavskikh, 2006, Gusev, 2016 a, b) and spontaneous changes in plant habitats, including soil reproduction (Lisetskii, 2012, Prikhodko and Manakhov, 2014, Lisetskii et al., 2016b and Terekhin and Chendev, 2018).

The ecological and biological characteristics of the species growing in difficult conditions of gully complexes may be of particular interest as a potential bioresource used to select plants, to obtain starting materials of herbal origin and to improve ecosystems stability. The present paper was aimed to study the floristic composition of natural communities of gully complexes with carbonate outcrops towards the south-west of the Central Russian Upland (on the example of the east of the Belgorod region). We estimated the species composition of the *Fabaceae* Lindl. family as a possible starting material for selection.

MATERIALS AND METHODS

Study area: Research was conducted in the Belgorod region in 2002-2016. The climate of the study area is temperate continental. Its main particular features as follows: large annual temperature range, relatively mild winters with frequent thaws and snowfalls, sunny and long summers, moderate and not quite stable moisture with summer precipitation being prevailed (Table 1).

Table 1. Average long-term weather conditions at the site where the studies were conducted according to the nearest weather stations

Indicators	Weather station	
	Valuyki	Alekseevka
Rainfall, mm	568	545
Average summer temperature, °C	+20.0	+20.0
Average winter temperature, °C	-7.0	-7.5
Duration of the frost-free period, days	155	155
The sum of the air t for the period with stable t > 10 °C	2750	2700
The average depth of soil freezing in winter, cm	60	60

Methods: Natural communities were studied in the course of route studies using the method of intermittent transects and geobotanical descriptions in accordance with the field geobotany procedures (Lavrenko and Korchagin, 1972).

Transects of 400 m^2 (2×200 m) in size were established diagonally to the direction of the gully under study. Four hundreds transects were established in total. P.F. Mayevsky (Mayevsky, 2006) determined the species composition of the plants. Throughout any transect we additionally set up permanent fixed plots with an

area of 100 m^2 (only 400 sites) for geobotanical descriptions, these plots were also used for further geobotanical descriptions of the communities with *M. sativa* L. and *M. varia* Mart. Each species is linked to a certain life form according to the system Raunchier (Raunkiaer, 1937). The florocoenotypes of the *Fabaceae* Lindl. family and their life forms have been classified of in relation to water (Kolchanov et al., 2012). The species have been distributed with respect to calcium substrate according to V.I. Malyshev (Malyshev, 1965). The occurrence of a species has been defined as a percentage of related geobotanical descriptions. The species names are given in S.K. Szerepanov (Szerepanov, 1981).

RESULTS AND DISCUSSION

The conducted analysis has shown that the floristic composition of phytocenoses of gully complexes consists of 65 families, which include 274 genera. The species from three families: *Asteraceae* Dumort., *Fabaceae* Lindl. and *Poaceae* Barnh. take the lead in the taxon hierarchy. They account for 161 species (32.8%) in the floral spectrum, ten leading families include 329 species or 67.0% of their total number (495 species) (Table 2). The number of families represented by one species is 22, those of two species – 13 and those of three species – 4. Each of six families contains four species, two families – five species each and 2 another families – seven species each. One family has nine, ten, eleven and twelve species, and two families contain thirteen species each. As for the number of genera, the leading triad of families include *Asteraceae* Dumort., *Brassicaceae* Burnett. and *Poaceae* Barnh. – they account for 77 genera (28.1%) (Table 2).

A study of ecological features, coenotic and geographical relations has shown that the examined bean species belong to five groups according to geographical confinement. There is a predominance of 20 plant species (44.4%) of Palearctic areal and 16 species (35.5%) of steppe area. The European-Caucasian, European and adventive types of geographical areas account for four (8.8%), three (6.6%) and two (4.4%) species respectively.

The generic coefficient (the number of species that per genera) is quite high and it is equal to 1.81, which reflects the diversity of the floristic composition of the communities of the studied gully complexes.

It has been found that the *Fabaceae* Lindl. species are quite numerous (they come second in the first triad of families). The *F.* Lindl. family is a valuable source of food and feed protein; it also produces soil symbiotic nitrogen. These species are distinguished by longevity, an exceptional ability to grow in a variety of natural conditions, multipurpose use, soil fertility recovery through atmospheric nitrogen fixation, and a number of other useful economic and biological features. The wild relatives of cultural species become of particular importance as sources for improvement of various economically useful features (Dzyubenko, 2015).

As follows from the analysis of the species composition of the bean component of the phytocenosis of gully complexes 45 species have been detected, i.e. about 58% of the diversity of the regional flora families (Table 3).

A study of general habit and life cycle duration has shown that taproot redevises tend to prevail among leguminous grasses. There is maximum 13.2% of annual and biennial plants and 17.6% of shrubs, sub-shrubs and dwarf sub-shrubs in gully complexes. A variety of ecological conditions, which have formed bean family species in gully complexes, is quite sufficiently shown by the spectrum of life forms. We have revealed that the majority of species (84.5%) belongs to hemicryptophytes – perennial herbaceous plants with aboveground sprouts being died away by wintertime. Their reproduc-

Table 2. Spectrum of the leading families of natural communities of ravine complexes (2002-2013)

Family	Family rank	Number of genera	Number of species	
			pieces	%
Asteraceae Dumort.	1	38	72	14.6
Fabaceae Lindl.	2	17	45	9.1
Poaceae Barnh.	3	22	44	9.0
Lamiaceae Lindl.	4	21	37	7.5
Brassicaceae Burnett	5	23	34	6.9
Rosaceae Juss.	6	17	25	5.1
Scrophulariaceae Juss.	7	8	22	4.5
Caryophyllaceae Juss.	8	11	19	3.9
Ranunculaceae Juss.	9	10	18	3.7
Boraginaceae Juss.	10	9	13	2.6
Total	65	274	495	100

Table 3. Species composition of the family Fabaceae in gully complexes with chalk outcrops (2002-2013)

No	Latin species name	Life form[a]	florocenotic[b]	Environmental group[c]
1	*Anthyllis vulneraria* L.	HC	MD	MX
2	*Astragalus albicaulis* DC.	HC	ST	X
3	*Astragalus austriacus* Jacq.	HC	ST	MX
4	*Astragalus cicer* L.	HC	MD	MX
5	*Astragalus danicus* Retz.	HC	ST	MX
6	*Astragalus dasyanthus* Pall.	HC	ST	MX
7	*Astragalus glycyphyllos* L.	HC	GB	M
8	*Astragalus onobrychis* L.	HC	ST	X
9	*Astragalus pubiflorus* DC.	HC	ST	X
10	*Astragalus sulcatus* L.	HC	MD	X
11	*Astragalus ucrainicus* M. Pop. et Klok.	HC	MO	X
12	*Astragalus varius* S.G. Gmel.	HC	SAN	MX
13	*Caragana frutex* (L.) C. Koch	PH	MD	X
14	*Chamaecytisus austriacus* (L.) Link	PH	CH	MX
15	*Chamaecýtisus ruthénicus* (Fisch. ex Woloszcz.) Klaskova	PH	SAN	MX
16	*Coronilla varia* L.	HC	ST	M
17	*Genista tanaitica* P. Smirn.	HC	CH	M
18	*Genista tinctoria* L.	PH	MD	MX
19	*Hedysarum grandiflorum* Pall.	HC	CH	MX
20	*Lathyrus pannonicus* (Jacq.) Garcke	HC	ST	MX
21	*Lathyrus pallescens* (Bieb.) C. Koch	HC	ST	MX
22	*Lathyrus pratensis* L.	HC	MD	M
23	*Lathyrus tuberosus* L.	HC	SYN	MX
24	*Lotus corniculatus* L.	HC	MD	M
25	*Medicago falcata L. subsp. romanica* (Prodan) Schwarz et Klinkovski	HC	ST	MX
26	*Medicago lupulina* L.	HC	SAN	M
27	*Medicago varia* Mart.	HC	SYN	MX
28	*Melilotus albus* Medik.	HC	ST	MX
29	*Melilotus officinalis* (L.) Pall.	HC	ST	MX
30	*Onobrychis arenaria* (Kit.) DC.	HC	ST	MX
31	*Ononis arvensis* L.	HC	MD	M
32	*Oxytropis pilosa* (L.) DC.	HC	ST	X
33	*Robinia pseudoacacia* L.	PH	SYN	MX
34	*Trifolium alpestre* L.	HC	MD	MX
35	*Trifolium arvense* L.	T	MD	MX
36	*Trifolium fragiferum* L.	HC	MD	MH
37	*Trifolium hybridum* L.	HC	MD	MH
38	*Trifolium medium* L.	HC	MD	M
39	*Trifolium montanum* L.	HC	MD	MX
40	*Trifolium pratense* L.	HC	MD	M
41	*Trifolium repens* L.	HC	MD	M
42	*Trifolium aureum* Poll.	HC	MD	MX
43	*Vicia cassubica* L.	HC	FR	M
44	*Vicia cracca* L.	HC	MD	MX
45	*Vicia villosa* Roth.	T	MD	MX

Note: a Life form: HC – hemicryptophytes (hemikriptophyte), T – therophytes (terophyte), PH – phanerophytes (fanerophyte). b florocenotic: ST– steppe, SYN – synanthropic, MD – meadow, FR – forest, GB – types of fields and shrubs, SAN – sandy habitat species, CH – types of chalk downs. c Environmental group: M – mesophyte, MX – mesoxerophyte, MH – mesogigrophyte, X – xerophyte

Table 4. Distribution of Fabaceae species in gully complexes depending on relief elements (2008-2013)

Sampling point	mesophyte	mesoxerophyte	mesogigrophyte	xerophyte
Slopes of beams with steppe phytocenosis	1	7	-	3
Slopes of beams with lime-loving association	-	4	-	4
Ravine mouths with alluvial cone	4	8	-	-
Alluvial cone acting ravines	6	7	2	-
Total	11	25	2	7

ing buds are located on the soil surface and protected by leaf litter. Phanerophytes and Therophytes are generally represented by 15.5% of species in the regional gully complexes. The bean species distribution in gully complexes is largely determined by soil moisture. An analysis of species-to-moisture factor ecological spectrum has shown that the species (55.5%) predominantly belong to mesoxerophytes, i.e. plants, which are able to tolerate a short-term drought. As for the number of species mesophytes, xerophytes and mesohygrophytes take the second, third and fourth places, respectively.

Various manifestations of calcephilia, which is related with calcium excess and substrate carbonate content have made it possible to distribute bean species by plant confinement to calcium substrate according to the classification by V.I. Malyshev (Malyshev, 1965). The bean gully complexes (73.3%) are prevailed by facultative calciphile plants, which are quite common on carbonate soils but can also grow outside. A group of calcium-indifferent bean plants (17.8%) include the species which are either unobviously confined to carbonate substrates or fail to be calciphobes. We have found 8.9% of obligatory calciphile plant, which grow almost exclusively on the calcium substrate. Under severe environmental conditions a combination of resources in a particular ecotope point is of critical importance for coenopopulations. The gully complexes have a well-defined micro-relief, which has impact on spatial distribution of species. 60.0% of mesophytic bean species are concentrated on the rank soils in ravine mouths and existing gully cone deltas, i.e. in more humid living environment. On gully slopes with steppe plant formations there are 20.0% of xerophytic and mesoxerophytic species whereas slopes with calciphilous communities have 15.5% of them (Table 4).

In general, in the conditions of gully complexes the ecological features of bean plants are characterized by the predominance of mesoxerophytes and facultative calciphile plants as regards ecotope conditions and steppe florocenotype hemicryptophytes by life form.

The landscape conditions of the gully complex ecotopes with cretaceous outcrops tend to create conditions for the introduction of new synanthropic species and their active formation. Many species may be of high bio-resource value. The most striking example include species of the *Medicago* genus: *M. sativa* L. and hybrid *M. varia* Mart. species.The history of wide distribution of these species, which are mainly of Central Asian and Mediterranean origin in the conditions of the Central Russian Upland, goes back about one hundred years. It is noteworthy that there is not a single record of blue-flowered and variegated-flowered *M. sativa* L. and *M. varia* Mart. species ("native" type alfalfa) in the detailed geobotanical descriptions of gully complexes with cretaceous outcrops which were made > 100 years ago V.I. Taliev (1904a) (Taliev, 1904b, Juan et.al., 1993).

Currently the *M. sativa* L. and *M. varia* Mart. species make an essential and integral component of natural herbaceous phytocenoses. The studies conducted in 2008-2013 and repeated in 2014-2016 showed that an average increase in the occurrence of these species had made up to from 3.2 to 4.5% for all geobotanical descriptions, with the most significant increase being demonstrated by the plant communities located on the Cretaceous outcrops (Table 5). In 2008-2013, the steppe areas and meadows were characterized by discrete (incomplete) local populations, which are mainly represented by old-aged blueing and non-blueing individual plants. However, in 2014-2016 some even-aged young plants were also reported to appear for seed regeneration.

In vegetation communities of chalk, outcrops the *M. sativa* L. and *M. varia* Mart. coenopopulations are subject to habitual areas, which have been earlier connected with human economic activities: locations of former farms, marginal plots on fields used for farm and soil conservation crop rotations.A more detailed study of the occurrence of blue-flowered alfalfa in gully complexes

Table 5. Occurrence of *M. sativa L. and M. varia Mart.* in natural association, %

Association	Years of research	
	2008-2013	2014-2016
Meadow steppes	2.0	3.6
Real steppes	3.0	3.5
Calciphilous steppes	4.0	6.2
Chalk outcrops	5.0	7.1
Meadows	2.0	2.0
Average	3.2	4.5

Table 6. Occurrence of *M. sativa* L. and *M. varia* Mart. in ecotopes of ravine complexes, %

Ecotopes	Study years	
	2008-2013	2014-2016
Slopes of beams with steppe association	0.0	0.2
Slopes of beams with lime-loving association	3.0	2.5
Ravine mouths with alluvial cone	8.0	11.2
Alluvial cone acting ravines	7.0	9.1
Average	4.5	5.6

and its dynamics has made it possible to identify the ecotopes with the most favourable conditions for the development of *M. sativa* L. and *M. varia* Mart. and for the formation of their stable populations (Table 6).

The occurrence of blue-flowered alfalfa is higher in lower gullies (in the existing gully cone deltas and in the ravine mouths with cone deltas) for a number of possible reasons. Firstly, due to the transfer of seeds through ecotonic corridors being gully slopes. Secondly, due to the particular features of the soil substrate (chalk residues), which forms high-degree crushing, low projective cover and, therefore, low distribution of cereals and lower inter-species competition. Thirdly, due to the influence of natural selection of sustainable forms in the hybrid populations which have formed in the ravine mouths with the varieties of *M. sativa* L. and *M. varia* Mart. and local forms of *M. falcata* L. which were previously cultivated on the arable land.

Local coenopopulations of *M. sativa* L. and *M. varia* Mart. are most complete in ravine mouths and they often have species distributed by age groups on a continuous basis. We have found a wide variation of all

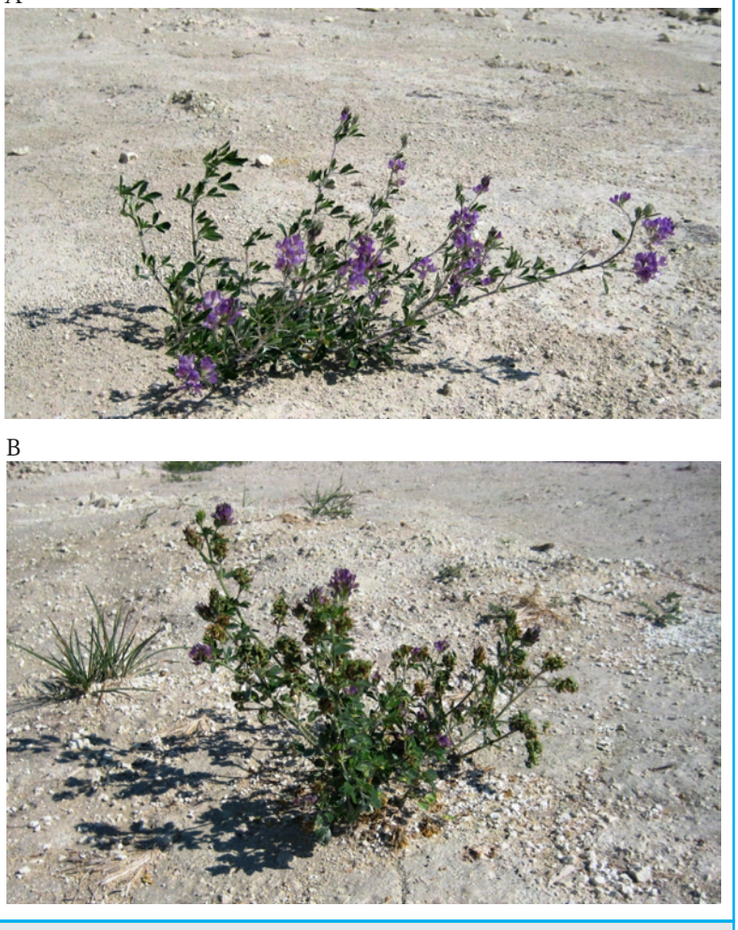

FIGURE: Species *M. varia* Mart. "Carbonate" environmental type in the flowering phase (A) and fruit formation (B) on alluvial cone ravines

main morphometric parameters, which proves alfalfa's intrapopulation heterogeneity and ecological plasticity. Hence, these two species provide material for the selection of competitive forms. Adaptive transformations are oriented towards the preservation of individual plants accompanied by a specific increase in leaf coverage, a reduction of generative organs with increased numbers of stemmed beans, bean curls and their dissemination. There has been observed the formation of *M. sativa* L. and *M. varia* Mart. coenopopulations with individual plants of "carbonate" ecotype, which is close to cultural forms by a number of morphological features but has a pronounced stress-tolerant type of adaptive strategy (Figure). The upper slope seeds (these are usually fields of crop rotations) used to be transferred by water flow to the bottom of the gullies where they fell on rubble chalk eluvium. A vegetation cover with low projective coating was formed in the cone deltas. There alfalfa found favourable conditions for its growth and development in conditions of low competition with cereals. Against the background of negative influence produced by carbonate substrate, primarily, due to iron deficiency, the population have quite promptly eliminated unstable forms and generated M. sativa L. and M. varia Mart. coenopopulations with high level of preservation of individual plants having a complete spectrum of adaptive changes. They have become the basis for microevolution transformations under extreme ecotope conditions. The local populations were reported to have high occurrence of forms carrying multifoliolate recessive mutation (mf-mutations). Externally, it is expressed by the formation of complex leaves with 4-7 leaflets in individuals instead of three. These forms are positioned as sources of high-quality material for the selection of high-quality cultural alfalfa (Juan, 1993, Petkova, 2010, Odorizzi et al., 2015).

The researchers consider the emerging multi-leafness in leguminous grasses as a throwback, i.e. atavism, which is most strongly manifested in the conditions of disturbed habitual areas when they grow outside the phytocenosis, on poor rocky soils, rock outcrops and in the areas being far from the optimal conditions (Plennik, 2002).An estimation of the populations by the method of transfer into favourable conditions have shown that the share of mf-mutant forms is from 3.3 to 53.3% in different populations. With seed renewal, the proportion of species with mf-mutation (the offspring of the first plant) is from 5 to 55% in families.

It is important that the *M. sativa* L. and *M. varia* Mart. forms, which have been found in the habitual areas of gully complexes, have a number of valuable crop selection features: good leaf coverage, high protein content and high seed production and others.

CONCLUSION

We have found a high variety of plant species with high biological potential in the gully complexes in the southern-west part of the Central Russian Upland. It is possible to have accumulations of stable viable recessive forms of individual species, which can be used to generate initial selection material. The example of *M. sativa* L. and *M. varia* Mart. was used to establish the formation process for coenopopulations of certain sustainable ecotype, which is close to cultural forms, by a number of morphological and qualitative characteristics. The populations studied are characterized by high occurrence of mutant forms (recessive mf-mutation). This phenomenon becomes obvious in the course of development of natural local populations along the boundaries of species distribution areas.

ACKNOWLEDGEMENTS

The study was supported within the intra-university grant of Belgorod State National Research University to support the creation and development of scientific departments – centres of excellence.

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Chemical
Communication

Biosci. Biotech. Res. Comm. 12(2): 211-221 (2019)

BBRC
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Biosorption of copper (II) ions in aqueous solution by microwave-synthesized starch-graft-N-methyl-N-vinyl acetamide

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ABSTRACT

A novel adsorbent starch-graft-N-methyl-N-vinylacetamide (starch-g-NMVA) was synthesized using microwave assisted graft copolymerization. Monomers of N-methyl-N-vinylacteamide (NMVA) was grafted to potato starch using microwave irradiation and potassium peroxydisulfate (KPS) as free radical initiator. The highest % grafting (%G) obtained was 10.03% under the optimum conditions of 0.55 M NMVA and 90 seconds exposure time, keeping the initiator, starch, and microwave power fixed at 0.0014M, 0.1 g, and 1200W respectively. FTIR and SEM analyses confirmed that the monomer was grafted successfully. The graft copolymer was investigated for its efficiency in removing Cu(II) ions from aqueous solutions at different concentrations. Using the Box-Behnken method the most favorable pH, adsorbent dose, and initial Cu(II) concentration generated for the adsorption was 5.5, 100 mg and 100 ppm, respectively. The resulting adsorbent is able to remove 52.9% of Cu(II) ions in an aqueous solution at these conditions. The Langmuir isotherm best described the adsorption property of the system with a correlation coefficient (R2) of 0.9361. On the basis of this model, the maximum adsorption capacity (Qo) of the starch-g-NMVA was calculated to be 46.9749 mg/g. This study indicated that starch-g-NMVA is a good alternative adsorbent for the removal of Cu (II) from aqueous solution. Further desorption and optimization studies will help us know the applicability of this adsorbent in removing metals from a simulated wastewater setup.

KEY WORDS: STARCH, GRAFT COPOLYMERIZATION, COPPER (II) IONS, BOX BEHNKEN DESIGN, N-METHYL-N-VINYLACETAMIDE

ARTICLE INFORMATION:

Corresponding Author: aangcajas@xu.edu.ph
Received 25th March, 2019
Accepted after revision 5th May, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/2

INTRODUCTION

Technological and industrial advancements have always posed danger to the environment as it is associated to increase in heavy metal contamination in water and other parts of the ecosystem. Mining operations, fertilizer production, batteries, pesticides, and paper industries may directly or indirectly discharge heavy metal wastewaters into the environment (Mrinalini *et al*, 2015). Many of these heavy metal ions, *i.e*, ions of zinc, copper, nickel, mercury, lead and chromium, are toxic and non-biodegradable (Zhu *et al*., 2012). Because of their non-biodegradability, heavy metals tend to accumulate in living organisms, causing various diseases and disorders (Hasret *et al*., 2012). Copper, although an essential compound to plants and animals, becomes toxic when biological requirements are exceeded. Some toxic effects of copper in the body is potentially damaging protein, lipid, and DNA (Moroncola *et al,* 2016). Removal of heavy metals like copper from waste waters is important to maintain a suitable quality of water. Filtration, adsorption, and chemical precipitation are some methods used in removing heavy metal ions from wastewater or aqueous solution. Adsorption technology has recently become a real alternative to traditional wastewater treatment due to its relative simplicity and efficiency (Paksamut *et al*., 2018).

This involves a mass transfer process by which a substance is transferred from the liquid phase to the surface of the solid, and becomes bound by physical and/or chemical interactions (Zhu *et al*., 2012). Chemical adsorption, also called activated adsorption, results from the chemical interaction between the adsorbent and the adsorbate. Chemisorption occurs only as a monolayer and substances chemisorbed on solid surface are hardly removed because of stronger forces (Mehta *et al*., 2014). High adsorption capacity can be observed from biosorbent with abundant amino and carboxyl groups (Zhang et al., 2017). The presence of functionalities such as hydroxyl groups (-OH) on the surface of the adsorbent allows introduction of several heavy metal groups.

The amount of adsorbed ions onto the adsorbent could depend on the acidity of the medium (pH), initial heavy metal ion concentration, and adsorbent dose. Other factors include temperature and contact time. The acidity of the solution or pH is one of the most important parameters controlling the uptake of heavy metals from wastewater and aqueous solutions since it determines the surface charge of the adsorbent and the degree of ionization and speciation of the adsorbent (Abdel *et al*., 2011). In a study done by Tumin *et al*. (2002), the uptake of copper increased significantly when the pH was at 4 to 5 and adsorption capacity decreased slightly in pH range of 6 to 9. Using a Box-Behnken design, it was observed that % metal adsorption increased as the pH of the metal-containing aqueous solution increased from 2.5 to 5.5 (Ocreto et al., 2019).

Adsorbent dosage is another important parameter which influences the extent of metal uptake from the solution. Studies report that percent metal removal increases as the adsorbent dosage increases due to the introduction of more adsorption or binding sites and the availability of more surface area for metal attachment (Onundi *et al*., 2010). Manivannan *et al*. (2015) reported that the amount of copper increased with the increase in adsorbent dose and reached a maximum value after a particular dose. Additionally, adsorption capacities increased as the dosage of natural bioadsorbents and contact time increased during Cu (II) removal (Paksamut et al., 2018).

The use of synthetic polymer as toxic metal ion adsorbent is a possible approach for preventing environmental pollution and recycling metals. Synthetic adsorbents are mostly composed of petroleum-based polymers which are usually non-renewable and non-biodegradable. Generally, synthetic adsorbents are discarded in landfills or treated by incineration after the adsorption process for metal ions. Natural polymer such as starch is a more attractive raw material for industrial applications because it is renewable, abundantly available, and fully biodegradable. It also has numerous hydroxyl groups that can be chemically modified to design adsorbent materials of interest (Singh *et al*., 2012).

In a study of Cankaya (2016), starch methacrylate, prepared by esterification of primary -OH group of starch, was grafted with N-cyclohexyl acrylamide and methyl methacrylate monomers via free radical polymerization. Chemical modification of natural polymers via grafting can be achieved through the use of microwave radiation to generate free radical sites on the natural polymer backbone (Mostafa *et al*., 2013). Fosso-Kankeu et al. (2016) reported a 107.1 % conversion to guar gum-graft-poly-ethyl acrylate during copolymerization of ethylacrylate and guar gum by microwave irradiation at 900 MW and 3 minutes exposure. Reaction variables, such as initiators, composition of reaction mixture, microwave power, and exposure time played a key roles during grafting copolymerization using microwave irradiation (Karthika et al, 2014).

This study involves the synthesis of potato starch grafted with N-methyl-N-vinylacetamide and its application in the removal of copper in copper solutions. Box-Behnken design under response surface methodology was used to identify the optimized adsorption condition (Tarangini *et al*., 2009). The grafted starch was characterized and the amount of copper removed was quantitatively analyzed using Atomic Absorption Spectroscopy.

MATERIALS AND METHODS

Potato starch, N-methyl-N-vinylacetamide (NMVA, 98%), reagent grade potassium peroxydisulfate (KPS, ≥ 99%), ethanol (≥ 99.5%), and copper sulfate pentahydrate (≥ 99.5%) were purchased from Sigma-Aldrich (Singapore) and were used without any pretreatment.

NMVA grafting onto potato starch under microwave irradiation: The grafting procedure employed in this study was adapted from Pandey *et al*. (2012) with slight modification. Potato starch (0.1g) was dissolved in 25 mL distilled water in a 150 mL open-necked flask. To this solution, calculated amounts of KPS (0.0014M) and NMVA (0.35 – 0.55M) were added together and the total volume of the resulting solution was adjusted to 25 mL. The flask was then exposed at 100% (1200 W) microwave power for 90 and 120 seconds in a Samsung domestic microwave oven (Samsung, MW73B, Malaysia) with a microwave frequency of 2450 MHz. Potato starch-graft-N-methyl-N-vinylacetamide (starch-g-NMVA) was precipitated by pouring the reaction mixture in ethanol to dissolve homopolymers of NMVA. The mixture was centrifuged and the separated copolymer was oven-dried at 50°C up to a constant mass. Grafting parameters such as % grafting, % grafting efficiency, % conversion, and % homopolymer were then calculated using the following equations:

$$\text{Percent grafting (\%G)} = \frac{W_1 - W_0}{W_0} \times 100 \quad (1)$$

$$\text{Percent grafting efficiency (\%GE)} = \frac{W_1 - W_0}{W_2} \times 100 \quad (2)$$

$$\text{Percent conversion (\%C)} = \frac{W_1}{W_2} \times 100 \quad (3)$$

$$\text{Percent homopolymer (\%H)} = 100 - \%GE \quad (4)$$

where W_0 is the weight of starch, W_1 is the weight of the grafted starch, and W_2 is the weight of NMVA.

Partial characterization of starch-g-NMVA: The synthesized starch graft copolymer (starch-g-NMVA) was subjected to partial characterization using FTIR and SEM to evaluate its physical and chemical properties. The IR spectra of potato starch and starch-g-NMVA samples were obtained with a Fourier Transform Infrared Spectrometer (Perkin-Elmer, Spectrum 100, USA) and using the range 500-4000 cm^{-1} to provide the proof of grafting. Surface morphology of starch-g-NMVA was analyzed using a Scanning Electron Microscope (JEOL, JSM 5300, USA).

Optimization of Cu(II) removal: Adsorption conditions such as initial pH (1.5 to 5.5), initial concentration of Cu(II) (5-100 ppm) and adsorbent dose (10-100 mg) were optimized simultaneously. The optimization experiments were carried out at a working volume of 25 mL, and agitated in the shaker apparatus with a speed of 120 rpm at room temperature for 240 minutes. After agitation, the mixtures were filtered through Whatman 0.45 mm filter paper and the amount of adsorbed metal ions was determined using the Atomic Absorption Spectrophotometer (Perkin-Elmer, AAnalyst 400, USA). Control experiments were carried out to show that no sorption occurs on either glassware or filtration systems. The pH of the reaction mixture was adjusted to the desired value using either 0.5 M hydrochloric acid or 0.5 M sodium hydroxide.

Box-Behnken design: Box-Behnken statistical experiment design and the response surface methodology by Minitab Version 17.1.0 was employed to investigate the combined effect of pH, adsorbent dose, and initial copper concentration. Each independent variable was studied at three different levels: low, medium and high, coded as -1, 0, +1, respectively. The center point of the design was replicated three times for the estimation of error.

The experimental data were analyzed by fitting to a second order polynomial model, which was statistically validated by performing Analysis of Variance (ANOVA) and lack-of-fit test to evaluate the significance of the model.The performance of the process was evaluated by analyzing the response (y), which is the percent removal of copper, that depends on the input factors X_1, X_2,..., X_k, and the relationship between the response parameters is described by

$$y = f(X_1, X_2, \dots, X_k) + \varepsilon \quad (5)$$

where f is the real response function the format of which is unknown and is the residual factor associated with the experiment. The surface represented by $f(X_i, X_j)$ is called a response surface. The response can be represented graphically, either in the three-dimensional space or as contour plots that help visualize the shape of the response surface.

For RSM, the most commonly used second order polynomial equation developed to fit the experimental data and determine the relevant model terms can be written as:

$$y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (6)$$

Where β_0 is the constant coefficient, β_i is the slope or linear effect of the input factor. $\beta_i X_i$ is the linear by linear interaction effect between X_i the β_i input factor and, is the quadratic effect of input factor.

Adsorption Isotherm Study: Adsorption data was fitted to the Langmuir and Freundlich isotherms. The Langmuir isotherm was expressed in the linear form as with the equation:

$$\frac{C_e}{q_e} = \frac{1}{(bQ_o)} + \frac{C_e}{Q_o} \quad (7)$$

where C_e is the equilibrium concentration (mg/L) and q_e the amount adsorbed at equilibrium (mg/g). The Langmuir constants Q_o (mg/g) represent the monolayer adsorption capacity and b (L/mg) relates the heat of adsorption.The essential feature of the Langmuir adsorption can be expressed by means of R_L, a dimensionless constant referred to as separation factor or equilibrium parameter for predicting whether an adsorption system is favorable or unfavorable. R_L is calculated using the following equation:

$$R_L = \frac{1}{1 + bC_o} \quad (8)$$

where C_0 is the initial Cu(II) concentration (mg/L). If R_L values lies between 0 and 1, the adsorption is favorable.

The Freundlich isotherm describes the heterogeneous surface energies by multilayer adsorption and is expressed in linear form as given:

$$\ln q_e = \ln K_f + \left(\frac{1}{n}\right) \ln C_e \quad (9)$$

where Kf (mg/g),is roughly an indicator of the adsorption capacity and 1/n is the adsorption intensity.

RESULTS AND DISCUSSION

Starch was grafted with N-methyl-N-vinylacetamide (NMVA) by microwave assisted method using potassium peroxydisulfate (KPS) as a free radical initiator. Grafting was carried out in aqueous medium. The microwave energy absorbed by the water molecules resulted to dielectric heating of the reaction medium. Moreover, microwaves have lowering effect on Gibbs energy of activation of reactions. With these effects of microwave in the reaction medium, peroxydisulfate decomposed into sulfate ion radicals quickly. The resulting sulfate ion radicals interact with water to give free hydroxyl radicals. The primary free radicals (sulfate ion radicals and/or the hydroxyl radicals) combine with vinylacetamide to give monomer free radicals. Since homopolymerization is reported to be faster than graft copolymerization, the growing homopolymer free radical abstracts hydrogen from the starch molecule to result in a macro radical to which more NMVA moieties become attached to form a chain. This chain will grow until it combines with other chain to give the graft copolymer (Singh et al., 2007). A proposed reaction mechanism of graft copolymerization involving starch (StOH) and free radical species ($R^\bullet$ or SO_4^- and OH^-) introduced by Kalia *et al.*, 2013 was shown below.

$$S_2O_8^{2-} \xrightarrow{mw} 2SO_4^{-\bullet}$$

$$2SO_4^{-\bullet} + H_2O \xrightarrow{mw} HSO_4^- + OH^\bullet$$

$$StOH + R^\bullet \rightarrow StO^\bullet + RH$$

$$StO^\bullet + CH_2 = CH_2X \rightarrow StOCH_2HC^\bullet X\,RH \xrightarrow{\substack{monomer\\molecules}} \text{graft copolymer}$$

The resulting graft, starch-g-NMVA, was gel-like upon adding ethanol. After drying, the graft copolymer was hard and plastic-like. The powdered form of the graft copolymer is shown in **Figure 1**.

FIGURE 1. Dried starch-g-NMVA copolymer

B. Optimum grafting conditions

The effect of varying the concentration of the NMVA monomer along with the effect of exposure time on grafting parameters were studied.

B.1 Effect of monomer concentration

The effect of NMVA on grafting percentage was investigated by varying its' concentration from 0.35 to 0.55 M. Results showed that % grafting increased with increasing NMVA concentration (**Table 1**).

The grafting percentage (%G) indicates the increase in weight of original starch subjected to grafting with a monomer while grafting efficiency percentage (%GE) is the fraction of monomer converted to graft polymer. In general, the grafting efficiency depends on the monomer concentration. This is true with the result obtained in this study as presented in Table 1. The increase in monomer concentration resulted to increase in %GE. However, the increase in %GE obtained in this study is lower compared to other studies (Nadiah *et al.*, 2016). Low %GE means that less monomer was used in grafting and most was wasted in side reactions and homopolymer formation (Nadiah *et al.*, 2016). The increase in %GE observed is an indication that important functional groups is present on the starch backbone and these groups will play an important role in the adsorption process.

Percentage conversion (%C) is taken as the ratio of the weight of the grafted copolymer to the weight of the monomer. It is observed that % C decreased from 0.45 to

Table 1. %Grafting and %Grafting Efficiency as a function of monomer concentration of 0.0014M potassium peroxydisulfate (KPS), starch (0.1g/25mL), at 2450 W and 90 seconds exposure time

N-methyl-N-vinylacetamide (NMVA)	% Grafting	%Grafting Efficiency	%Conversion	%Homopolymer
0.35 M	7.780.064	1.850.064	25.640.015	98.150.015
0.45 M	9.440.085	2.300.085	26.580.100	97.710.010
0.55 M	10.00.036	2.410.014	26.380.241	97.600.014

Values are average of five individual samples (n=5), expressed as mean ± standard deviation.

0.55 M concentration of NMVA. This may be due to the large ratio of NMVA to initiator KPS. In this case, KPS is relatively deficient to the superabundant NMVA monomers. Hence, though the absolute amount of grafted copolymer increases, as evidenced by the increase in %G, %C is observed to decrease. The same observation was made by Liu *et al.* (2005).

This direct relation between the percent graft yield and monomer concentration within the experimental range studied can be attributed to the molecular collisions triggered by the increased NMVA population in the vicinity of starch molecules. Hence, grafting increased. On the other hand, the percentage of homopolymer (%H) showed a reverse trend with respect to %GE. This behavior can be attributed to accumulation of monomer at close proximity of the starch backbone. (Castañeda *et al.*, 2012)

B.2 Effect of exposure time

To investigate the effect of time on graft copolymerization, the reaction was carried out using the exposure times 90 and 120 seconds. As shown in **Figure 2**, an increasing trend of grafting percentage was observed after reaction of starch with different concentrations of NMVA (0.35 M, 0.45 M, 0.55 M) at fixed microwave radiation exposure time of 90 seconds.

However, at longer exposure of 120 seconds, there was a decrease in the grafting percentage. This can be attributed to the depletion of initiator concentrations as the reaction proceeds. Also, more homopolymers are formed compared to the graft copolymer at higher exposure time. When homopolymerization increases, the viscosity of the reaction medium also increases which creates hindrance in the movement of the free radicals

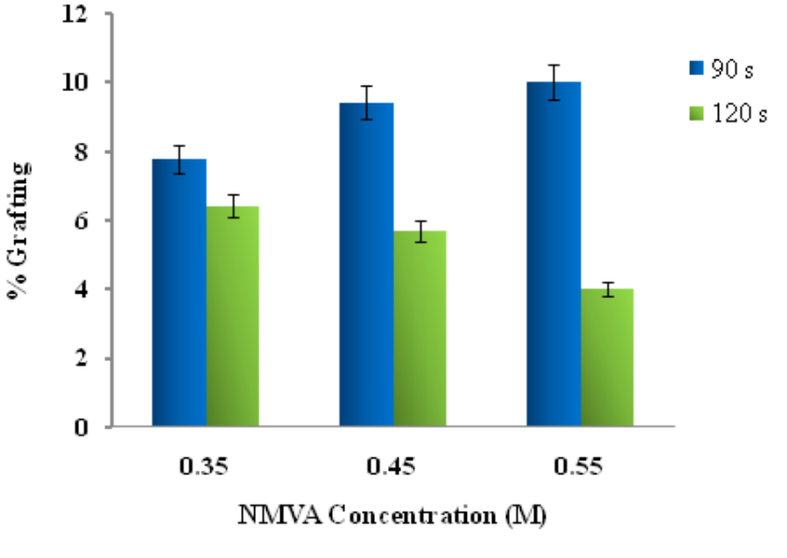

FIGURE 2. Effect of different exposure time on % grafting. Values are average of five individual samples (n=5), expressed as mean ± standard deviation

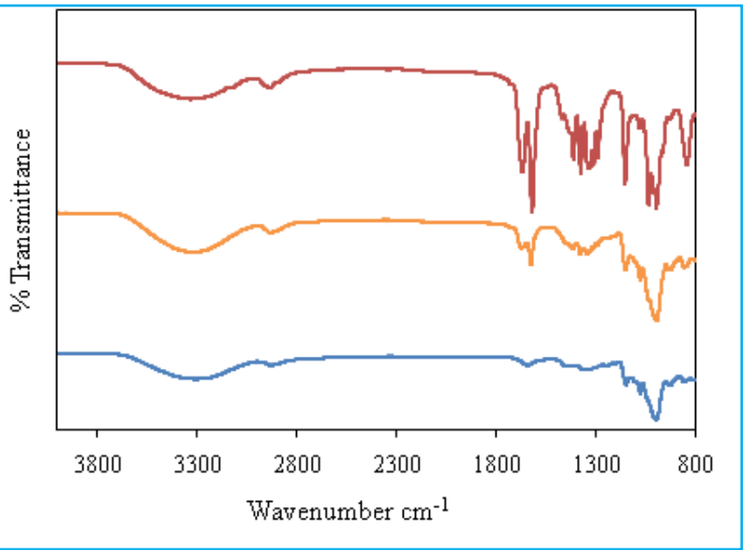

FIGURE 3. IR spectra of starch (blue), NMVA (red), and starch-g-NMVA (yellow)

toward active sites, resulting in less grafting percentage (Lakshmi *et al.*, 2011).

The highest %G which is 10.03% was obtained by exposing a reaction mixture containing 0.55 M of NMVA, 0.0014 M of KPS initiator and starch (0.1 g) to 1200W microwave power for 90 seconds. The starch-g-NMVA representative sample containing the maximum %G was characterized using FTIR and SEM analysis and was used for the adsorption experiments.

C. Characterization of the starch-g-NMVA

Potato starch, NMVA, and starch-g-NMVA were subjected to FT-IR to examine the functional groups present. As shown in **Figure 3**, the IR spectrum of starch (blue) showed a wide band at around 3300 cm^{-1}. This can be attributed to the O-H stretching of starch. This result verifies the polyhydroxy nature of starch molecule. Furthermore, the appearance of adsorption band at around 2934 cm^{-1} can be attributed to the asymmetric stretching of CH groups.

In the spectrum of NMVA (red), the peak at 1670 cm^{-1} confirms the carbonyl functional group in the structure of NMVA. This carbonyl group is reported to enhance metal adsorption (Ozturk *et al.*, 2015). The peak at 1620 cm^{-1} confirms the vinylic carbon present in the monomer while the 1337 cm^{-1} is for the C-N stretching of the amide group. In the case of starch-g-NMVA spectrum, it is observed that there is variation in the intensity of C-H stretching vibration and shifting of peak from 2940 cm^{-1} to 2980 cm^{-1}. Additional peaks at 1337cm^{-1} and 1620 cm^{-1} indicate the added functionality of the graft copolymer. However, there is a peak appearing at 1620 cm^{-1} indicating C=C groups present. This can be due to the impurity of the grafted copolymer. The % homopolymer is large (97.6%) thus there are still unreacted monomer molecules which were not removed thus accounting for the C=C peak appearing in the spectrum of starch-g-NMVA.

In the study of Kalia *et al.* (2013), the surface of the pure starch appeared to have oval granules that have smooth surfaces but are irregular in size and shape. The SEM image (**Figure 4**) obtained after grafting of our starch and exposure to microwaves showed aggravation in the surface of the starch granules. Irregular rough surface can be seen where the monomers of NMVA were grafted. The microwave irradiation employed caused the heating energy to penetrate deep inside the gran-

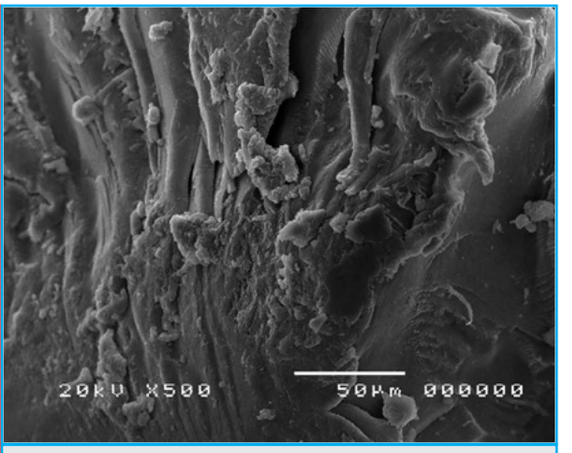

FIGURE 4. SEM micrograph of starch-g-NMVA at 500x magnification

Table 2. Box-Behnken results for Cu(II) adsorption onto starch-g-NMVA.

Run Order	Biosorption Parameters			% Cu (II) Adsorbed	
	P	A	C	experimental	theoretical
1	-1	0	+1	7.1416	5.1876
2	0	0	0	8.9305	10.6168
3	+1	-1	0	24.0418	23.5302
4	+1	0	+1	32.1254	34.8879
5	0	0	0	12.2139	10.6168
6	0	+1	-1	15.2749	17.5257
7	0	-1	+1	16.8189	14.5681
8	0	-1	-1	7.5415	6.0991
9	-1	0	-1	2.9289	0.1664
10	0	0	0	10.7059	10.6168
11	-1	-1	0	6.4854	10.6902
12	0	+1	+1	23.5426	24.9849
13	-1	+1	0	7.1829	7.6945
14	+1	+1	0	52.5741	48.3693
15	+1	0	-1	22.0267	23.9808

P = pH(-1=1.5, 0=3.5, +1=5.5)
A = Adsorbent dose (-1=10mg, 0=55mg, +1=100mg)
C = initial Cu(II) concentration (-1=5mg/L, 0=52.5mg/L, +1=100mg/L)

ules causing them to bursts outside and formed indents in the surface. The same result was observed from the study conducted by Nadiah *et al.*(2016) on the effect of microwave heating on potato and tapioca starches in water suspension.

D. Optimization of adsorption parameters

In this study, only the parameters adsorbent dose, pH, and initial Cu(II) concentration and its effect on the efficiency of Cu(II) removal by starch-g-NMVA were studied. Optimization of the removal of Cu(II) ions from aqueous solutions for three parameters were carried out with Box-Behnken statistical design. Using the Minitab 17.1.0 software, a total of 15 experiments were generated and the theoretical percentage removal of Cu(II) are compared with the experimental values, as shown in **Table 2**. The theoretical % removal was also generated by the Minitab software based on the experimental results.

By applying multiple regression analysis on the design matrix and the responses given, the following second-order polynomial equation was established to explain the Cu(II) removal efficiency which can then be used to obtain the theoretical percentage removal by substituting the values of the parameters from the specific runs.

$$\% \text{ Removal} = 20.14 - 9.06\,[P] - 0.461\,[A] + 0.067\,[C] + 1.527\,[P]^2 + 0.00289\,[A]^2 - 0.000296\,[C]^2 + 0.0773\,[P^*A] + 0.0155\,[P^*C] - 0.000118\,[A^*C] \quad (10)$$

Regression analysis suggests strong correlation between theoretical and experimental percentage Cu(II) removal. The experimental results fit to the Box-Behnken model; hence this model can explain the relationship of the different parameters considered in this experiment. With this, the Box-Behnken model was reliable enough to be used in estimating the best parameter conditions. Analysis of variance (ANOVA) was conducted to test the significance of the second-order polynomial equation for the experimental data. The *F-test* and *p-value* determines the significance of each process parameter on the Cu(II) adsorption onto starch-g-NMVA. A *p-value* of less than 0.05 and a calculated F-value higher than the tabulated F-value would indicate statistical result.

The quadratic model was highly significant, as was evident from the low p-value of 0.004. Furthermore, the calculated F-value (F_{cal} = 15.95) was found to be greater than the tabulated F-value ($F_{\alpha,df,(n-df-1)} = F_{0.05,9,7} = F_{tab}$ = 3.68) at 5% level, indicating that the computed Fisher's variance ratio at this level was large enough to justify a high degree of the quadratic model and also to indicate that the treatment combinations or runs are highly significant, as similarly reported by others *(54)*. Since $F_{cal} > F_{tab}$ (15.95 > 3.68), The Fisher's F-test concluded with 95% certainty that the regression model explained a significant amount of the variation in the dependent variable.

All the three parameters pH, adsorbent dose, and initial Cu(II) concentration are significant. However in the 2-way interactions, only the interaction between pH and adsorbent dose was significant. Thus signifies that pH and adsorbent dose have considerable contribution in the model. The high value of the coefficient of determination (R^2 = 0.9663) as determined by the Minitab software indicates that 96% of the variability in the response is explained by the model.Metal uptake depends on pH and is related to both the functional groups on the adsorbent surface and the metal chemistry in solution, which affects the surface charge of the adsorbent and the degree of ionization of the adsorbate (**Tumin *et al.*, 2008**). In **Figure 5A**, it can be observed that % removal increased with pH. At pH 1.5, the adsorption capacity is low due to the increase in positive charge density on the surface sites, and thus electrostatic repulsion occurs between the metal ions and the edge groups with positive charge on the surface. At pH 5.5, the surface charge of the graft copolymer becomes negatively charged,

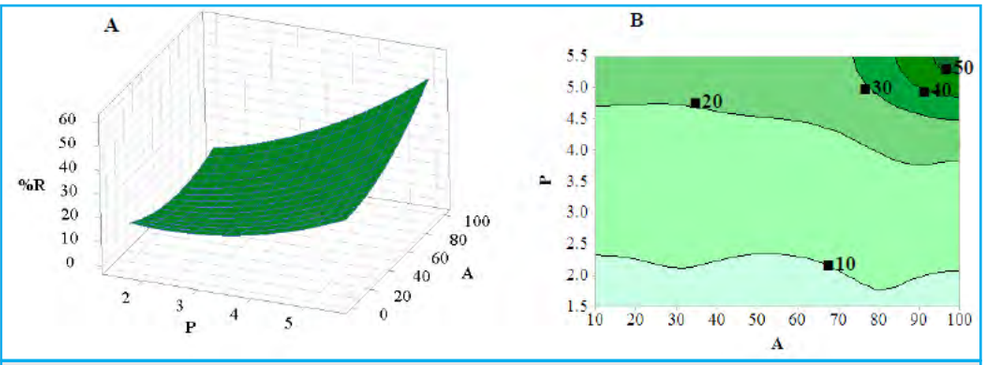

FIGURE 5. (A) 3D surface plot and (B) contour plot exhibiting the interactive effects between pH (P) and adsorbent dosage (A) on the % removal of Cu(II) (%R)

which enhances Cu(II) adsorption through electrostatic attraction (Tumin *et al.*, 2008).

It was observed that an increase in the removal of Cu(II) ions is caused by an increase in initial Cu(II) concentration. At lower Cu(II) concentration, the ratio of number of moles of metal ion to the available adsorption sites is low, and therefore the amount adsorbed per unit adsorbent increases slowly. With increasing metal ion concentration, there is an increase in the amount of metal ions adsorbed due to increased driving force of the metal ions toward the active sites on the adsorbent (Abdel Salam *et al.*, 2011).

The effect of adsorbent dose on the % removal of Cu(II) ions is shown simultaneously with pH (**Figure 5**) . The % removal was observed to increase as the adsorbent dose increased from 10 to 100 mg. At a low dose of 10 mg starch-g-NMVA, there is tight competition between the Cu(II) ions due to the limited number of available binding sites; hence a low % removal was attained. An increase in adsorbent dose to 100 mg would cause a corresponding increase in % removal due to more adsorption sites that are available for Cu(II) uptake (Tumin *et al.*, 2008). **Table 3** summarizes the best parameters obtained from the statistical software.

E. Adsorption isotherm studies

Adsorption isotherms are critical for design purposes since it describes how the adsorbate and the adsorbents interact with each other. It can be made into a model equation where it expresses the relation between the amount of solute adsorbed and the concentration of the solute in the fluid phase. For any adsorption operation the correlation of equilibrium data using an equation is essential. In this study, two isotherm equations were adopted: the Freundlich isotherm equation and the Langmuir isotherm equation.The adsorption of Cu(II) onto the starch-g-NMVA adsorbent fits the Langmuir model. The plot of $1/q_e$ against $1/C_e$, as shown in **Figure 6**, gives a straight line with r^2 value of 0.9381. The best fit line obtained from plotting log qe against log Ce for a Freundlich model (**Figure 7**) gives an r^2 value of only 0.8914.

Table 3. Best adsorption parameter values for maximum Cu(II) removal.

Adsorption parameters	Best values
% Removal	52.90
pH	5.5
Adsorbent dose (mg)	100
Initial Cu(II) concentration (mg/L)	100

The Langmuir equation assumes that during maximum adsorption a saturated mono-layer of adsorbate molecules formed on the adsorbent surface, the energy of adsorption is constant, and there is no transmigration of adsorbate in the plane of the surface (Zenasni *et al.*, 2012). A large value of the Langmuir adsorption equilibrium constant, *b* implies strong bonding of Cu(II) to the graft copolymer. This is due to the chemical modification by grafting N-methyl-N-vinylacetamide on starch, thus, increasing its metal binding abilities. Additionally, the characteristic equilibrium parameter, K_L, which is found to be 0.0735, indicates favorable adsorption for all initial concentration (C_o) studied as it lies between the values of 0 and 1. The Langmuir monolayer adsorption capacity (Q_o) has a value of 46.97 mg/g which is the amount of the metal required to occupy all the available sites per unit mass of the sample (Zenasni *et al.*, 2012).

The ability of Freundlich model to fit the experiment data was also examined. For this case, the plot of log q_e vs log C_e (**Figure 7**) was employed to generate the intensity of adsorption (*1/n*) and adsorption capacity K_f which were calculated from the slope and intercept of the plot respectively. The Freundlich constants K_f and *n* obtained from this study were 0.6240 and 1.2571 respectively.

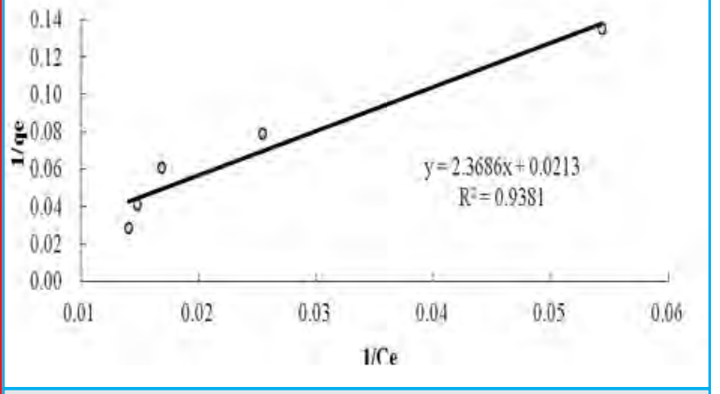

FIGURE 6. Langmuir plot for the removal of Cu(II)

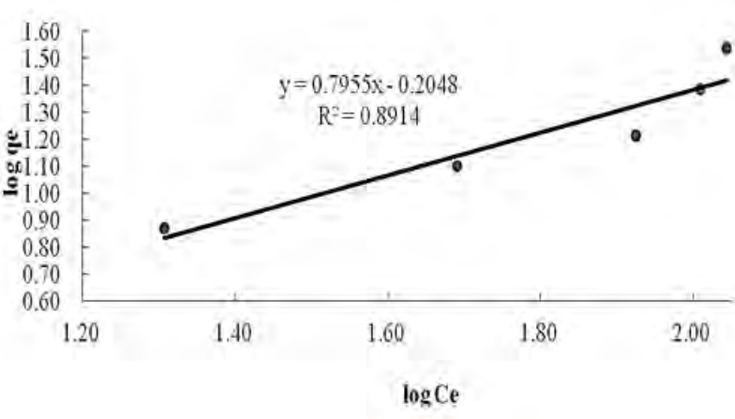

FIGURE 7. Freundlich plot for the removal of Cu(II)

The intercept K_f value is an indicator of the adsorption capacity of the adsorbent while the slope *1/n* indicates the effect of concentration on the adsorption capacity and represents adsorption intensity. The magnitudes of K_f and *n* values obtained show easy separation of Cu(II) ions from the aqueous solution and indicate favorable adsorption. As seen from **Table 5**, *n* value was found to be good enough for separation as it is greater than 1.

Consequently, the sorption process of metal ions on starch-g-NMVA follows the Langmuir isotherm model, where the metal ions are taken up independently on a single type of binding site in such a way that the uptake of the first metal ion does not affect the sorption of the next ion.

Table 4. Langmuir and Freundlich isotherm parameters for the adsorption of Cu(II) onto starch-g-NMVA.

Isotherm	Parameter	Value	R^2
Langmuir	K_L	0.07350	0.9361
	Q_o	46.9749	
	b	0.0504	
Freundlich	K_f	0.6240	0.8914
	n	1.2571	
	1/n	0.7955	

CONCLUSION

In this study, starch was grafted with monomers of N-methyl-N-vinylacetamide (NMVA) using microwave irradiation and potassium peroxydisulfate (KPS) as radical initiator. Variation of the monomer concentration and exposure time yielded the highest % grafting which is 10.03%. It was obtained at 0.55 M of NMVA and 90 seconds exposure time keeping the starch, KPS, and microwave power at 0.1g, 0.0014M, and 1200W

respectively. The graft copolymer was also investigated for its efficiency in removing Cu(II) ions from aqueous solutions at different concentrations. Using the Box-Behnken method, the best parameter conditions of 100 mg/L initial Cu(II) concentration, 100 mg adsorbent dose and a pH of 5.5 were predicted to yield percent Cu(II) removal of 52.90%. The adsorption data were also fitted to both Langmuir and Freundlich isotherms and it was found out that Langmuir isotherm best described the equilibrium data with $R^2 = 0.9361$, which signifies that a homogeneous adsorption takes place between Cu(II) ions and starch-g-NMVA. Starch-graft-N-methyl-N-vinylacetamide was proven to be an effective adsorbent for the removal of Cu(II) ions from aqueous solution with an adsorption capacity of 46.9749 mg/g.

CONFLICTS OF INTERESTS

The authors have no conflict of interest to declare.

ACKNOWLEDGEMENT

The authors wish to acknowledge the Kinaadman University Research Office (KURO) of Xavier University-Ateneo de Cagayan for the research assistance provided through the Kinaadman Support for Student Research (KSSR).

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Horticultural Communication

Biosci. Biotech. Res. Comm. 12(2): 222-229 (2019)

BBRC Bioscience Biotechnology Research Communications

TYMV induced gene silencing of TrxG family gene *BrULT2* affects the leaf shape of chinese cabbage

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ABSTRACT

The SAND domain protein *ULTRAPETALA1 (ULT1)* and its paralog *ULT2* define a small family of closely related plant proteins, both of them physically associated to form heterodimers of trxG (Trithorax group) factors. Although trxG gene *ULT1* has well-defined roles in controlling gynoecium patterning and cell fate decisions of plants, relatively little is known about the specific functions of *ULT2*. In this study, bioinformation analysis revealed that the *BrULT2* gene of *Brassica rapa* encodes a protein of molecular mass of 26.37 KDa, an isoelectric point of 7.9. qRT-PCR was performed to detect the expression profiles of the *BrULT2* gene of Chinese cabbage, showing that the transcription level of BrULT2 gene was significantly down-regulated during the vegetative stage. We silenced the trxG family gene *BrULT2* with optimized TYMV induced gene silencing, the TYMV-derived vector pTY was recombinated to infect *Brassica rapa*. After twenty days of inoculation, the infected plants showed abnormal phenotypes: leaf rolling, smaller and increased number leaves, thin stem, branch outgrowth. We detected that the expression level of *BrULT2* gene of infected plants was down-regulated meanwhile the transcriptional level of *BrULT1* gene was up-regulated. Overall, our analysis introduce a novel mechanism that mediated *BrULT* family genes control the morphogenesis of *Brassica rapa*.

KEY WORDS: *BRULT1*, *BRULT2*, CHINESE CABBAGE, LEAF SHAPE, VIGS

ARTICLE INFORMATION:

Corresponding Author: changweizh@njau.edu.cn
Received 17th April, 2019
Accepted after revision 18th June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/3

INTRODUCTION

The epigenetic factors Trithorax group (trxG) and Polycomb group (PcG) proteins act as antagonistic regulators to maintain gene in transcriptional repressed and active states through their histone lysine methyltransferase (HKMT) activity during plant developmental processes (Bernadett and Jürg, 2006, Maria et al., 2015). Historically, the two major and best-characterized PcG proteins are Polycomb repressive complex 1 (PRC1) and PRC2 (Zheng and Chen, 2011), several proteins with trxG-like functions also have been identified in plants: ARABIDOPSIS HOMOLOG OF TRITHORAX1 (Alvarez-Venegas et al., 2003), ATX2 (Saleh et al., 2008), ATXR3/SDG2 (Alexandre et al., 2010, Lin et al., 2010), ATXR7/SDG25 (Berr et al., 2009, Tamada et al., 2009), and the SAND-domain DNA binding proteins ULTRAPETALA1 (ULT1) and ULT2 (Carles et al., 2005). Previous reports have demonstrated that the *Arabidopsis* trxG gene *ULT1* can switch the key floral homeotic gene *AGAMOUS (AG)* locus from repressed state to active state through restricting the deposition of H3K27 methylation, which was mediated by *CURLY LEAF (CLF)* containing the PRC2 complexes (Carles et al., 2009, Goodrich et al., 1997). The *ULT1* gene also acts antagonistically to *KAN1* to pattern adaxial-abaxial polarity of gynoecium and rosette leaves (Pires et al., 2014, Pires et al., 2015). The trxG gene *ULT2* have a very similar amino acid sequence and mRNA expression pattern with *ULT1* (Carles et al., 2005), but there are little researchs focus on the specific biological function of *ULT2* gene. To gain insight into the molecular mechanism of *ULT2* activity in plant, the optimized VIGS technology was performed.

Virus-induced gene silencing (VIGS) is an RNA mediated specific degradation mechanism based on highly conserved nucleic acid levels. By using the viral vector carrying the cDNA fragment of the target gene to infect plant, VIGS can specifically induce the sequence degradation or methylation of the homologous gene along with the replication and transcription of the virus, that will generate changes in plant phenotype or physiological indicators in favour of identifing the functional gene (Burch-Smith et al., 2010, Vaghchhipawala et al., 2011). In 1995, Kumagai constructed a VIGS vector based on the tobacco mosaic virus (TMV) for the first time, then the VIGS was successfully performed to silencing the expression of phytoene desaturase gene *PDS* in tobacco (*Nicotiana benthamiana*). Nowadays, VIGS system has been successfully established not only on model plants such as *Arabidopsis* (Manhães et al., 2015), *Nicotiana benthamiana* and *Solanum lycopersicum* (Velásquez et al., 2009), but also on wheat (Bennypaul et al., 2012), cotton (Ye et al., 2014), rice (Kant et al., 2015). Turnip yellow mosaic virus (TYMV) is a higher plant virus with a positive RNA strand, which can infect many cruciferous plants (Martínezherrera et al., 1994). Recently improved VIGS system based on TYMV-derived vector has been exploited, which has been successfully performed by gun powder with synthesized 80-nt fragment identical to target gene (Yu et al., 2018).

Previous study have indicated that the trxG genes *ULT1* and *ULT2* have overlapping roles in regualting shoot and floral stem cell accumulation during the reproductive period, however, they have not been sufficiently characterized during the vegetative growth stage. Here, we characterized the role of *BrULT2* gene of *Brassica rapa*, optimized VIGS system was deployed to silenced the *BrULT2* gene, qRT-PCR was performed to decideted the expression level of *BrULT* gene of innoculated and wild type Chinese cabbage, and the morphology of *BrULT2*-silenced transgenic plants was investigated. The collective results of our study will provide some basic information related to the meshanism of leaf growth of Chinese cabbage.

MATERIALS AND METHODS

Plant Materials and VIGS Assay: *Brassica rapa* L.CV. heading type 'Bre' and non-heading type '49 caixin' were grown in soil (1:1:1 mixture of perlite:vermiculite: topsoil) and placed in artificial climate room at 22 with 16h/8h light/dark photoperiod cycle, 50% relative humidity. When the cotyledons and the first 3 true leaves have emerged, the empty vector pTY-S and recombined vector pTY-PDS and pTY-BrUL T2 were transformed into plants by applying 10μL 2mg cm^{-3} plasmid to leaves which was injured advanced by rubbing with quartz sand. Three weeks post-inoculation, leaf samples were collected directly from the control plant and *BrULT2*-silenced plant, it was frozen with liquid nitrogen and stored at -80 until used. All experiment were conducted with three biological replicates.

Plasmid Construction: The sequence of *BrULT2* gene was downloaded from the *Brassica* database (http://brassicadb.org/brad/), total RNA was extracted from the tissue of heading type 'Bre'. The open reading frame (ORF) of *BrULT2* gene was amplified by PCR, using first-strand cDNA as template and gene-specific primers which are listed in Table 1. The PCR conditions were: 95 for 5 min, followed by 35 cycles at 95 for 30 s, 55 for 30 s and 72 for 1 min, with a final extension at 72 for 10 min. The PCR products were detected by 1.2% agarose gel electrophoresis and recovered with AxyPrep DNA gel extraction kit (AxyPrep, China), then they were cloned into the pMD19-T vector (TaKaRa, Japan) and verified by sequencing. 40-nt fragment identical to *BrULT2* gene was selected for plasmid construction (5'-TTAGGGTTTTCTCAGATGGAGACCTCCAAATCACTT-

Table 1. Sequences of the specific Primers used in gene cloning.

gene name	Sense Primer	Anti-sense Primer
BrULT2	5'-ATGGAGAGAGAATGCGGGTCGA-3'	5'-TCAAATGGGTTTGGTGTTGG-3'

GCCA-3'), specific method has been described in Yu et al (2018). The fragment was self-hybridized and the empty vector pTY-S was digested with SnaBI, then the 80-nt inverted-repeat fragment of *BrULT2* was inserted into the linearized vector pTY-S at 3:1 ratio. Afterwards, the recombined vector BrULT2-pTY, pTY-PDS and the empty vector pTY-S were transformed into *Escherichia coli* strain DH5α cells and subjected to sequencing. The empty vector and recombinant vector were isolated using maxiprep-quality plasmid (Tiangen, China) and concentrated to 2mg cm $^{-3}$ for VIGS assay.

Bioinformatics Analysis of *ULT* family genes and Conserved Motif Analysis: A phylogenetic tree of the *ULT* family genes was generated using the MEGA (version 6.0) program with the neighbor-joining method based on multiple alignments of their protein amino acid sequences, the internal branch support was estimated using 1,000 bootstrap replicates. The *ULT* genes of *Arabidopsis thaliana* were obtained from the TAIR database (http://www.arabidopsis.org/), the *ZmULT1* gene was searched from NCBI database (https://www.ncbi.nlm.nih.gov), and the *BrULT* gene of *Brassica rapa* and *BolULT* gene of *Brassica oleracea* were downloaded from *Brassica* database (http://brassicadb.org/brad/), all accession numbers are listed in Table 2. Multiple sequences alignment of BrULT1 protein and BrULT2 protein was performed by using DNAMAN software (version 5.0). The EMBOSS Pepstats software was used to calculate statistics of protein properties. Conserved motif analysis used the Multiple Expectation Maximization for Motif Elicitation (MEME) (http://meme.nbcr.net/meme/) search tool. The protein secondary structure was predicted by predictprotein (https://www.predictprotein.org/), gene interaction model was analysised by SMART (Main page http://smart.embl-heidelberg.de/).

Isolation of RNA and cDNA Synthesis: Total RNA was extracted from the leaves of BrULT2-pTY and pTY-S inoculated plant by using the RNAeasymini kit (TIANGEN, China). Subsequently, the first-strand cDNA was synthesized with 1μg of total RNA according to the instructions of the manufacturer's PrimeScript ™1 st Strand cDNA Synthesis Kit (TaKaRa, Japan).

Gene Expression Analysis by qRT-PCR: The Gene-specific primers were designed based on the gene sequences of *BrULT1* and *BrULT2* using the Becon Designer v. 7.9, that are listed in Table 3. The analysis was performed on 7500 Fast Real-Time PCR System (Applied Biosystems, USA) following the instruction manual for the SYBR Green (TaKaRa, Japan). The thermocycling conditions

Table 2. ULT family genes identified in *Brassica rapa*, Arabidopsis thaliana, *Brassica oleracea*, *Zea mays* and the sequence characteristics.

Gene name	Locus ID	Chromosome	Start	Stop	Isoelectric point	Molecular weight (KDa)
BrULT1	Bra026276	A01	10038908	10040319	7.0849	27397.08
BolULT1-1	Bol020015	Scaffold000127	1378942	1380353	6.8382	26993.52
BolULT1-2	Bol042381	C06	44651418	44652931	7.7838	26673.57
AtULT1	AT4G28190	Chr4	13985178	13987442	7.2871	26744.24
ZmULT1	NC_024466.2	Chr8	177291861	177295258	7.8795	26486.92
BrULT2	Bra036504	A07	33825	34786	7.9079	26375.91
BolULT2-1	Bol001467	Scaffold000442	29537268	29540037	6.9431	8982.30
BolULT2-2	Bol001263	Scaffold000457	120188	121156	7.6502	26378.92
AtULT2	AT2G20825	Chr2	8966867	8969756	7.5056	26097.69

Table 3. Sequences of the specific primers used in quantitative real-time PCR.

gene name	Sense Primer	Anti-sense Primer
CP	TCCACCCTCACCACCTTC	GGGACAGACCTCGCTAACT
BrULT1	ATGTGACCAAGACAAGTT	TTCTCCTCCAACGATAAC
BrULT2	GTGTTCATTGAGGGAGAC	CCGTTGGAGTTATTCTTCA
BrActin	TTGCTATTCAGGCTGTTCT	CACCATCACCAGAGTCAA

were as follows: initial polymerase activation at 94 °C for 30s; followed by 45 cycles at 94 °C for 10s , at 60 °C for 30s; and finally a melting curve was performed (60 cycles at 65 for 10 s). Each reaction was performed in triplicate. The *Actin* gene *(Bra028615*) was used as endogenous control (Dheda et al. 2004), and the relative expression levels of *BrULT1* and *BrULT2* gene were calculated using the $2^{-\Delta\Delta CT}$ method as described by Schmittgen and Livak (2008).

RESULTS AND DISCUSSION

Expression of BrULT2 during the Vegetative Stage of Chinese Cabbage

In *Arabidopsis thaliana*, the *ULT1* and *ULT2* gene are expressed coordinately in shoot and floral meristems, developing stamens, carpels and ovules, the *ULT1* transcripts could also be detected in vegetative meristems and leaf primordia during the vegetative stage, while the *ULT2* expression is specific to the reproductive developmental stage, which can be detected only in inflorescences, pollen and siliques (Carles et al., 2005). In this study, qRT-PCR was performed to determine the distribution of *BrULT2* mRNA transcripts in *Brassica rapa* L.CV. heading type 'Bre', the specific primer pairs are shown in Table 3. The analysis results showed that the *BrULT2* mRNA transcripts can be detected in the wild type leaves during vegetative stage, and it decreased significantly from the second leaf stage to fourth leaf stage with more than 80% drop at the middle and basal region of the leaf, 50% drop at apical portion (Fig. 1).

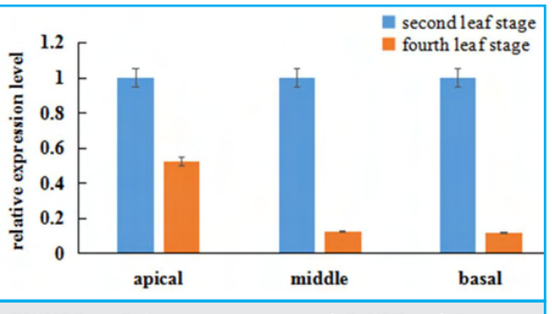

FIGURE 1. Relative expression of *BrULT2* of *Brassica rapa* L.CV. heading type 'Bre' under normal condtions, in different apart of leaf, at the different time point.

Sequences Analysis of BrULT genes

Bioinformatics analysis of *ULT* family genes showed that the predicted molecular weights (MW) of the ULT1 and ULT2 proteins ranged from 27.39 kDa to 8.98 kDa, and the theoretical isoelectric points (pIs) varied from 7.90 to 6.83 (Table 2). The cDNA sequence of *BrULT2* gene was 639bp in length, the calculated molecular mass of the protein encoded by *BrULT2* was 26.37 kDa with theoretical pI of 7.90. Multiple sequence alignment showed that the sequences of BrULT1 protein and BrULT2 protein were basically the same (Fig. 2a). To better understand the similarities and differences of *ULT* family genes between *Brassica rapa* and other plants, an unrooted phylogenetic tree was generated, the analysis result showed that the *ULT* family genes were highly conserved among the development of *Arabidopsis thaliana, Zea mays*, *Brassica rapa, Brassica oleracea* (Fig. 3a), and the *BrULT2* (*Brassica rapa*) and *BolULT2-2* (*Brassica oleracea*) clustered on the same branch. A total of 8 motifs were identified by analysis amino acid conserved domains of the *ULT* genes, showing that the conserved domain structure and positions were homogeneous (Fig. 3b). The prediction of secondary structure of *BrULT2* gene-encoding protein showed that BrULT2 protein has multiple RNA and protein binding sites (Fig. 2b), which

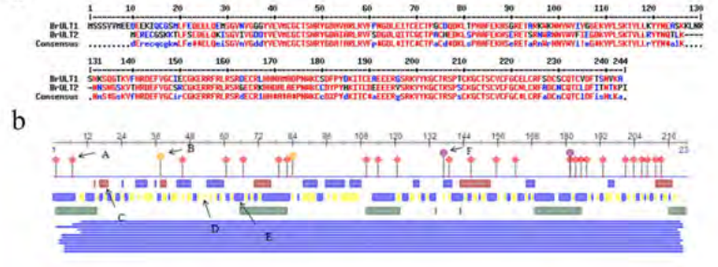

FIGURE 2. Protein analysis of BrULT1 and BrULT2. (a) Multiple sequence alignment of the BrULT1 and BrULT2 protein of *Brassica rapa*. (b) The predicted secondary structure of BrULT2protein in Chinese cabbage. A: protein binding region; B: DNA-binding region; C: α-helix; D:buried proteins; E: exposed proteins; F: RNA-binding region.

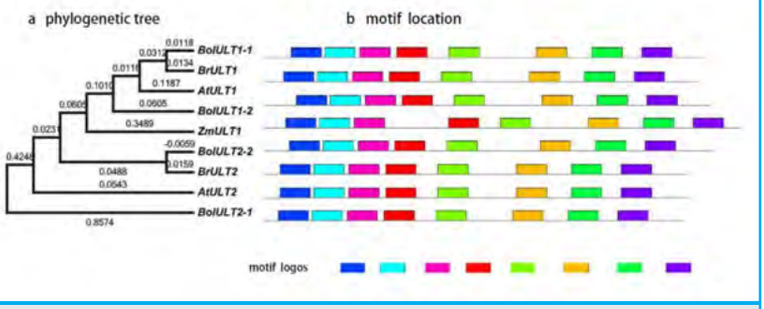

FIGURE 3. Phylogenetic tree and conserved motif analysis of ULT proteins. (a) Alignments are based on amino acid sequences of ULT proteins ofchinese cabbage and other plant species. The phylogenetic tree was constructed by the neighbor-joining method using MEGA 6.0 software with 1,000 bootstrap replicates. (b) Motif analysis of ULT proteins of *Brassica rapa*, *Arabidopsis thaliana*, *Brassica oleracea*, *Zea mays*. Motifs of the ULT proteins were elucidated by MEME. The box length corresponds to motif length. Different motif types are represented by different color blocks. The same color in different proteins indicates the same group or motif.

may facilitate its interaction with multiple transcription factors and contribute to its close regulation of a variety of growth and development-related genes.

VIGS of *BrULT2* was Successfully Performed in *Brassica rapa*

Comparing with genetic research methods such as transgene, gene knockout, and antisense inhibition, VIGS hardly require genetic transformation and mutant acquisition with a short research cycle, according to its outstanding merits: low cost, simple to operate, which has become one of the most attractive technological approaches in functional genomics research (Becker and Lange 2010). In this study, the optimized VIGS was performed to gain insight into the specific function of *BrULT2* gene.

The Phytoene desaturase (PDS) protein is a key enzyme in the carotenoid synthesis pathway, plants will lose the photoprotective effect of carotenoids and thus exhibit a whitening effect when the expression of *PDS* was blocked (Velásquez et al., 2009). In present study, the specific 40np of *BrULT2* and *BrPDS* CDS of the *Brassica rapa* was selected to construct intronic hair pin silencing vector to targeting the native *BrULT2* and *BrPDS* transcripts, the antisense construct generated dsRNA hairpins as a key elicitor for gene silencing (Christophe et al., 2003), the recombinate vector pTY-BrPDS was transformed into plants to confirming the visual success of

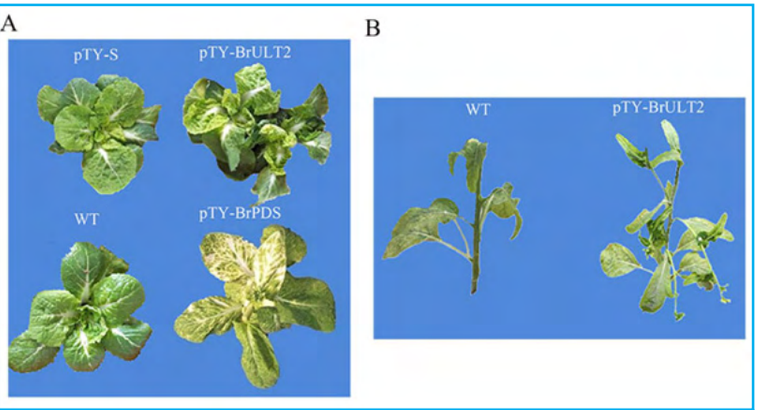

FIGURE 4. VIGS of *BrULT2* affects the leaf growth of *Brassica rapa*. (A) Growth performance of *Brassica rapa* L.CV. heading type 'Bre' inoculated with pTY-S and pTY-BrULT2, pTY-BrPDS. (B) The Phenotypes of *Brassica rapa* L.CV. non-heading type '49caixin' inoculated with pTY-s and pTY-BrULT2.

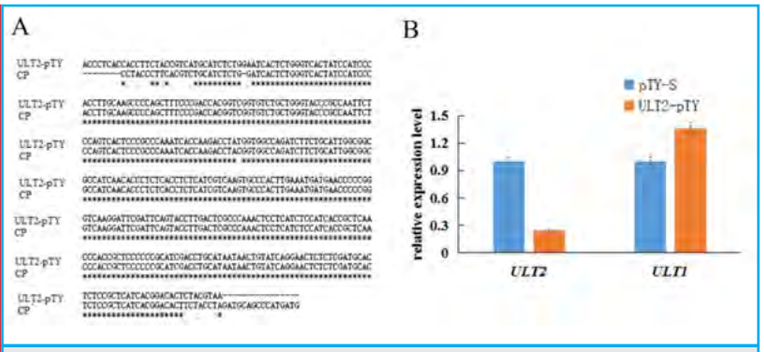

FIGURE 5. (A) Sequence alignment of PCR product amplified with specific primer of virus CP gene from inoculated plant leaves. (B) Relative expression level of *BrULT2* and *BrULT1* gene in the leaves of *Brassica rapa* L.CV. heading type 'Bre' inoculated with pTY-BrULT2.

VIGS. After three weeks of inoculation, photo-bleaching was observed in the leaves of pTY-BrPDS plant (Fig. 4A). Total RNA was extracted from the diseased 'Bre' leaves and reverse-transcribed into cDNA, which was used for the template of PCR with specific primers of virus coat protein *(CP)* gene. By sequencing, we verified that the *CP* mRNA was significantly abundant in the diseased 'Bre' plants (Fig. 5A); which implicated that TYMV virus had successfully infected Chinese cabbage.

Twenty days post-inoculation, the heading type 'Bre' infected with pTY-BrULT2 exhibited an obviously irregulate growth state with an increased number leaves comparing with control plant (pTY-S), showing a distinctive phenotype with smaller and curled rosette leaves (Fig. 4A). The non-heading type '49 caixin' infected with pTY-BrULT2 virus also showed abnormal phenotypes along with slender and irregularly curved stem, additionally the leaves number increased significantly with an increased branch outgrowths (Fig. 4A). To confirm the silencing efficiency at molecular level, the *BrULT2* gene transcripts in the leaves of heading type 'Bre' was investigated by reverse transcription qPCR. The analysis result showed that the transcription level of *BrULT2* gene dropped by 75% approximately, which indicated that the *BrULT2* gene has been silenced successfully(Fig. 5B). Those novel phenotypes observed in the inoculated plant indicating that the ectopic *BrULT2* expression alters the plant growth state leading to the modification of leaf shape of Chinese cabbage, additionally it also functions to regulate the stem and branch growth.

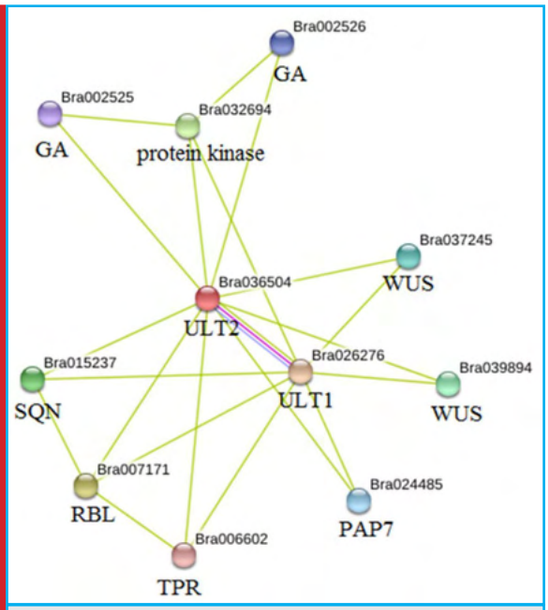

FIGURE 6. Predicted interaction networks of BrULT proteins.

In present study, we also detected that the expression level of *BrULT1* was up regulated 35% simultaneously when the *BrULT2* gene of heading type 'Bre' was silenced (Fig. 5B). An interaction network predicated by using SMART software, as shown in Fig. 6, the BrULT2 protein was predicted to interact with BrULT1 and other proteins: GA, WUS, PAP7, SQN, RBL, TPR. So we hypothesis that the *BrULT1* and *BrULT2* gene interact genetically during the vegetative growth stage of Chinese cabbage, and the *BrULT1* gene have similarly function with *BrULT2* to regulate the rosette leaf development, with the pTY-BrULT2 mutants display abnormal phenotypes: curly leaves and ectopic branch outgrowth. The results were in line with many studies. For example, Monfared et al. (2013) reported that the *ULT1* and *ULT2* genes function redundantly to specify the apical-basal polarity axis of the gynoecium, indicating that they may function in differentiating tissues, and in a study

by Monfared and Fletcher (2014) showed that the *ULT2* gene play a minor but overlapping role with *ULT1* in the regulation of shoot and floral stem cell accumulation.

Plant leaf shape is closely related to the photosynthetic efficiency and lodging resistance, exploring the leaf-shaped genes will contribute to the genetic improvement of excellent traits: disease resistance, stress resistance, high photosynthetic efficiency, leafy head formation. Previous report have demonstrated that class 1 *KNOTTED1-LIKE HOMEOBOX (KNOX)* genes are the common targets of *ULT1* and *ULT2* (Monfared et al. 2013), its ectopic expression may sculpt leaf morphogenesis with an auxin-directed mechanism controlling (Hay and Tsiantis, 2006). Nonetheless, future work will be required to exploring the hormone-related pathway of dramatic change in leaf shape for understanding the details of *BrULT* genes regulation.

CONCLUSION

In this study, we demonstrated that the optimized VIGS can be effectively performed to study specific gene function of *Brassica rapa*, and we firstly reveal that the *BrULT2* gene act as an important epigenetic factor to regulating the leaf shape development of *Brasssica rapa*. Meanwhile, we hypothesis that the closely related *BrULT1* and *BrULT2* gene may play overlapping roles in the regulation of leaf curl status and other vegetative phenotypes. Our study provide some important information for exploring the specific functions of *ULT* family genes during vegetative stage of plants.

ACKNOWLEDGEMENTS

We thanked for providing Antoine Bouteilly (Centre National dela Recherche Scientifique) plasmid pTY-S. This work was supported by the grants from the Fundamental Research Funds for the Central University (KYZ201826), Jiangsu Agricultural Science and Technology Innovation Fund (CX(18)3068), and Jiangsu Agricultural Industry Technology System (JATS[2018]273).

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Technological Communication

Biosci. Biotech. Res. Comm. 12(2): 230-238 (2019)

BBRC Bioscience Biotechnology Research Communications

Measuring patient experience in real time using iBeacon technology

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ABSTRACT

In recent years, there has been an increasing interest in patient experience measurement. The goal is to determine the shortcomings in healthcare services and improve the overall care paradigm to meet patient needs. Traditional methods for measuring patient experience have many limitations such as, survey length, infrequent sampling frequency, slow feedback, and a failure to integrate results into improving care. In this paper, we develop a location aware system, called PJM system, to measure patient experience in real time. It facilitates the analysis of results by stakeholders in order to highlight shortcomings in the service. We use a Bluetooth Low Energy based iBeacon. Our system consists of a smartphone application that senses the user location through iBeacon technology to deliver relevant content based on the location. It also includes a web-based platform with two interfaces: one for the patient experience admin and the other is for the patient experience decision-maker. The system gives the stakeholders the ability to design the patient's journey through the healthcare service. Also gives decision makers the ability to view survey results in a visual way. Visualisation is adopted as a means of presenting the survey results in an effective and efficient manner. This helps the decision makers obtain information in a rapid and efficient manner, thus enabling the identification of issues requiring rectification or improvement. Performance and user acceptance tests showed that the proposed system has many benefits.

KEY WORDS: HEALTHCARE, IBEACON, PATIENT EXPERIENCE, REAL-TIME

ARTICLE INFORMATION:

Corresponding Author: ialturaiki@ksu.edu.sa
Received 12th March, 2019
Accepted after revision 12th June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/4

INTRODUCTION

Patient experience is deemed an integral component when measuring health outcomes or evaluating the quality of the care provided. Current research suggests patients who have a good healthcare experience usually form more positive attitudes towards the healthcare system, which increases patient compliance, enhances care continuity, and improves overall healthcare outcomes Doyle et al. (2013). The first step in improving a healthcare system is to determine what a patient need are and how those needs are best met; this is the foundation of patient centred healthcare M.D & Lundeen (2015). Measuring patient experience is a crucial component in the goal of assessing the quality of healthcare. It allows health organizations to determine the shortcomings in service, therefore, improve the overall care paradigm pursuant to meeting patients needed Jenkinson et al. (2002). Yet, understanding and evaluating patient experiences can be challenging and they may not be easily incorporated into reality, as the answers are often subjective and specific. Traditional methods used to gather the views of patients and service-users such as surveys, focus groups, interviews, and complaints Doyle et al. (2013) have been criticised for having a variety of deficiencies and limitations: survey length, infrequent sampling frequency, slow feedback, and a failure to integrate results into improving care Robert & Cornwell (2013).

Another problem with these methods is that none of these options measure the patient experience in real-time. The more time that passes between a service being rendered and a patient being asked to opine about the service, weakens the accuracy of the findings derived from questionnaires and other forms of information-gathering Stull et al. (2009), Bjertnaes et al. (2012). The patients may forget how they felt at the time that the service was being provided, or subsequent events may have coloured their memory of it.

This work therefore proposes to develop an approach to measure patient experience while they are in the process of receiving healthcare services, i.e. in real time. The task involves developing a web-based platform and a mobile application. The platform gives the patient experience admin the ability to map the patients journey through healthcare and identify points of pain or frustration in the process. The mobile application senses the location of the patient by using iBeacon technology, then presents the relevant questions based on their location. The general concept is that when the patient enters the clinic and comes close to the proximity of the pre-installed beacon, the application will be able to ask some questions to the patient about a service provided in that region. The goal is to help stakeholders to identify which aspects of care are in most need of improvement.

Presenting a solidified definition of the term patient experience is complex, as it has until now been defined ambiguously and diversely by the various institutions and research studies that have attempted to understand it Wolf et al. (2014). Thus, it has no formal definition. In addition, there are multiple terms employed in health care, and thus only experts can know the distinctions between them (e.g., satisfaction, engagement, perceptions, and preferences), which makes conceptualizing this term difficult. The healthcare environment is simultaneously undergoing rapid changes, which causes definitions to change over time Wolf et al. (2014). Finally, it is relatively new as an area of focus M.D & Lundeen (2015). The Beryl Institute, which is a global leader on improving the patient experience in healthcare, defines patient experience as the sum of all interactions, shaped by an organizations culture, that in- fluence patient perceptions across the continuum pf care Institute (2019). This definition has since been incorporated (with or without adaptations) into a number of healthcare facilities globally as their own definition of patient experience Wolf et al. (2014). Cleveland Clinic, which is a pioneer in the patient experience, defines the patient experience as, first, providing safe care; second, delivering high-quality care; third, in an environment of exceptional patient satisfaction; and, finally, in a value-conscious environment M.D & Lundeen (2015).

The patient journey originates from a set of interactions between a patient and hospital staff, irrespective of whether they are medical or administrative staff. This journey is strictly personal and implies the patient's involvement on different levels (rational, emotional, sensory, physical, and spiritual) Trebble et al. (2010), Trebble & Hydes (2011). The journey is worked out based on mapping a consecutive series of touch points between the patient and the service where the patient experience has been actively shaped Bate & Robert (2006), Bessant & Ma- her (2009). It is a multi-stage journey with many different channels and touch points along the way Druckenmiller (2016). In the context of healthcare, the touch points of the patient journey are classified into three stages: the before, during, and after 1. In the before stage, the assessment is based on the ease of making contact with a clinic, booking an appointment, etc. While in the during stage, it is based on an appraisal of their health condition, care, treatment, etc. Lastly in the later stage, it is based on the process of arranging their next appointment and appointment reminders, etc.

Beacons can be described as devices which transmit radio signals at regular, defined intervals. Bluetooth beacons characteristically use Bluetooth Low Energy (BLE), a short-range radio technology which is both effective and useful because it uses extremely low levels of power. Any smartphone or tablet which is in range

of the Bluetooth beacon's transmission area can pick up BLE signals. Powering a beacon can be managed using two processes: (1) using of a fixed power source and (2) using batteries with a typical lifespan of between six months and two years He et al. (2015). When the iBeacon is activated, it sends a data packet along with the signal it is transmitting. This data packet is frequently called an advertisement packet and is regularly transmitted by the iBeacon, providing location and identity information, namely, UUID (Universally Unique Identifier), Major and Minor values. These values can then be used to place a number of beacons in the most appropriate positions for the layout of a shop or a supermarket. The manufacturers of the beacons give the values themselves, and they can be amended relatively easily, by using the Software Development Kits (SDK) of the manufacturer. In this work, we used a Bluetooth Low Energy-Based Beacon (BLE) to measurement of patient experience. We choose Beacon technology since it overcomes certain drawbacks associated with other technologies such as: radio-frequency identification (RFID) and near- field communication (NFC). For example, it does not require any extra device, such as a scanner for scanning cards barcode systems. In RFID the card must to be brought near to the reader for it to be scanned manually; however, the use of beacons system does not require any manual action. In NFC the tag should be brought to within the range of 5cm whereas a BLE Beacon range is up to 100cm.

Patient experience is a primary quality outcome for health services, which may be utilised to enhance quality, governance, public accountability and patient choice Ahmed et al. (2014). In this section, we highlight certain research pertinent to the measurement and enhancement of patient experience.In 2014, Benson et al. Benson & Potts (2014) adopted a novel approach for measuring patient experience, called howRwe. This is a questionnaire-based method for rapidly and effectively collecting data concerning patients assessment of their professional medical care. howRwe is designed to diminish the necessary survey reaction time, while tightly empha- sising criteria that may be adopted to enhance the patient experience. It comprises of just 29 words and easy readability, therefore it may be quickly read and comprehended by almost all readers. Wheeler et al (2015) described how, in Ontario Canada, cancer patients care during the early treatment stages has been improved through the adoption of Diagnostic Assessment Programmes (DAPs). DAP's fundamental concept is patient navagation. The objective of navigation is to assist and support patients who have recently received a cancer diagnosis, guiding them through medical and administrative processes that are occurring during a time when they and their families are potentially vulnerable and confused. In DAPs, every patient is provided with an individual professional contact, known as their navigator, whose function is to guide and inform the patient during their transition from diagnosis into treatment and care, thus facilitating this process.

Yang et al. Yang et al. (2015) proposed adopting an iBeacon-based indoor positioning system in hospitals, assisting patients with discovering their departments or wards, thus enhancing their treatment experience. This was designed according to the three-layer architecture of the Internet of Things: a network layer, facilitating data transmission through- out the location; a perceptual layer, interpreting individual user requirements, as well as an application layer, which delivers and displays this information on the receiving device. A final aspect is that Floyd Floyd (1962), the shortest distance algorithm, is used to identify the patient's nearest department or ward. Presented as a result of the experiment, hospital indoor positioning can be realised through the system, with its application saving both time for patients and required manpower, as well as conserving hospitals material resources.

Additionally, Lin et al. Lin et al. (2015) implemented this technology (iBeacon) in National Taiwan University's emergency room. In this case, the system was adopted for continually monitoring patients location in the facility. Additionally, the system displayed the patients clinical information and information about the staff attending them. Received Signal Strength was used to estimate the patients locations. This uses a signal generated by mobile devices to allow systems to determine their distance from the receiver, which may be used to deliver push notifications, in this case location and navigation information. The systems accuracy was determined by applying a 95% Confidence Level, resulting in a range between 95.9% and 98.55%. This provided an overall net accuracy of 97.22%, which is satisfactory for indoor location determination. The tested system comprised of four elements: System Server (storing information); monitoring system (processing information); an app installed on patients devices, as well as the iBeacons. Having reviewed the previous literature, we found that there is a lack of research in measuring patient experience through technology. No study has measured patient experience through the use of technology in real time; except using an iPad for assessing patient experience at the end of the journey Benson & Potts (2014). Therefore, we decided to devise a system for hospitals that can present location-based specific questionnaires to patients.

MATERIALS AND METHODS

The main aim of this solution is to develop a location aware system, PJM system which consists of: mobile

application that delivers surveys based on the patient's location and a website which calculate specific statistics from the retrieved information of these surveys. The intended users of the proposed system can be classified into three types:• Admin: who is responsible of managing the system and the patent journey as well.• Patient: who is responsible of answering the system surveys in their phone.• Decision Maker: who is responsible to make decision regarding patents journey based on the calculated statistics from the retrieved information from surveys.The main features of the system are presented below according to the beneficiary user of the Service: • Admin: Admins manage the PJM through our system website (admin home page). the main page for the admin, there are seven choices: add patient and decision maker, which allows the administrator of the system to add a new patient or decision maker to the database; manage patient and decision maker, which allows the admin to edit, and delete patient/decision maker and their information; add PJM, which allows the admin to create a new patient journey map (PJM) and adds new touch point if he did not find it within existing PJM elements; manage PJM, which allows the admin to edit, delete and archive a PJM; and the final choice is edit admin profile. In add PJM, the admin can add survey and beacon ID by clicking on each touch point. See Figures 1, 2, and 3.• Decision maker: our system gives decision makers the ability to display patients' feed- back in three types of charts which are pie, bar and stacked bar on the system website (decision maker home page). More specifically, the decision maker home page displays all PJMs to select particular PJM for viewing its feedback. The system allows the decision maker to print a feedback. See Figure 4. Patient: Patients first log in to the mobile application using a unique identification (pa- tient ID and password) which is stored in the system database. After a successful login, a pop-up notification

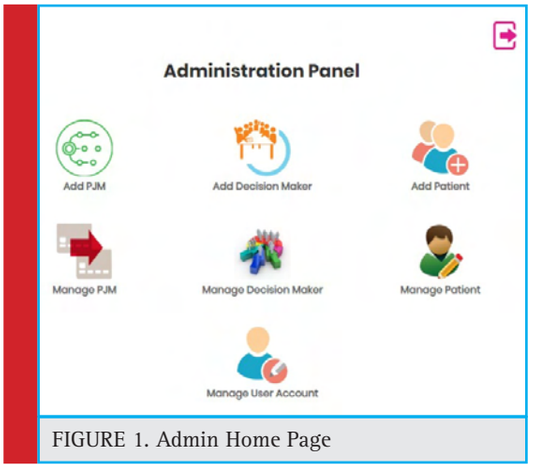

FIGURE 1. Admin Home Page

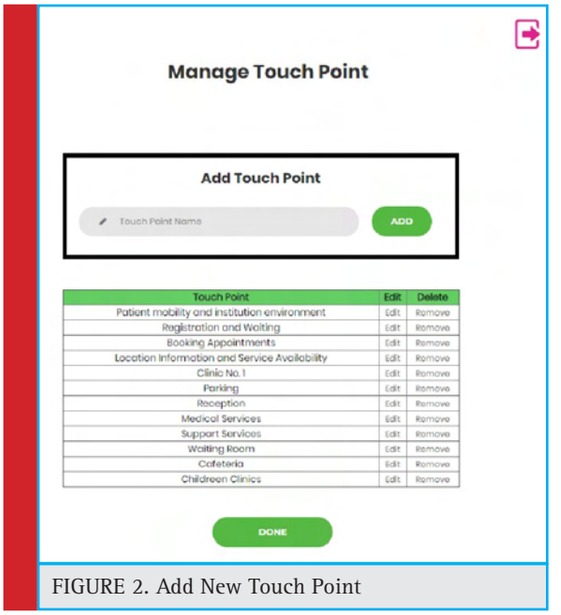

FIGURE 2. Add New Touch Point

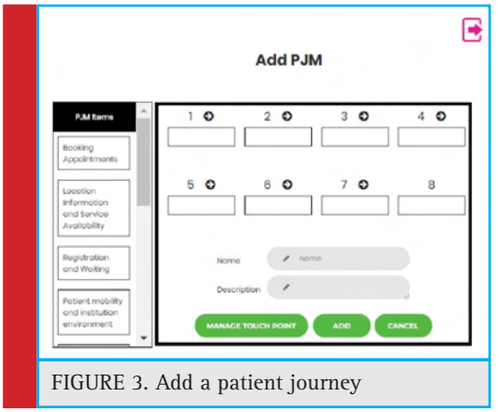

FIGURE 3. Add a patient journey

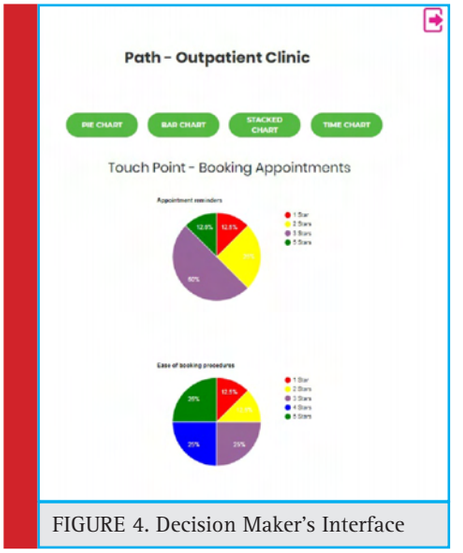

FIGURE 4. Decision Maker's Interface

FIGURE 5. Mobile App Interface

will be sent asking permission to enable Bluetooth connectivity on the patient's mobile phone, and once this is enabled the device interacts with the BLE beacon signal and sends the iBeacon ID to the server. Finally, the server sends the specific survey to the patient mobile. See Figures 5 and 6.

We present the design and implementation processes of PJM system based on Socio-technical approach Sommerville (2010). Socio-technical approach in systems development pertains to theory regarding the social aspects of people and technical aspects of organizational structures. The framework of the Socio-technical approach as suggested by Sommerville (2010) consists of several stages, which rely on gathering system requirements from the end-users of the system and considering the organization structure and administration processes. The requirement also should consider the hardware in the deployment environment. Socio-technical approach assumes huge systems and complex environments. In our system, we have a simple environment which has several iBeacons installed along the patient's journey path, a sever, and a few mobile phones connected to the server

FIGURE 6. Beacon Detection

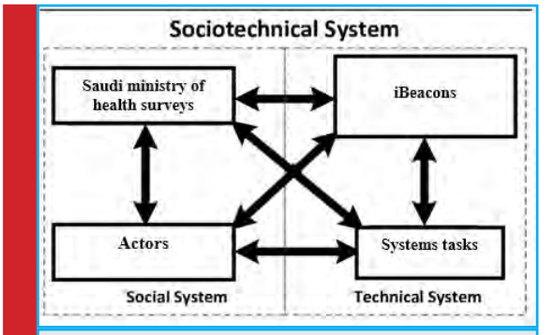

FIGURE 7. Sociotechnical approach for PJM system

at a time. However, it is essential to capture the relation between the iBeacon, the patient, and the server. For that, we choose the Socio-technical approach, but we adopt a simpler version, which focus on gathering requirements considering end users, hardware locations, and the patient journey surveys provided by the Saudi ministry of health, see Figure 7. Then, the following phases of analysing requirements, system implementation, deployment and the system testing will be considered. For system architecture, we adopt the client-server architecture as our system consists of mobile phones connected to iBeacons and website. Our architecture including the main components, which are the iBeacon, mobile application for the patient, platform for admin/decision maker and system server. These components aggregate information regarding the location of the patient and provide content based on patient location. The iBeacon devices are deployed at each PJM item such as Reception, Waiting Room, Clinic and Pharmacy. When the patient enters the iBeacon range, they can receive and interpret radio signals from it. The installed smartphone application sends the patient login information and the iBeacon information (iBeacon ID) to the server. The server determines the location of the user according to information from the pre-configured iBeacon, and then sends the specific survey, based on location, to the patient.

The application is developed to work under the Android platform. The programming language used for developing the application is Java. In addition, MySQL is used as a database tool and PHP as the API to allow the Android application and website to communicate with the project server. JSON parser is used for data interchanging between application and server.

RESULTS AND DISCUSSION

This section covers the process of testing our system. The principal objective of the testing process is to assess the system's quality and to ensure that it does not con-

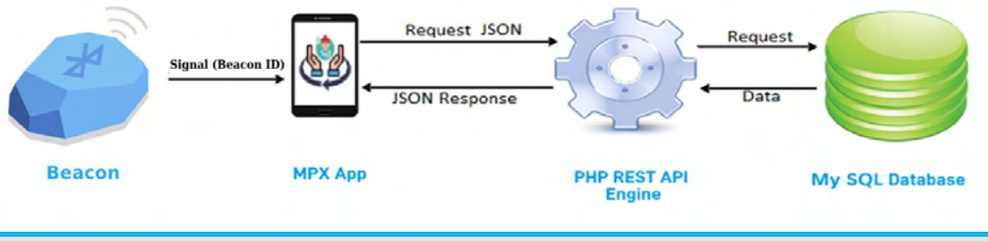

FIGURE 8. System Implementation

tain bugs or errors A number of tests have been run, including both performance and acceptance testing. All possible test cases and their results have been provided.

Performance Testing: The process of assessing the effectiveness or speed of a device, computer, network or piece of software is known as performance testing. This may consist of conducting quantitative test in a laboratory, for example to determine the system's response time or the number of millions of instructions per second that it works at. This process could also involve tests to assess qualitative attributes such as interoperability, reliability or scalability. Stress testing is often carried out at the same time as a system's performance is being assessed. We have tested the performance of our application by using the tool SystemPanel on an Android mobile phone. SystemPanel is an app that allows to view and manage device activity with simple visualizations SystemPanel (2019).• CPU usage: The total CPU utilization that have is about 1.73% as a minimum value when the application does not receive any data. On the other side, it can reach 3.20%.• Memory usage: The memory consumption of our application is 2.48 MB of the disk storage. This means the application is acceptable compared with other applications. In fact, it is considered as one of the applications that uses the least memory storage.

User Acceptance Testing : User acceptance testing (UAT), sometimes known as End-User Testing, is one of the most important elements of the testing phase of any software development process. This is be- cause, throughout this phase, the actual users for whom the software is intended will test its functions. These tests are undertaken in order to ensure that the software can successfully execute the tasks and functions which may otherwise have been overlooked in real world scenarios. Therefore, this is an essential process and must be undertaken prior to the release of the software to the real-world market and its installation for the final clients Hambling & Goathem (2013).

Effectiveness, efficiency and satisfaction are the criteria which will be considered in order to measure the degree of usability. This will be based on the number of errors which occur for each function. This information serves to disclose the effectiveness of these functions. In addition, the time that the user takes to perform a specific function is recorded and quantified in order to measure the efficiency of a given function. Finally, at the conclusion of the tests, the end users will complete a questionnaire so that their impressions of the system may be considered Hambling & Goathem (2013).

Admin and Decision makers

In order to test the efficiency of the system, the average number of errors and the average length time that users took to complete the task were calculated and recorded for both the website and application users. Firstly, the groups and their functional tasks were defined. Following this, the acceptance tests were conducted with 6 users represented the admins and decision-makers in terms of their tasks on the website. The participants are aged between 20 to 35 years old. Tables 1 and 2 show the results for the user acceptance tests for the two users. A survey is made to measure the user satisfaction with the system. The survey results showed that the interfaces and menus are flexible, and the color and fonts are clear enough to most of the participants. Most of the participants found the navigation between web- site/application screens is easy and clear. They also indicated that accessing the system and learning what it offers is easy. The majority of the participants also indicated that finding the commands required to complete a task is easy and the options names made sense to them. In general, they think that the functions provided by the system are efficient and effective. The majority of the participants find the procedure of the function is simple and require a minimum number of steps and the time required to complete it is reasonable. All of the participants agree about using the system in the future.

We have conducted an experiment at Qassim Regional Dental Center in Buraydah to evaluate the proposed application from the patient's point of view. To enable the measurement of patient experience in outpatient clinics at the Ministry of Health in the Kingdom of Saudi Arabia, 6 touch points have been identified. These

Table 1. User Acceptance Tasks and Results for the Admin

Task	Number of errors (Average)	Time in Seconds (Average)	Results
Login	0	11.37	pass
Add a new patient	0	25.5	pass
Delete a patient	0	14.6	pass
Add a new PJM	0	37.4	pass
Edit PJM	0.33	50.25	pass
Logout	0	3.08	pass

Table 2. User Acceptance Tasks and Results for The Decision Maker

Task	Number of errors (Average)	Time in Seconds (Average)	Results
Login	0	10.22	pass
view	0	2.22	pass
Print	0	3	pass
Logout	0	2	pass

include: appointment booking, location information and service availability, registration and waiting, patient mobility and facility environment, medical services, support services such as the laboratory, radiology and pharmacy procedures. Two touch points were excluded, medical services and support services. For medical services, some patients will spend far longer than an hour at the clinic, for treatments like dental implants, and as it is not required by all patients, support services were also excluded. Three beacons were deployed at different locations, in the reception, waiting room and hallway. Participants The total number of participants in the experiment was twelve, three were male and 9 were female. The participants were aged between 20-50 years old with the majority of them in their 40?s. Half of the participants held a bachelor's degree or higher, and the other half possessed a high school diploma. All of the participants have experience with using smartphone applications.

A test plan comprised of three stages was developed. Stage 1 introduction: includes welcoming participants and providing a brief description of the app with verbal instructions for the testing procedure. Then, the participants were asked to complete a demographic questionnaire to collect background data regarding their gender, age, and experience in using smartphone apps. Stage 2 app testing: we sought to test the technical effectiveness of the app (i.e., whether or not the user could complete a given task). To achieve this, participants were given 5 tasks to complete (Table 3). First, we asked the participants to log in then respond to the app notification to evaluate the service using a five-point rating scale (Likert scale). And so on, for every touch point in their journey. Stage 3 user satisfaction: we examined the user's satisfaction with the app by completing the PJM app experience questionnaire. Prior to testing, the PJM app was installed on an Android mobile (HTC U Play). The app was tested to ensure that it had downloaded

Table 3. Description of Tasks for the Patient

Task number	Task Name	Task Description
Task 1	Login	Login with patient's ID and password
Task 2	Evaluate Location information and reception services (appointments booking)	Score the service based on the five points rating scale.
Task 3	Evaluate Registration and waiting	Score the service based on the five points rating scale.
Task 4	Evaluate Patient mobility and facility environment	Score the service based on the five points rating scale.
Task 5	Logout	Terminate the session.

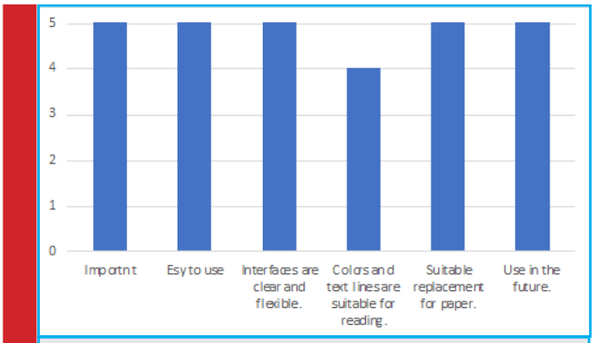

FIGURE 9. Patient satisfaction survey results

correctly, was functioning without error, and was connected to a WiFi network. All tasks were completed successfully with the users commenting on how easy the app was to use. However, two participants did record experiencing difficulties with the apps logout process. The participants commented that they had to return to the main interface to logout. This is because the user is required to answer all touch point questions, when this is done the return button will become available.

The user satisfaction survey revealed that all participants rated the app as being important and easy to use. Moreover, most of the participants stated that they were willing to use the app again. There were two recommendations raised by the users to improve the app. First, the colours used needed to be brighter and second, the text should be larger (Figure 9). Patients reported that they strongly agree that the application is a suitable replacement for paper.

CONCLUSION

In this paper, we proposed a system that uses a Bluetooth Low Energy based iBeacon for measure patient experience. Patient can assess healthcare services via Android devices in real time. This reduces the time needed and produces more accurate results compared to the traditional hand written approaches. The intended users of the proposed system are classified into three types: admin, decision maker and patient. For admin, a platform that allows the patient experience admin to create and manage a new patient journey map is developed. In addition, decision maker is able to retrieve feedback from patients in a visual way to identify deficiencies in the service and speed of processing. For the patient, a mobile application that allows them to complete a survey based on their location is developed. After developing the proposed system, the usability is measured using three criteria: effectiveness, efficiency and satisfaction. The results showed that the system gave satisfactory performance and achieved the usability requirements. The system can be improved in future by implementing it on other operating system like iOS. The same technology can be used in various other applications such as banking services, museum, restaurants services, retail stores etc.

ACKNOWLEDGEMENTS

This research project was supported by a grant from the "Research Center of the Female Scientific and Medical Colleges Deanship of Scientific Research, King Saud University.

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Bacteriological
Communication

Biosci. Biotech. Res. Comm. 12(2): 239-244 (2019)

BBRC
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Assessment of bacteriological quality of drinking water from North Kerala, India

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ABSTRACT

The objective of our study was to monitor bacteriological contamination in drinking water from northern districts of Kerala (Malabar) was carried out and also to detect the suitability of water for drinking purpose. Total coilforms can be detected by most probable number method and quantitative analysis through total Viable Count. Sixty drinking water samples were analysed both qualitatively and quantitatively. The total viable count varies from 90 to 8 x10^6 CFU/ml and three samples have MPN more than 1600/100ml. About 10^5 bacterial isolates obtained from 60 samples comprised of eight species such as *Staphylococcus aureus* (18.1%), *Bacillus Spp.* (18.1%), *Pseudomonas Spp.* (17.14%), *Klebsiella Spp.* (17.14%), *Enterobacter Spp.* (10.48%), *Citrobacter Spp.* (9.52%), *E.coli* (8.57%), and *Shigella Spp.* (0.95%) respectively. And the distribution of *Escherichia coli* in both public water supplies as well as in well water found to be 15.6% and 19.04% respectively. This reveals drinking water in this area is contaminated. So an urgent action is needed to eliminate this issue by conducting planned bacteriological assessment regularly and it helps to provide safe drinking water to public.

KEY WORDS: BACTERIOLOGICAL ASSESSMENT, DRINKING WATER, ESCHERICHIA COLI, MPN, NORTHERN KERALA

ARTICLE INFORMATION:

Corresponding Author: drds@uoc.ac.in
Received 5th May, 2019
Accepted after revision 25th June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
Crossref Clarivate Analytics
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/5

INTRODUCTION

Large number of cases are reported annually due to consumption of unsafe drinking water because of limited access to safe drinking water and poor sanitation (Hunter et al., 2001). The United Nations identifies improving water quality as one of the eight Millennium Development Goals (MDGs), and its target is to reduce the number of people without access to safe water by 50% in 2015 (Pandey et al., 2014). Even though waterborne outbreaks have been declining dramatically since the 1900s, the global burden of infectious waterborne disease is still considerable. Moreover, the numbers of outbreaks underestimate the real incidence of waterborne diseases (Leclerc et al., 2002). So there is an urgent need to take an action to control the cases of waterborne diseases. In India, contaminated water consumption plays an important role in many waterborne diseases outbreaks occurrence. Coliforms are major contaminants in surface and ground water in developing countries and are the representative of important group of indicator bacteria as a measure of water quality, (Chitanand et al., 2010, Chauhan et al., 2017, Joseph et al., 2018).

Ground water is the major source of drinking water and the quality of water threatened by number of parameters including microbiological and chemical contamination, (Kolbel-Boelke et al., 1988). Drinking water is a major source of microbial pathogens in developing regions. Waterborne infections are those which are transmitted through ingestion, airborne or contact by wide variety of infectious agents such as bacteria, virus, protozoa and helminthes. Water contaminated with infectious and toxigenic microorganisms has been a major public health concern throughout the world. Heterotrophic bacteria is common in ground water mainly because of their phenotypic plasticity. Ground water examination shows prevalence of *Pseudomonas spp*. in many samples (Leclerc, 2003). When there is fecal or other contamination, dominance of pathogenic bacteria increases.

The major health risk from drinking water is caused by the presence or introduction of coliforms in the drinking water supply which may come from the non-treated sewage systems sited nearly the water source or distribution system as well as overflow from them. Water analysis mainly focuses on coliforms, thermo tolerant coliforms and *E. coli* is used as an indicator of fecal contamination of water. Fecal coliforms (or thermo tolerant coliforms) are coliforms which can ferment lactose at 44.5 °C, (Craun, 1978), (Grabow, 1996, Rompré et al., 2002, Payment et al., 2003). And the presence of faecal coliforms indicate recent contamination of water sources with human and animal wastes and this 'indicator organisms' indicate possible presence of other potential pathogens, (Cabral, 2010, Rodríguez et al., 2012).

Total coliforms are Gram-negative, oxidase-negative, non-spore forming bacilli and are facultative anaerobes ferment lactose with gas production at 35–37°C, after 48h and it comprised of *Escherichia*, *Klebsiella*, *Enterobacter* and *Citrobacter*. But the significance of total coliforms as sanitary significance is very disparate because it also contain on soil and on vegetation. There is no relation between total coliform count and fecal pollution. The use of the coliform group as an indicator of fecal contamination is subject to strict governmental regulations. *E. coli* is the major coliform among the intestinal flora of warm blooded animals and its presence is associated with fecal contamination, therefore no *E.coli* is allowed in drinking water. Thus, detection of indicator organism is considered as the best method to detect the effectiveness of disinfection process and also recent and frequent fecal contamination of water (Rodríguez et al., 2012), (Tharannum et al., 2009).

Accurate identification of bacteria is the next most important issue. Recently,many workers used different carbon sources utilization pattern (Biolog) and cellular fatty acids profiling using Microbial Identification System (MIDI) for bacterial identification (Holmes et al., 1994), (Müller and Ehlers, 2005), (Slabbinck et al., 2009).

MATERIALS AND METHODS

The study was conducted in Northern Kerala which consists of 5 districts in Kerala such as Kasaragod, Kannur, Calicut, Wayanad and Malappuram. The geographical location of Kerala is on the southwest coast of India. Kerala is situated between latitude 10°00 North and longitude 76°25 East. Kerala shares its state borders with Tamil Nadu on the east and Karnataka on the north. It is flanked by the Arabian Sea on the west. In Kerala well water is the main drinking water source.

Drinking water samples from different sources such as well water, bore well water and tap water samples were

FIGURE 1. Northern Kerala map showing sampling locations

randomly collected aseptically from Malabar region of Kerala between the period of January 2015-August 2016.

Standard plate count: 0.1 ml of pre enriched water samples were inoculated onto nutrient agar through spread plate technique for the isolation of total viable bacteria. Plates were incubated at 37°C for 24 hours. For the final identification, all the isolates were identified by using primary as well as secondary identification methods such as Gram's staining, biochemical methods according to Bergey's Manual of Systematic Bacteriology (Garrity et al., 2007).

Detection of total coliforms using most probable number: The media used was single and double strength phenol red lactose broth for presumptive test, tubes that were positive for gas production after 24 hrs incubation at 35°C were inoculated into brilliant green lactose bile broth for confirmed test and positive tubes were used to calculate the most probable number using statistical table [8,9]. In completed test, the samples from positive brilliant green lactose bile broth from the confirmed test are streaked onto eosin–methylene blue,nutrient agar slant and lactose broth. Streaked nutrient agar slant, was used to establish that coliforms were present in the sample using primary and secondary methods such as Gram's staining, biochemical methods according to Bergey's Manual of Systematic Bacteriology (Garrity et al., 2007).

Selective isolation of *E.coli*: By using membrane filter method, 100ml samples were filtered through 0.45-µm filter and filter paper was transferred to nutrient broth.Subcultured in Eosine methylene blue media and observed the characterestic colonies with green metallic sheen, the isolates were confirmed by biochemical methods according to Bergey's Manual of Systematic Bacteriology (Garrity et al., 2007).

Selective isolation of *Shigella Spp.*:A 100ml samples were filtered through 0.45-µm filter and filter paper was transferred to nutrient broth,after incubation, subcultured to Deoxy cholate Citrate agar and observed the pale colored colnies and the isolates were confirmed by using biochemical reactions according to Bergey's Manual of Systematic Bacteriology (Garrity et al., 2007).

Selective isolation of Salmonella Spp.: By using membrane filter method, 100ml samples were filtered through 0.45-µm filter and filter paper was transferred to buffered peptone water and overnight incubated [pre enrichment]. 0.1ml of the culture was transferred to 10 ml of Rappaport–Vassiliadis Broth [selective enrichment] and incubated at 420°c for 24 hrs, and then subcultured to DCagar and observed for colonies with black centres and further confirmed by biochemical reactions according to Bergey's Manual of Systematic Bacteriology (Garrity et al., 2007).

Selective isolation of *Vibrio cholerae*.: For the isolation of *Vibrio cholerae* membrane filtration of 100 ml sample was carried out and incubated in alkaline peptone water at 37°c for 18-24 hrs and then subcultured to thiosulphate citrate bile salt sucrose agar and observed the characterestic yellow colored colonies, then the isolates were confirmed by by biochemical reactions according to Bergey's Manual of Systematic Bacteriology (Garrity et al., 2007).

Selective isolation of *Pseudomonas aeruginosa*.: For the isolation of *Pseudomonas aeruginosa* membrane filtration of 100 ml sample was carried out and incubated in nutrient broth ay 37°c for 18-24 hrs and then subcultured to King's B agar and observed the characterestic green fluorescent colonies, then the isolates were confirmed by by biochemical reactions according to Bergey's Manual of Systematic Bacteriology (Garrity et al., 2007).

Selective isolation of *Aeromonas hydrophila.:* For the isolation of *Aeromonas hydrophila* membrane filtration of 100 ml sample was carried out and incubated in alkaline peptone water at 37°c for 18-24 hrs and then subcultured to Starch ampicillin Agar and incubated at 30°c for 24-48 hrs [Characterestic yellow to honey colored colonies and after addition of 5ml of Lugols iodine colonies surrounded by clear halo], then the isolates were confirmed by biochemical reactions according to Bergey's Manual of Systematic Bacteriology (Garrity et al., 2007).

Selective isolation of *Yersinia enterocolitica*.:For the isolation of *Yersinia enterocolitica* membrane filtration of 100 ml sample was carried out and incubated in Yersinia enrichment broth and incubated at 10°c for 10 days and then subcultured to Yersinia selective agar and incubated at 30°c for 2 days [Characterestic transluscent colonies with dark pink centre],then the isolates were confirmed by biochemical reactions according to Bergey's Manual of Systematic Bacteriology (Garrity et al., 2007).

RESULTS AND DISCUSSION

The standard plate count which indicates total microbial count in drinking water was in the range of 90 to 8 x 10^6 CFU/ml. Obviously drinking water samples are seriously contaminated in these region. The presumptive coliform counts of the test samples were presented in table 1 & 2. In the present study, drinking water from this area is found to be highly unsatisfactorily with MPN up to >1600/100ml. About 32 well water samples analysed 16 got unsatisfactory results and in tap water (n=21) 11 were unsatisfactory , bore well water (n=7) no unsatisfactory results obtained.

Drinking water samples were seriously contaminated in these regions. Many reviews suggested these results

Table 1. Presumptive coliform count of total water samples tested

Grade of water sample	Presumptive coli form count/100ml	Number (%) of water samples (n=60)
Excellent	0	28
Satisfactory	01-03	1
Suspicious	04-10	4
Unsatisfactory	>10	27

Table 2. Occurrence of presumptive coliforms in water samples

Source	No. of samples analysed (n=60)	Excellent	Satisfactory	Suspicious	Unsatisfactory
Well water	32	13	1	2	16
Tap water	21	8	0	2	11
Bore well water	7	7	0	0	0

in various regions in Kerala and India. In many surveys drinking water samples in India as well as Kerala is found to be unfit for drinking purposes and need regular monitoring for microbial contamination(Tyagi et al., 2014), (Sidhu et al., 2016), (Jain et al., 2010, Mahath and MophinKani, 2016). Borah *et al* (Borah et al., 2010) reported, water samples containing varying levels of coliforms ranging from 10 to 2.8 x 10^3 cfu/100ml in Golaghat district, Assam. According to Suthar et al. the microbial load in drinking water from Rajasthan as measured though standard plate count (SPC) varied greatly from 8.3 × 10^4 to 28.3 × 10^4 Suthar *et al* found many bacterial species prevalent in those area comprised of both Gram positive and Gram negative. (Suthar et al., 2009)

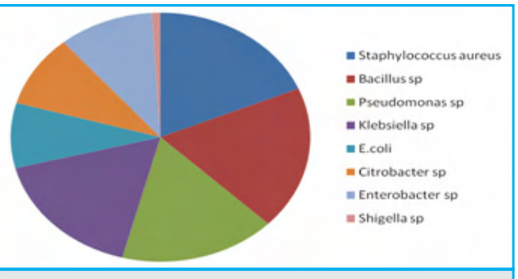

FIGURE 2. Occurrence of bacterial species in water samples

A total of 105 bacterial isolates comprised of eight bacterial species were identified in these samples. The organism isolated were found to be *Staphylococcus aureus* (18.1%), *Bacillus spp* (18.1%), *Pseudomonas spp* (17.14%), *Klebsiella spp*(17.14%), *Enterobacter spp* (10.48%), *Citrobacter spp* (9.52%), *E.coli* (8.57%), and *Shigella spp* (0.95%) respectively (Fig. 2).

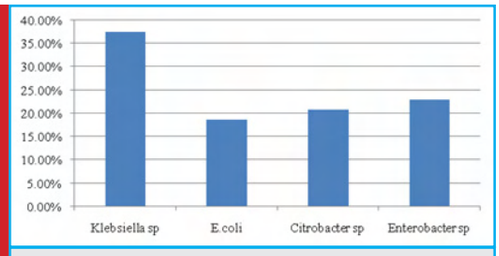

FIGURE 3. Distribution of coliforms

Out of the 60 samples screened, 36 (60%) were positive for the presence of coliforms (number of coliforms=48). Among which *E. coli* accounts to be 18.75%, *Citrobacter sp* (20.83%), *Enterobactersp* (22.91%), and *Klebsiella sp.* (37.5%) respectively (Figure 3). The indicator organism *E.coli* was present in a total of 9 samples among which 5 undergoes well water samples and 4 tap water, and in bore well water no *E.coli* was found. (Table 3).

Table 3. Distribution of *E.coli* in samples

Sample	Number of samples analysed	E.coli isolated (%)
Well water	32	5 (15.625)
Bore well water	7	0(0)
Tap water	21	4(19.04)

In a study by Ahmed et al. the indicator bacterium *Escherichia coli* were detected in 32% using MPN method (Ahmed et al., 2015) from Dhaka metropolis. Sidhu S et al. (Sidhu et al., 2016) conducted a study in Northern Indian Schools and found that 39.8% samples were non potable. And a study by Chitanand MP et al. revealed high populations of *Escherichia coli*, followed by *Citrobacter freundii, Citrobacter diversus, Enterobacter aerogens* and Klebsiella species from six sites along the bank of the river Godavari (Chitanand et al., 2010). A study conducted by Rajendran et al revealed 37% of coliform contamination from tsunami hit areas of Kanyakumari, Tamilnadu (Rajendran et al., 2006).In a study conducted in different zones of Delhi to detect drinking water quality sold by road side vendors , all the samples were found to be coliform contaminated with a MPN value ranges from 14 - >1600 per 100 ml of sample, with 61% of *E.coli* contamination followed by *Salmonella* , *S. aureus* and *P. aeruginosa* contamination respectively, (Chauhan et al., 2017).

In a study by Mahath et al. (2016), conducted in Kollam district, Kerala, total coliforms and fecal coliforms were detected in household water samples and it was found to be 60% and 50% respectively. And in our study in Northern Kerala showed the presence of both total coliforms (53.3%) and fecal coliforms (15%) indicates the presence of fecal contamination. As compared with southern Kerala, the prevalence of total coliforms and fecal coliforms was lower in Northern Kerala. Recent reports says more than three million people in the world die of water related diseases due to contaminated water each year, including 1.2 million children (Hunter et al., 2001), (Cabral, 2010), (Fenwick, 2006). Many developing regions suffer from the lack of safe drinking water for their population. About 800 billion people in Asia and Africa are living without access to safe drinking water. Consequently this has caused many people to suffer from various waterborne diseases (Tanwir et al., 2003). Continuous consumption of these samples causes infection especially in children and infants.

CONCLUSION

The study concluded that the standard plate count in 60 samples was in the range of 90 to $8x10^6$CFU/ml. A total of 60% of the tested samples were contaminated with coliforms. Prevalence of *E.coli* in different water sources (8.57%) indicates the presence of faecal contamination. Consumption of contaminated water will cause serious health problems to the public. Routine monitoring will help solve this problem. Consistent and periodical examination of drinking water samples and disinfection process should be done periodically to prevent the spread of pathogenic microbes. Thus there is an urgent need for an awareness program to the people to decrease the cases of water borne diseases. The Government of India has already launched Swatchh Bharat Mission on 2nd October 2014, with an aim to eradicate open defecation by 2019. The mission with the help of its partners like UNICEF is looking at the challenge of Open Defecation very seriously.

ACKNOWLEDGEMENTS

The authors acknowledge funding support from the Department of Science and Technology (DST) INSPIRE Fellowship, Govt. of India.

Conflict of Interest Authors have declared that no competing interests exist.

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Biotechnological Communication

Biosci. Biotech. Res. Comm. 12(2): 245-250 (2019)

BBRC Bioscience Biotechnology Research Communications

The mathematical modelling of algal growth for the production of biofuels

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ABSTRACT

The mathematical modelling of the growth of microalgae (*Nannochloropsis*) was developed under flue gas in a photobioreactor. Carbon dioxide (CO_2) was the major growth parameter used to model algal growth. The critical SO_x concentration and CO_2 mass transfer rate were considered under the growth inhibitory model (GIM) which played a big role in pH based inter-conversion of the bicarbonate. The *Nannochloropsis* profile was validated at 0.04% of CO_2 in air and in a range of 2% to 12% (v/v) CO_2 and the predicted values were observed consistent with the measured values. Sensitivity analysis was used to justify the constants used in the GIM. The growth inhibitory model was defined as $\pm 0.5 Lmin^{-1}$ of the calibrated flow rate of 3.0 $Lmin^{-1}$. The growth model was used to predict the growth of microalgae under flue gas to generate biomass as a feed stock for the production of biofuels.

KEY WORDS: MICROALGAE, NANNOCHLOROPSIS, GROWTH INHIBITORY MODEL, SENSITIVITY ANALYSIS

INTRODUCTION

The generation of biofuels from microalgae is as a result of a high thrill for alternative energy sources. The increasing population which has led to the increase number of vehicles and high energy demand has left the world market with scarcity of fossil fuels (Minneapolis, MN, USA, 2011). Biodiesel can be obtained from any vegetable oils like sunflower, cotton seeds, peanut, safflower and microalgae lipids, but microalgae has the highest lipid yield (Demirbas and Demirbas, 2013). Algae cultivation is done in open ponds and bioreactors, but to control the growth parameters, algae is cultivated in photobioreactors. *Nannochloropsis* are algae species of

ARTICLE INFORMATION:

Corresponding Author: kkchandrika@kluniversity.in
Received 6th April, 2019
Accepted after revision 15th June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/6

cold-water marine, with high triglycerides (triglyceride is an ester derived from glycerol and three fatty acids) and high growth rate (Archambault, 2014) and therefore, it is most opted as the source of biofuel, (Islam et al., 2013, Mondal et al., 2017 and Sharma et al., 2019).

Factors affecting microalgae growth include light intensity, PH, presence of nitrogen (Danesi et al., 2002; Colla et al., 2007; Celekli and Yavuzatmaca, 2009), presence of contaminants (Vonshak, 1997), initial biomass concentration (Costa et al., 2002), population density (Vonshak, 1997), temperature, salinity and growth dynamics and much more. All these parameters can be controlled and improved only when the algae cultivation is done in a photobioreactor, instead of an open pond.

It is important to understand growth modelling of organisms and also its behaviour under vast different environmental conditions which include PH, temperature, nutrients (Zwietering et al., 1990; Celekli et al., 2008; Celekli and Yavuzatmaca, 2009) and light intensity.Differentmodels like Gompertz model were used to describe the curve of microbial growth and biovolume production (Zwietering et al., 1990; Celekli et., 2008). These models are also used to predict microbial biomass production and optimizing the growth conditions (Costa et al., 2002; Celekli et al., 2008; Celekli and Yavuzatmaca, 2009). Gompertz model is modified to give lag time, specific rate of growth and stationary phase (maximum population biovolume) (Zwietering et al., 1990; Celekli et al., 2008). Other growth models have been put on the table to describe growth during the cultivation (Rangel-Yagui et al., 2004; Chojnacka and Noworyta, 2004, Mondal et al., 2017 Singh and Singh, 2019).

With the escalating climate change, global warming has been a major concern worldwide. Presence of much greenhouse gas concentrations in the atmosphere is the major cause of ozone layer depletion that leads to global warming, in which carbon dioxide takes a lion's share with an estimation of 76.7% (v/v) and this concentration has been increasing rampantly from industrial revolution(Sharma et al., 2019). It is estimated that emission of carbon dioxide from coal thermal plants is about 7% (v/v) of global CO_2 emission and that emitted from flue gas of power plants is 10% (v/v) to 15% (v/v), and these exclude the emissions from transportation sector (automobiles)(Natural Gas Production, 2012).

As the population increases, there is an increase in the number of vehicles with high demand of petroleum products which contribute to the emission of greenhouse gases (CO_2 in particular). This has brought the attention to the scientist and researchers to look for ways of mitigating the concentrations of CO_2 from the atmosphere (Sharma et al., 2019).

It's has been found in some studies that growth of microalgae is much more influenced by carbon dioxide and sunlight; nutrients are also required for biomass production (Mondal et al., 2017). The concern of this paper is to prepare a model that maximizes biomass production from microalgae by use flue gas, for the production of bio-fuel, (Majid et al., 2014).

Flue gas is used to make this model. In flue gas, toxins like SO_x and NO_x have to be put into consideration, the presence of these toxins inhibit algal growth. The use of flue gas as a source of CO_2 for algal growth would reduce the content of emitted CO_2 from automobiles and industries, in the atmosphere. Air contains less percentage (0.04%) of CO_2 and the growth of microalgae that gets CO_2 from the air would be gradual and biomass production minimal. On the other hand, high concentrations of CO_2 would drive microalgae to death phase (Singh and Singh, 2019).

Hence, determining the maximum concentration of $CO_2$for the growth of algae is more preeminent.

MATERIALS AND METHODS

Organism and culture conditions for model development

Nannochloropsis limnetica 18.99 was obtained from a culture collection of Algae, SAG, Germany. The culture was grown in a basal medium (Shen and Yuan, 2012) at 30°C and a pH of 8.0. In all experiments, the initial cell density was maintained at 0.01 g L^{-1}. Cool white fluorescent lamps were mounted on either side of the glass reactor (bubble column: 30 L; 220 mm dia; 3:1 H/D) with 100 µmol $m^{-2}s^{-1}$ light intensity. The temperature, pH, and algal growth were monitored every 24 hours. Algal growth was estimated in terms of cell count using a Neubaur chamber (Rohem, India) .The number of cells was converted to equivalent dry weight values by the regression equation.

$$a = 4.5469b - 0.2751, R^2 = 0.99 \quad (1)$$

Where is the dry is weight (g L^{-1}); is the cell number expressed in cells L^{-1}.

Effective growth rate and SO_x inhibitory parameters

A series of experiments were made to determine effective growth rate (μ_{eff}), consisting CO_2 (% v/v) inputs at different concentrations of SO_x. The PH was adjusted in Erlenmeyer flasks (250mL) with dilute H_2SO_4 (18.4M) to simulate the effect of SO_xin the growth medium, therefore different concentrations of SO_4^{2-}(0-150 mg L^{-1}) were generated. The effective growth rate (μ_{eff}) under a concentration of SO_4^{2-}(M_{SOx}) was measured in each experimental flask by recording cell concentration with respect to time.

A linear plot logarithmic cell concentration versus time was then used to derive μ_{eff} from the slope of the curve. A supplementary linear plot, comprising $\mu_{eff,}$ versusSO_4^{2-} concentration wasalso used to derive the constant c which is equivalent to specific growth rate () from the Y-intercept and the SO_x inhibitory constant from the slope of the plot following Eq. (10). The critical SO_4^{2-} concentration (M_t) was also determined from the same plot where the SO_4^{2-} concentration corresponding to the μ_{eff} = 0 was recorded to be the M_t for the algae.

Growth Model development

The model consists the following factors: (i) The growth inhibition in the presence of flue gas that depends on partial pressures of CO_2 and SO_x;(ii) The substrate (HCO_3^-) concentration is influenced by the pH of the medium; (iii) The effective growth rate (μ_{eff}) is influenced by critical toxin (M_t) in the medium.

To minimize the complexity, some of the assumptions were made; no external mass transfer influence due to a diffusion process from the head space into the culture, the total pressure of the system is constant at operational gas velocities; the light intensity, temperature and O_2 inhibitory were not considered as but kept constant. The CO_2 in the feed was assumed to be consistent throughout stages of growth and sequestered CO_2 is consistent during the entire exponential phase.

Gas phase CO_2 and SO_x mass transfer and their solubility

The rate of mass transfer of CO_2 and SO_x into the liquid is estimated with an assumption of a steady state and the mass balance of the inlet and outlet volumetric flow rate of flue gas comprising mole fraction of CO_2 and SO_x is as below;

$$Q_{CO_2} = \frac{(mol.w)_{CO_2}.60.H.Pt.\left((Gyco_2)_i-(Gyco_2)_o\right)}{TRV} \quad (2)$$

also:

$$Q_{SO_X} = \frac{(mol.w)_{SO_2}.60.H.Pt.\left((Gyso_x)_i-(Gyso_x)_o\right)}{TRV} \quad (3)$$

Where and are the mass transfer rates of CO_2 and SO_x across air-water interface; G is gas the flow rate, and are the inlet mole fraction of CO_2 and SO_x respectively; and are the outlet mole fraction of CO_2 and SO_x in the flue gas; $(mol.w)_{CO2}$ and $(mol.w)_{Sox}$ are molecular weights of CO_2 and SO_2 respectively; H is the total sparging time of flue gas in a day; Pt is total pressure; and R,T&V are universal constant, absolute temperature and total volume of the liquid respectively.

The concentration of the liquid phase of CO_2 and SO_x in equilibrium with that of CO_2 and SO_x in the flue gas feed is described as follows:

$$M_{Lm} = Pt.y_{CO_{2i}}.He \quad (4)$$

Where M_{Lm} is CO_2 concentration at equilibrium; Pt is the total pressure; is the mole fraction of gas component; He is Henry's constant for CO_2.

Similarly;

$$M_{SO_X} = Q_{SO_X}.R.T.He \quad (5)$$

Where is mass transfer of from gas to liquid medium; R, T and He are universal gas constant, absolute temperature and Henry's constant of respectively. Initial substrate (M_L) is an important variable influencing growth rate in the model development, and an assumption of steady state between absorption and consumption of the substrate was made, while CO_2 transfer from the flue gas was driven by M_L gradient in the liquid which is aided by the mass transfer coefficient of the system.

Therefore;

$$M_L = M_{Lm} - (Q_{CO_2} / K_L a_{CO_2}) \quad (6)$$

Where the volumetric mass is transfer coefficient; is the equilibrium concentration of CO_2 and is the concentration of dissolved CO_2 at a defined time.

The was estimated experimentally by using the static method.

$$\text{Hence;} \qquad K_L a_{CO_2} = K_L a_{O_2}\sqrt{\frac{D_{CO_2}}{D_{O_2}}} \quad (7)$$

where and were the diffusivities of CO_2 and O_2 respectively

Inhibitory growth kinetics

To model the effective growth rate appropriately, the effects of CO_2 and SO_x inhibition have to be combined.

Knowing μ_{CO2},

$$\mu_{co_2} = \mu_M\left[\frac{M_L}{\left(K_S+M_L+\frac{(M_L)^2}{K_I}\right)}\right] \quad (8)$$

The growth time slows down when SO_4^{2} accumulates during the transfer of SO_xinto the liquid medium, while considering SO_x inhibition along with then the growth rate as a function of is modeled as;

$$\frac{dx}{dt} = qx[1 - f(M_{SO_X})] \quad (9)$$

Where is the dissolved concentration of SO_x in the form of SO_4^{2}, q is the SO_x inhibitory constant.

When SO_x linearly decreases growth rate,

$$\frac{1}{x}\frac{dX}{dt} = q(1 - rM_{SO_X}) \quad (10)$$

Also $\frac{1}{x}\frac{dX}{dt}$ is equal to μ_{eff} and q equivalent to μ_{CO2}; therefore

$$\mu_{eff} = \mu_{co_2}(1 - bM_{SO_X}) \quad (11)$$

The critical time for SO_x inhibition

The SO_x in the flue gas is toxic to the algal cells when the reduced pH reaches the critical concentration. Thus, it's of a great significance to correlate the defined mass transfer rate of SO_x in the liquid medium and the time at which the critical concentration is reached.Therefore, toxic time and stationary phase are modelled as follows;

$$t_c = (M_t/M_{SO_x}) \quad (12)$$

Where is the critical SO_x concentration, is the dissolved SO_x (SO_4^{2-}) concentration at a defined time.

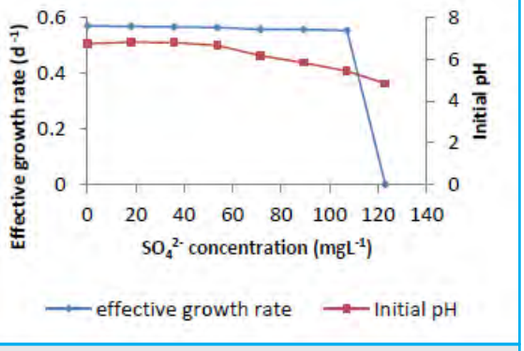

FIGURE 1. The effect of SO_4^{-2} concentration and initial pH in various independent experiments on the effective growth rate of algae.

Table 1. List of best fit parameter constants and their comparison with literature

Parameter	*Best fit value	Comparable values from literature
μ_M	0.82 day^{-1}	1.0 day^{-1}; Forján et al. (2007)
K_I	1.02 g L^{-1}	10 mM@; Silva and Pirt. (1984)
K_S	0.07 g L^{-1}	0.12 gL^{-1}; Chen et al. (2010)
C_t	123mg L^{-1}	300 ppm@ (gas phase); Lee et al. (2000)

*The best fit value is the base value in sensitivity analysis; @: K_I and Ct equivalent to 0.44g L^{-1} and 133mg L^1 (liquid phase concentration) respectively.

RESULTS AND DISCUSSION

Toxic components in the flue gas have influence on algal growth, high concentration of CO_2; residual SO_x drastically affect the growth rate.

Figure 1 shows graphical interpretation of effective growth rate against SO_x (SO_4^{2-}) concentrations with the corresponding values of initial pH. The active growth was under alkaline condition with the pH of 6.8, as SO_x concentration increases, the effective growth rate (μ_{eff}) decreases linearly with a constant . The effective growth rate fluctuates slightly from 0.48 to 0.43 per day and suddenly to 0.0 at SO_x critical concentration (M_t=123 mgL^{-1}), and this leads to substrate (HCO_3) depletion and thus affecting the and μ_{eff} . The change in effective

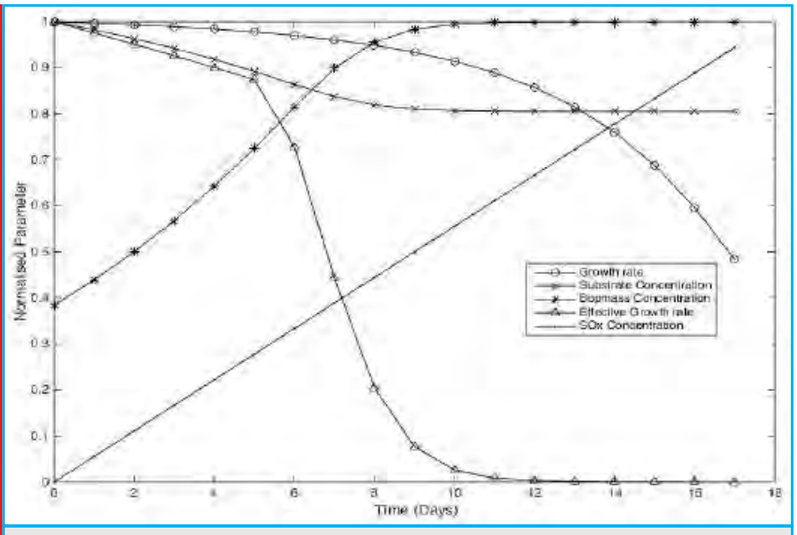

FIGURE 2. Normalized profiles of various predicted parameters (CO_2 growth rate (μ_{co2})), substrate concentration (M_t), Biomass concentration (X), effective growth rate (μ_{eff}), and SOx concentration (M_{SOx})) in the modelling of algal growth.

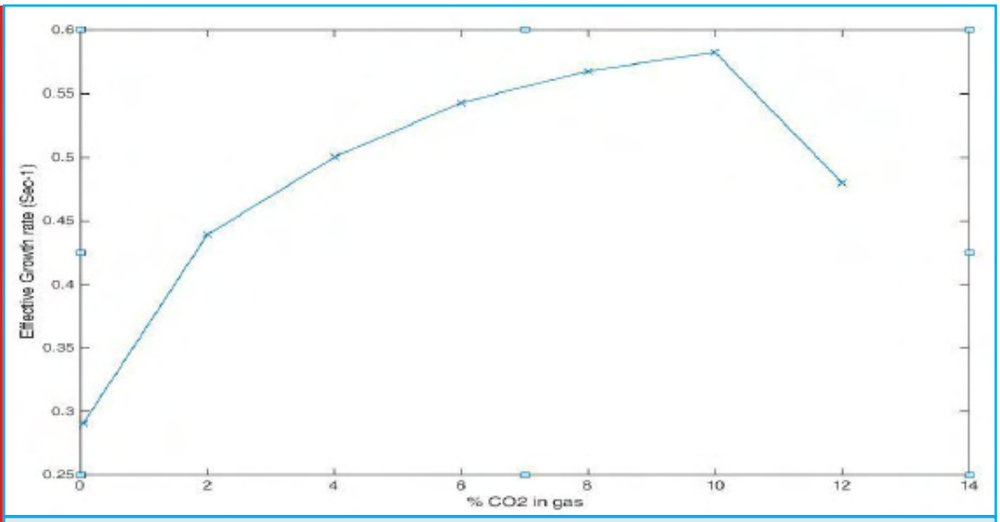

FIGURE 3. Analysis of effective growth rate (μ_{eff}) with increase in CO_2 concentration

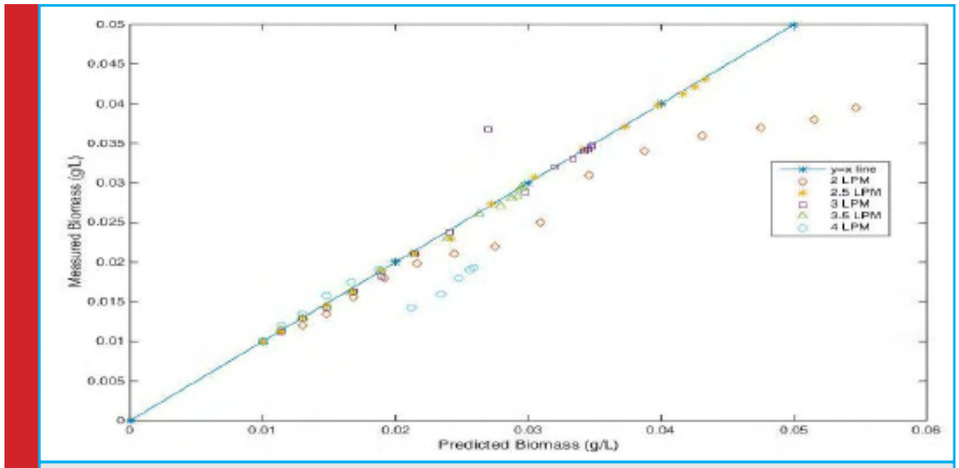

FIGURE 4. Validity and scope of the model for the prediction of biomass under flue gas at various flow rates.

growth rate profile before reaching M_t is insignificant in comparison to μ_{eff} at M_t. This supports theoretical evidence that is constant before reaching M_t with less effect of toxin (SO_4^{2}), whereas sudden drop in at M_t, toxin concentration has a great effect on μ_{eff}.

In figure 2, CO_2growth rate (), substrate concentration (M_t), Biomass concentration (X), effective growth rate (), and SO_x concentration (M_{SOx}) were taken as normalized parameters against time. Growth kinetics like effective growth rate and CO_2growth rate were plotted with their maximum inputs against time. The is stable until reaching critical SO_x concentration (M_t) and then declined drastically after reaching, affecting the , according to Eq. (11). Biomass concentration increases with time until it reaches the phase of decline and then remains constant. Substrate (HCO^-_3) concentration depletes as it is consumed during algal growth and becomes constant when the algal growth stops. Since the SO_x concentration is not utilized during the algal growth, it keeps on increasing as flue gas is supplied to the algal culture.

Air contains 0.04% of carbon dioxide and the toxins like SO_x and NO_x are negligible and therefore, there is no inhibition of SO_x to the algal growth. The algal increases as CO_2percentage in the flue gas increases. The increase in the is seen when carbon dioxide ranges from 0.0004% to 10%. Continuous supplying of CO_2 to the algal culture, beyond 10% of CO2 leads to the decline in the effective growth rate and eventually, to the death phase.

Flow rate is one of the operating parameters and it shows a direct impact on biomass production. Six different flow rates were used to check the validation of the model (Figure 4) by using predicted and experimented biomass concentrations. From the profile shown in figure 4, for the validity of the model for the prediction of biomass, higher deviations are observed at high flow rates of >3.5LPM and at low flow rates of <2.5LPM. The model is valid at the region of flow rates ranging 2.5LPM to 3.5LPM.

CONCLUSION

In this model, certain amount of carbon dioxide can be reduced from the atmosphere as flue gas from industries or vehicles is used for algal growth. The cell growth was directly affected by SO_x concentration which also influenced the pH dependent kinetics. The validity of this model was observed as $\pm 0.5 Lmin^{-1}$. Little variation ($\pm$10%) of the base value of constants used in GIM was observed in sensitivity analysis. It was also found out that up to 10% (v/v) of CO_2 is required for this model and beyond this CO_2 concentration, effective growth rate leads to decline phase.

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Medical
Communication

Biosci. Biotech. Res. Comm. 12(2): 251-257 (2019)

BBRC
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Practice of therapeutic exercise, physical therapy modalities and pet therapy in negotiating with depression in adolescent, adults and elderly population: A narrative review

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ABSTRACT

Depression is a mental stage which affects the quality of life in people around the globe. It's a phase in one's life characterized by low mood, reluctance toward performing activities which greatly affect behavior, thought process, short and long term feeling, temporary and permanent well being. A thorough search of the literature was carried out to identify studies investigating therapeutic interventions in treating patients with depression. All the studies were thoroughly searched for the relevant information contained in them. Electronic database, including Embase, Scopus, Pub Med, CINAHL, Web of science were searched for research articles published from the earliest of 2009 to latest of 2016 using keywords of "depression", "Physical therapy", "Therapeutic intervention", "pet therapy". The present study looked into various therapeutic interventions while writing the article. Earlier reported literatures have employed various interventions in treating adolescent, adults and geriatric populations with depression. Limited study have been performed on Pet therapy, the authors are directed to employ more time and efforts in studying new methods to treat depression.

KEY WORDS: DEPRESSION, RELAXATION, AEROBIC, STRENGTHENING, BALANCE, EDUCATION, PET THERAPY

ARTICLE INFORMATION:

Corresponding Author: mshaphe@jazanu.edu.sa
Received 11th April, 2019
Accepted after revision 18th June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
Crossref Clarivate Analytics
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/7

INTRODUCTION

Depression is a mental stage which affects the quality of life in people around the globe[1]. It's a phase in one's life characterized by low mood, reluctance toward performing activities which greatly affect behavior, thought process, short and long term feeling, temporary and permanent well-being. It is a syndrome which demonstrates sign and symptoms of darkness in thoughts, feeling of worthlessness, weakness in attitude and slowdown in confidence. Depression is greatly a negative impacting health problem which if ignored in the preliminary stages grows to deeper extent which in return can lead to adverse effects to the community (Cardona-Arias et al., 2015 and Rothon et al., 2010). In other words, it is the widest prevailing psychiatric disorder affecting nearly 121 million adults worldwide which is projected to become the highest epidemiology for a disease by the end of 2020, (Moussavi et al., 2007, (Ozturk et al., 2015, Kujawa et al., 2016 and Tasci et al., 2018).

Over the years, with extensive studies performed on depression by researchers from varied domains around the world, the widely used and favorable therapeutic interventions for depression have been delivered by either using single or combination of interventions (Riemer et al. 2012). The choice depends on the current stage of depression which is mild, moderate or severe. If the treatment is started in the early phase, single or two modes of interventions are sufficient and effective, while in moderate and severe depression, a collective therapeutic intervention are used to relieve from agony of depression.

As the authors have mentioned the common and worldwide accepted effective therapeutic interventions towards depression, in the current article the authors would like to concentrate on physical therapy, therapeutic exercises and Pet therapy interventions to treat depression to improve the overall quality of life. The association of exercise with psychological and physical health has been established in healthy along with in population suffering with long term health conditions like depression.

MATERIAL AND METHODS

A thorough search of the literature was carried out to identify studies investigating therapeutic interventions in treating patients with depression. All the studies were thoroughly searched for the relevant information contained in them. Electronic database, including Embase, Scopus, Pub Med, CINAHL, Web of science were searched for research articles published from the earliest of 2009 to latest of 2019 using keywords of "depression", "Physical therapy", "Therapeutic intervention", "pet therapy".

General precaution in dealing individuals with depression:

1. The individual's stage and age of depression should be the foremost point of consideration
2. A quiet, single handle environment should be created for inducing techniques of relaxation, breathing exercise.
3. Group classes should be focused for aerobic and strengthening exercises to create interest and develop patient's confidence which they shall gain by seeing other members performing training within the same group.
4. Individuals should be advised not to hold their breath during all interventions and so, inspire from nose and exhale via mouth.

Therapeutic Interventions in treating depression:

1. Relaxation

Instructions:
Foremost, the individual should wear loose and comfortable clothing. He should be put in a quiet space with eyes closed without any interruption and distractions for at least 30 to 45 minutes. The lights should be turned low. Soft background music of natural sounds like rainwater,

Table 1. Represents the widely used different therapeutic interventions

Modes of treatment	Studies performed with therapeutic interventions
Prevention	Gillham Jane et al., 2000, Boland & Keller, 2002 Buckworth et al., 2002 and Goodwin et al., 2003
Antidepressant	Arroll et al., 2009 and AHRQ 2012
Physical exercise	Trivedi et al., 2006, Blake et al., 2009, Rethorst et al., 2009 and Wideman et al., 2012, & Rimer et al., 2012,
Psychological treatment	Cuijpers et al., 2008, Ambresin et al., 2012 and Brakemeier et al., 2012 Klein et al., 2013 Schaefert et al., 2013 Emmelkamp et al., 2014, Trevis et al., 2016
Tai chi, Qigong and yoga	Saeed et al., 2010 Jahnke et al., 2010 Lavretsky et al., 2011 Li et al., 2011 Li et al., 2012 Rimer et al., 2012 Ryan Abbott, and Helen Lavretsky, 2013 Saeed et al., 2010
Pet therapy	Brooks et al., 2016 Abigail et al., 2017 Germain et al., 2018 Melanie et al., 2019

piano and wind can help to relax the person. The phone should be either switched off or put on silent mode. The individual can either select to sit or lie on his back. If sitting on chair, the spine should be straight, with the back and thighs well supported with feet's resting on a support. If lying supine the back should be rested on the mattress while the legs should be kept straight and uncrossed to feel the stream of energy.

Indicators: There are physiological, cognitive, behavioral and emotional responses which occur during relaxation of the body. These are decrease in muscle tension, lowering of heart and respiratory rate, reduction in blood pressure, constriction of pupils, little to no visible movement of the body, followed by closed eyes with flat facial expression and relaxed jaw and hands with the palms opened.

Techniques: a. Contrast Method

The physiology of this method is that a strong contraction of muscle is followed with a phase of equal relaxation for the same muscle. This is an excellent relaxation exercise for individuals with depression. The eyes can be either open or closed, depending on the preference of the individual. This technique consist a sequence of voluntary muscle contractions (induced by one's self) performed from distal to proximal for each limb or pair of limbs which is followed by relaxation. It can be done for upper and lower limbs. The stage of contraction is usually between 6 to 8 seconds followed by relaxation for 25 to 30 seconds. It should be noted that on relaxation the patient should feel warmth in the relaxed muscle.

b. Reciprocal Method: In this technique, the "antagonist muscles" (muscles which work in opposite direction to the actual working muscles) take the patient out from the tensed posture. To induce this mode of relaxation the individual is allowed to remain in the tensed posture either in lying or sitting position. The individual is taught to recognize his tension at any given time and relieve it without unnecessarily changes in his position. The sequence followed is usually proximal to distal, and each part of the body is specified with three instructions which are, move to open the tense position of the body which is followed by no movement and finally, letting the brain to appreciate the new attained posture and making the individual to think about his new posture, (Crone 1993) .The individual should be asked to take time to feel the instinct of relaxation and not be in hurry (Payne 2005)

c. Suggestion Method: This method is used for individuals who may not perform or not willing to perform much of muscle work. In this technique the physical therapist provides a comfortable relaxing environment with a warm well-ventilated area at ease support, low light and hypnotic tones. The individual is asked to think about each part of his body in turns like to think that his limb is weighty. The suggestions are made several times until the limb furnish the appearance of relaxation, e.g. when the lower limb is rolled out. The individual can be asked to raise his limb and feel as though it is floating. He is then instructed to direct his attention to the other leg following with to each arm in turn and ultimately to the whole body. Deep breathing exercises should be practiced and by the end of the session the individual will be found to have moved in to sleep.

d. Pendulum Swinging: This form of technique is used for relaxation of the shoulder, hip, knee and lumbar spine. Herein, single or both arm and leg are swung back and forth until they feel numb which imply that their sensory receptors have accommodated to the continuous movement. A 1/2 to 1 kg weight as either a dumble or weight cuff can be used in later stages i.e. grasped in the hand or fastened to the ankle.

Breathing techniques: a. Deep breathing exercises

In this technique, the individual is asked to sit with their head and neck on the head rest and back supported on the chair. He is asked to breathe in through nose, such that the belly is filled with air which is confirmed by touching the raised stomach. Simultaneously, he is asked to breathe out through nose following which the belly should be felt lowered. This mode of inspiration and expiration should be continued till the completion of treatment session. Four to five breathe are recommended in one go following which rest is advised for half a minute and same is repeated as per the convenience of the individual, (Valenza et al., 2014).

b. breathing technique : In this technique, the individual is asked to exhale completely through mouth by making use of tongue to produce a whooshing sound. Following which the individual is asked to close the mouth slowly and inhale silently via nose with count of four. He is taught to count mentally till 7 while holding breathe and subsequently exhale from count of 8 along while producing a audible whooshing sound.

3. Aerobic techniques: Activities like cycling, skipping, slow/medium/high speed walking, jogging, spot jogging, running, swimming, stair climbing, hiking, dance and aquarobics etc. are performed as aerobic exercise in individuals with depression. The activity is selected depending on individual's age, severity and level of fitness. These exercises benefit the body by improving cardiovascular fitness, maintenance of blood pressure which decreases risk for heart disease, diabetics, bowel cancer and osteoporosis, increase level of cognition and physical fitness, (Conn et al., 2010). In return these effects help the individual to elevate his mood which

plays a vital role in increasing social integration within the society, (Wegner et al., 2014).

4. Strengthening techniques: While devising a strengthening protocol for patient with depression, the Frequency Intensity Time and Type (FITT) principle issued by American college of sports medicine and centre for disease control and prevention should be used to design and implement a safe, efficient and enjoyable session (Chen et al., 2009). Frequency=Minimum of 2-3 days per week, Intensity = Mild depression (12-15 repetitions per body part), Moderate depression (5-10 repetitions per body part).Severe depression (2-5 repetitions per body part), Time = 45 minutes to 1 hour for mild to moderate depression, 2-3 sets per muscle group in each session.

Type = Individuals unfamiliar to any type of strength training in their past should be encouraged to exercise using mechanical machines as they reduces the risk of injury. Whereas, individual familiar to any kind of strength training, should be trained in present by making use of machines or free weights. Free weights offer strength training in conjugation with balance training as free weights require and exert higher level of balance. While performing these exercises the individual should be asked not to hold their breath while lifting weights, as holding breath may lead to increase in blood pressure leading to abnormal heart rhythms. In addition, individuals suffering from any cardiac diseases should perform one to two set of exercise per session.

5. Postural awareness: In standing: Individuals should be trained towards development and maintenance of an erect posture. A chin tuck, retracted shoulder, erect trunk, buttocks protruded, hip and knee joint aligned in straight line with ankle neutral, foot and toes slightly outwards should be practiced at regular intervals during the day.

In sitting: Individuals should be trained to sit in a relaxed posture by resting the head on headrest, shoulder resting on the upper back of the chair with the buttocks and thigh completely supported on the cushioned seat and the ankle falling directly under the knee with the foot and toes placed on the floor. Postural awareness helps to improve chest expansion, reduce chances of kyphosis, scoliosis, forward head posture, flat back and postural back pain (Fujino H 2012).

6. Balance training

Individuals with depression should be trained for balance by performing activities like standing on both feet, standing on one foot, standing on balance board with both foot (Li et al., 2015), standing on balance board with one foot and backward walking etc. Any kind of unstable, rubble surface should be avoided. The training area should be with ample of free space to prevent any physical injuries. A chair, wall or railing should be placed around the individual to be used in an emergency occurring due to lack of balance.

7. Educational interventions

All educational establishments (school, college and universities) should appoint professional councellors and physical therapist on regular basis. Students studying in higher secondary, college or university level should be evaluated for depression at regular intervals using universally accepted depression questionnaires and scales. Early detection of depression will prevent future devastating effects on the individual's psychology (Hassett, et al., 2009). In addition, students detected with depression should be evaluated for their physical fitness by physical therapist. Students desiring to be voluntaries can be trained to detect depression among their peer group and family members, thus, lend a helping by spreading awareness of implications, methods and prevention of depression by organizing camps at supermarkets, malls and social clubs etc as a step towards serving the society. Students should be perfected for preparing handouts in creative ways by using local language (Butler, et al., 2006).

8. Physical therapy modalities

Transcutaneous Electrical Nerve Stimulation (TENS), Microcurrent Electrical Nerve Stimulation (MENS), Shortwave Diathermy (SWD), Microwave Diathermy (MWD), LASER, Ultrasound, Infra Red Lamp, Interferential Therapy, Vacuum therapy biofeedback and hot packs are physical therapy modalities used to reduced pain, improve circulation which enhances healing of soft tissues to increase perception of relaxation, whereas Faradic, Di dynamic, High voltage and Russian currents can be used to improve the muscle strength the body during mild, moderate or severe phase of depression (Giggins et al., 2013) .

9. Pet therapy

Along with the above therapeutic interventions, the authors would like to encourage a recent, slowly getting popular mode of treatment for cases of mild, moderate to severe depression which is referred as 'Pet therapy'. "You just need a pet" may be a dog, cat, squirrel etc. Pets help humans in many ways (Beetz et al., 2012). They help to maintain a regular schedule of individual's getting up from bed, leaving home early morning and returning back by early evening and preparing food for their pet. These acts with time help to improve and gradually increase level of physical activity (Gee 2009), fitness and mental alertness. Pet therapy also has a equal and positive effect on an individual's psychology i.e. patting or stroking the animals promotes sense of calmness which

improves psychological mindset of the individual, going for slow or brisk walk with the pet helps in developing confidence and increase social encountering, (Cardoso et al., 2011). People, who have felt positiveness with pets while suffering with depression, can solely explain the positive feeling which evolves when you have someone to love and be loved all the time around you (Brooks et al. 2016). Hence, the authors encourage researchers from all over to perform research on short and long term benefits of pet therapy in curing mild, moderate and severe depression.

CONCLUSION

The present study looked into various therapeutic interventions while writing the article. Earlier reported literatures have employed various interventions in treating adolescent, adults and geriatric populations with depression. Limited study have been performed on Pet therapy, Therefore the authors are directed to employ more time and efforts in studying new methods to treat depression.

Conflict of Interest: NIL, **Source of funding:** NIL, **Ethical clearance**: N/A

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Biotechnological Communication

Biosci. Biotech. Res. Comm. 12(2): 258-265 (2019)

BBRC Bioscience Biotechnology Research Communications

CDKN2A/2B rs10811661 gene polymorphism and sedentary life style factors, their risk association with type 2 diabetes mellitus in Indian population–A case control study

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ABSTRACT

CDKN2A/2B is a major gene in pancreatic β-cell dysfunction and regeneration related with Type-2 diabetes, Obesity, Insulin resistance and CVD. Current study analyses the consortium of *CDKN2A/2B* polymorphism in 369 Type 2 Diabetes cases and 100 controls in Indian population. The study was done by PCR-RFLP technique to study the *CDKN2A/2B* polymorphism in the study subjects. Study contemplated that CDKN2A/2B C/T genotype distribution between cases and controls were found to be significant (0.0001). Higher CDKN2A/2B T allele frequency (0.31%) in cases as compare to control (0.13%). CDKN2A/2B CT and TT had 3.16 (1.84-5.42), 5.84 (1.75-19.45) Odd ratios with 95% class intervals. HbA1C, Cholesterol, HDL and LDL were responsible for disease, and significant results were observed in biochemical parameters. On comparing biochemical parameters with sedentary life style factors, it was observed that PPG and FPI, HDL exhibited positive relation with smoking at p(0.04), (0.02) and (0.07) respectively, alcoholism with HDL at p(0.05), Non vegetarian food with Triglycerides at p(0.005),and Exercise (not doing) with PPG, HbA1C, FPI, HDL, LDL and triglycerides at p (0.02, 0.0001, 0.04, 0.0001 and 0.0003) respectively. Remaining parameters manifest no significance. The epilogue of the study contemplates affiliation of *CDKN2A/2B rs10811661* gene polymorphism with possibility of Type 2 Diabetes Mellites. HbA1C, Cholesterol, HDL and LDL increases the risk of occurrence of T2DM in subject population. Significant association between biochemical parameters with sedentary life style factor increases the danger of Type 2 Diabetes Mellites in Indian population.

KEY WORDS: TYPE 2 DIABETES MELLITES; CDKN2A/2B GENE; ALLELE FREQUENCY; GENETIC ALTERATION; POLYMORPHISM

ARTICLE INFORMATION:

Corresponding Author: amitvrm999@gmail.com
Received 5th April, 2019
Accepted after revision 25th June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/8

INTRODUCTION

In the recent past the Prevalence of Diabetes mellitus has been increasing as major widespread chronic non communicable diseases worldwide, and with urbanization altered lifestyle characterised with reduced physical activity and obesity, the pace of occurrence has escalated. ("WHO | Diabetes programme," n.d.). The International Diabetes Federation (IDF) has confirmed that the pervasiveness of diabetes will growing from 451 million in 2017 to 693 million by 2045, (Cho et al., 2018). The pervasiveness of T2DM continue to increase worldwide with growing sedentary lifestyle of people and accounts for more than 80% of diagnosed cases currently (Murea et al., 2012). While incidents of T2DM varies with age, sex and population. It is estimated that worldwide age-standardized adult diabetes is >9.2%, and confirmed that >347 million adults have diabetes, (Danaei et al., 2011). T2DM can be diagnosed by FPG, PPG, HbA1C and many clinicopathological parameters which are associated with disease. Sedentary life style induced Physical inactivity and obesity also contribute to prevalence of T2DM (Committee, 2009, Mehramiz et al., 2018). Decrease insulin supply attributable to insulin resistance causes T2D mostly induced because of genetic and environmental factors in early phases, whereas Decline in β-cell function that cause tissues to become resistant to insulin is gradual event in the later phase, (Schäfer et al., 2011). Researchers have estimated that by 2030, ~552 million individuals globally will have diabetes, with Asians becoming the most affected group, (Chan et al., 2009; Whiting et al., 2011). Genetic linkage analyses and GWAS have confirmed variants in genes that contribute toward the development of T2DM, (Bao et al., 2012, Mehramiz et al., 2018 and Cho et al., 2018).

CDKN2A/2B rs10811661 Cycline-dependent kinase inhibitor-2A/B has been reported as one of the prospect gene responsible for type 2 diabetes based on its chromosomal position (9) and its vital task in beta cell function and revival. (Voight et al., 2010) *CDKN2A/2B* in T2D impairs immune system functioning which altered the cytokine creation and leukocyte turn on in tissues such as liver, adipose tissue, pancreas, and the vascular wall. Immune cells also regulate obesity induced glucose intolerance and insulin resistance in T2D, (Hannou et al., 2015). An interrelation between SNPs adjacent to *CDKN2A/B* gene and impaired first phase glucose-induced insulin production have been confirmed instead of deficiency in glucose tolerance or insulin sensitivity, (Hart et al., 2010; Hribal et al., 2011) indicating the connection of *CDKN2A/B* in pancreatic β-cell. Candidate gene approaches have also examined variants in this locus related with various types of cancer like breast cancer, acute lymphoblastic leukaemia, ovarian cancer, glioma, malignant melanoma, pancreatic cancer, glaucoma and nasopharyngeal neoplasm, presenting that the chromosomal area harbours genes involved in a few different physiological processes, (Foulkes et al., 1997). A polymorphism on chromosome 9p (rs10811661), located 125 kb upstream of the CDKN2B and CDKN2A genes has been related with type 2 diabetes in the genome-wide association (GWA) studies, (Scott et al., 2007; Voight et al., 2010; Zeggini et al., 2007 Mehramiz et al., 2018).

In the Danish inhabitants research study, comprising 5,970 adult individuals, it was confirmed that *CDKN2A/2B* C allele showed increased level of insulin generation in reciprocation to an oral glucose load compared with individuals having the TT genotype. (Grarup et al., 2007) similar outcomes were also obtained in a research work of 5,327 non-diabetic Finnish inhabitants, (Scott et al., 2007). These genotypic relation results was replicated in several ethnic groups including Danish, Norwegian, French, Korean, Japanese and Chinese participants. (Duesing et al., 2008; Grarup et al., 2007; Hertel et al., 2008; Lee et al., 2008; Wu et al., 2008) but not confirmed in African-Americans and Pima Indians, (Lewis et al., 2008; Rong et al., 2009). Thus, to acquire supplementary outcome on the consortium of the rs10811661 polymorphism with impaired glucose tolerance and pathophysiological quantitative traits, i.e. measures of beta cell function and insulin sensitivity, biochemical parameters we have conducted this particular study on well-characterised samples of Indian population.

MATERIAL AND METHODS

The present study on "patients-healthy persons" was completed at the Jamia Millia Islamia, New Delhi, in Medical Biotechnology Laboratory, New Delhi, where total of 469 individuals were taken. Out of 469 there were 369 newly diagnosed T2DM cases (patients) and 100 non-T2DM controls (Healthy persons). A proper inclusion/exclusion criterion for T2DM cases and healthy controls population was followed. The samples were collected after the ethical clearance from various institutional ethical committees, like Jamia Millia Islamia, New Delhi, Gurukula Kangri University, Haridwar and from all sample collection sites. Patient included in the present study were screened and sample collection was done after informed consent of the concerned individuals (diabetic/non-diabetic). Patient's information was collected in standardized pretexted questionnaires and it was entered later on in database. Patients Performa for recording clinical data of each patient and patient consent was maintained throughout the course of study. Written informed assent was obtained from

every individual involved in the research work. All the clinical factors such as fasting plasma glucose, post-prandial plasma glucose, cholesterol, triglycerides, were taken care off as per approved criteria. Total 2 ml of Blood samples were taken in EDTA coated vacutainer of Type- 2 diabetic patients as well of healthy persons. The non-diabetic (healthy persons) were taken as control for the present study. (The samples were collected in the hospital by the expert medical professional according to guidelines provided by ICMR/GCP.)

DNA extraction was done using phenol chloroform method from collected blood samples. Genomic DNA was examined on 2% agarose gel to confirm and observed under UV transilluminator. DNA quantity came out to be 45-50 ng. A260/A280 ratio came around 1.8 and perfect DNA band with no smearing was resulted after agrose gel electrophoresis. Which state that the extracted DNA was of pure, without impurity and intact. Extracted DNA was then amplified to determine the genotypes of *CDKN2A/2B* by a- specific primers forward: 5'-CCGGC-CCATTTTCTTTGTCA-3' and reverse: 5'-CAAAGCGCTGG-GATCATAGG-3' using thermos cycler. PCR was imple-mented in 20µl reaction volume having1 µl DNA, 1 µl primer each forward and reverse, 10 µl Taq polymerase and 7 µl nuclease free water (ddH_2o). The PCR was con-ducted with beginning denaturation at 94°C for 3 min-utes, come next with 35 cycles of denaturation's at 94°C for 30 seconds, annealing at 61°C for 30 seconds, initial extension at 72°C for 40 seconds and final extension at 72°C for 5 minutes. The PCR product of 232 bp was seen under UV trans illuminator. RFLP (Restriction Fragment Length Polymorphism) *CDKN2A/2B* polymorphism was done by digesting 6 µl PCR product (amplified genes) with restriction enzyme BspHI 2.5 units in 10 µl reaction mixture minimum 30 minutes or maximum overnight at 37° C. The restriction enzyme recognizes the sequence. Digested DNA was examined by 3% Agarose Gel Elec-trophoresis. DNA band showed 232bp C allele: uncut T allele: 164+68 bp.

Statistical analysis: Genotype frequencies between cases and controls were calculated using Chi-square test. The values <5 were analysed by Fisher exact test. The Fisher Exact test is a test of significance conducted for more accuracy of results in 2 by 2 tables, especially when the same size is small. Allele frequency was calculated by Hardy-Weinberg Equilibrium equation. The relation between *CDKN2A/2B* genotype and risk of T2DM were drawn by calculating the odd-ratios (OR) with 95% con-fidence intervals. In the parametric and nonparametric data, p value <0.05 was considered statistically signifi-cant. Analysis of Variance -One-Way ANOVA was used to compare the clinicopathological parameters geno-type of T2DM cases and control where p value <0.05 was considered significant. Comparison of biochemical parameters with sedentary life style parameters by one-way Anova test, p value <0.05 considered significant. Statistical analyses were carried out using SPSS soft-ware, version 19.0 (IBM Corp., Chicago, Illinois, USA).

RESULTS AND DISCUSSION

Biochemical characteristic of research subjects analysed in the present study has been summarised in Table 1: Among these characteristics Hip, weight, BMI, FPG, PPG, HbA1C, BP (s, d), Cholesterol, LDL and triglycerides are significantly associated with risk of T2DM as compare of means ± sd between cases and control (Student'-t test applied).

Table 1. Comparison of biochemical parameters among T2D cases and controls.

Variables	Cases (Mean+SD)	Controls (Mean+SD)	P Value
Hip	35.44+3.22	34.87+1.88	0.09
Waist	32.0+3.77	31.65+2.50	0.37
Weight	78.26+14.82	70.74+7.79	<0.0001
BMI	28.45+5.24	24.83+2.33	<0.0001
FPG	137.3+33.63	90.22+7.10	<0.0001
PPG	211.6+70.05	135+13.02	<0.0001
HbA1C	7.14+1.10	5.74+0.53	<0.0001
FPI	10.18+8.36	8.65+0.71	0.07
BP (systolic)	144.4+18.46	106.1+10.39	<0.0001
BP (diastolic)	104.5+44.41	75.85+10.91	<0.0001
Cholesterol	245.5+15.15	152.6+18.82	<0.0001
HDL	46.68+11.35	46.21+8.69	0.69
LDL	192.3+29.12	106.4+19.92	<0.0001
Triglycerides	357.9+99.10	141.0+5.52	<0.0001

*note: data presented as means ± sd; p-value<0.05 considered significant.

The difference seen in the genotype among patients and healthy person was found significant (p<0.0001) (Table 2). It was seen that high percentage of heterozy-gous CT 148 (40.10%) and mutant TT 41 (11.11%) genotype was found in patients compared to control heterozygous CT 20 (20%) and TT 03 (3%) while lower CC 180 (48.78%) geno-type in patients compared to control homozygous CC 77 (77%) genotype. The higher allele frequency of T allele (0.31) was observed in T2DM patients compared to con-trol (0.13).

Table 2. Genotypic distribution and allele frequencies of *CDKN2A/2B* gene among T2DM patients and Healthy controls.

CDKN2A/2B	CC (%)	TT n(%)	CT n(%)	p value	Allele frequency	
					C allele	T allele
Patients (369)	180	41	148	<0.0001	0.69	0.31
Controls (100)	77	03	20		0.87	0.13

*note: data presented as n (%); p-value<0.05 considered significant. genotype frequency by chi-square test & allelic frequency calculated by hardy weinberg equation.

Odd ratio with 95% confidence intervals was drawn for each group to estimate the degree of association between CDKN2A/2B genotype and risk of T2DM in Indian patients presented in Table 3. Compared to the CC genotype, the OR 3.16 (1.84-5.42) for heterozygous and OR 5.84 (1.75-19.45) for mutant homozygous were estimated suggesting a possible dominant effect of CDKN2A/2B polymorphism on T2DM risk.

Table 3. Risk of T2DM associated with CDKN2A/2B genotype.

CDKN2A/2B Genotype	T2D patients	Healthy controls	OR (95% CI)
CC	180	77	(ref)
TT	41	03	5.84 (1.75-19.45)
CT	148	20	3.16 (1.84-5.42)

*note: data presented as n (%) association estimates by computing odd ratio (or) with 95% confidence intervals.

The risk associated with various biochemical parameters like Waist- Hip ratio, weight, BMI, FPG, PPG, HbA1C, BP (s, d), Cholesterol, LDL and triglycerides have been calculated and presented in (Table 4). Analysis results of the relation linking CDKN2A/2B rs10811661 C/T polymorphism genotypes and the biochemical parameters have also been illustrated. The *CDKN2A/2B rs10811661* "TT" genotype exerts significant effect on HbA1C, Cholesterol, HDL-C and LDL-C level in both patients and controls (*P* values = 0.02, 0.04, 0.02 and 0.03 respectively). Individuals harbouring this genotype seem to have a significantly higher HbA1C, Cholesterol, HDL-C and LDL-C as compared to carriers of the CC genotype. No significant effect of the *CDKN2A/2B* rs10811661 genotypes on the FPG, FPI, PPG and Triglycerides was observed.

A Comparison of biochemical parameters with sedentary life style factors was conducted to assess the dominance of these factors in biochemical variables, by one-way Anova Test where, p value of <0.05 was considered significant. In this regard all the Lifestyle factors like smoking, consumption of Alcohol, physical activeness was assessed individually with all the biochemical parameters to identify the dominant association of these factors along with Biochemical parameters. On comparing the habit of smoking with biochemical variables it was observed that PPG and FPI, HDL showed significant association with smoking were p value of 0.04, 0.02 and 0.07 respectively was recorded as mentioned in (table 5). It was observed that the patients who smoke have higher PPG, FPI and HDL as compared to them who don't smoke. According to the observation made these 3 parameters

Table 4. The relation between *CDKN2A/2B* genotypes and the investigated clinical parameters.

Variables	CDKN2A/2B genotypes			P Value
	CC (Mean+SD)	TT (Mean+SD)	CT (Mean+SD)	
FPG	137.6+37.37	145.3+27.23	134.7+30.07	0.19
PPG	210.7+52.84	219.0+42.65	210.5+91.50	0.77
HbA1C	7.16+1.11	7.55+1.24	7.01+1.04	0.02
FPI	9.63+1.38	9.15+1.30	10.33+10.0	0.56
Cholesterol	247.5+14.21	243.9+15.36	243.5+15.97	0.04
HDL	47.82+12.51	48.80+8.91	44.72+10.18	0.02
LDL	192.1+29.75	182.1+35.01	195.3+25.96	0.03
Triglycerides	358.6+97.18	331.9+82.41	364.3+104.9	0.17

*note- note: data presented as means ± sd; p-value<0.05 considered significant. analysis of variance (anova) - one-way anova from summary data. abbreviations- fpg- fasting plasma glucose, ppg- post prandial plasma glucose, hba1c- haemoglobin a1c test, hdl- high density lipoprotein, ldl- low density lipoprotein.

Table 5. Comparison of biochemical parameters with sedentary life style factor (Smoking) among T2Dcases and controls.

Variables	Smoking			P value
	Current (Mean+SD)	Former (Mean+SD)	Never (Mean+SD)	
FPG	139.4+37.95	135.1+48.5	135.9+27.95	0.60
PPG	221.8+95.85	218.3+65.08	203.0+39.57	0.04
HbA1C	7.22+1.16	6.87+1.12	7.11+1.05	0.35
FPI	10.58+9.86	9.18+1.17	9.96+7.49	0.02
Cholesterol	246.7+14.92	244.4+21.81	244.7+14.55	0.45
HDL	47.66+10.95	41.70+12.72	46.43+11.43	0.07
LDL	191.7+29.97	194.2+35.55	192.5+27.88	0.92
Triglycerides	351.8+93.41	359.7+111	362.5+102.3	0.66

* note: data presented as means±sd; p-value<0.05 considered significant. analysis of variance (anova) - one-way anova from summary data.

increases with nicotine consumption which comes with smoking. However, rest of the biochemical parameters showed no significant association with smoking as all of them were above the significant p value of 0.05.

On comparing (Alcoholism) as one of the factors of lifestyle with all the 8 Biochemical parameters taken amongst cases and control samples, only 1 parameter showed significant association which was HDL Cholesterol (see table 6). The consumption of Alcohol reflected significant association with HDL cholesterol, were p value of 0.05 was found. The patients with increase alcohol consumption have increased HDL-C level. Rest of the biochemical parameters showed no significant association with sedentary life style factors the p value was more than 0.05.

Similarly, on comparing Non-Vegetarian food (as a factor of Sedentary lifestyle) habits among T2Dcases and controls with Biochemical parameters it was observed that Triglycerides with significant p value of 0.05 can be associated with consumption of Non- Vegetarian food (as shown in table -7). It means that patients who consume Non-vegetarian food as major part of their Diet have increased Triglycerides level. Rest of the biochemical parameters showed no significant association with sedentary life style factors.

Similarly, when Exercise factor of the Sedentary lifestyle was taken among T2Dcases/ controls and were compared with given 8 Biochemical parameters, 5 amongst them highlighted positive significant relation because the p value of all these 5 variables were observed to be < then 0.05) as presented in table -8). It was observed that exercise is associated with PPG, HbA1C, FPI, HDL, LDL and triglycerides, with significant p value of 0.02, 0.0001, 0.04, 0.0001 and 0.0003 respectively. The patients with less physical activity or less exercise habit have increased PPG, HbA1C, FPI, HDL, LDL and triglycerides level. Rest of the biochemical parameters showed no significant association with sedentary life style factor parameters since the p vale of remaining variables were recorded more than p value of 0.05.

GWAS have provided information's on a broad scale, to see the relation of many gene variants with complex

Table 6. Comparison of biochemical parameters with sedentary life style factor (Alcoholism) among T2D cases and controls.

Variables	Alcoholism			P value
	Current (Mean+SD)	Former (Mean+SD)	Never (Mean+SD)	
FPG	135.6+29.22	142.8+20.73	138.2+37.69	0.61
PPG	215+87.59	214.+27.47	208.5+55.07	0.67
HbA1C	7.16+1.11	7.62+1.26	7.09+1.07	0.61
FPI	10.98+12.67	9.75+1.50	9.56+1.33	0.74
Cholesterol	246.0+14.0	245.2+15.80	245.1+16.04	0.85
HDL	46.87+10.74	51.76+10.48	46.08+11.84	0.05
LDL	193.6+27.19	179.5+35.38	192.3+29.94	0.16
Triglycerides	364.5+89.81	347.1+104.2	353.6+99.10	0.52

* note: data presented as means±sd; p-value<0.05 considered significant. analysis of variance (anova) - one-way anova from summary data.

Table 7. Comparison of biochemical parameters with sedentary life style factor (Non-Vegetarian food habit) among T2Dcases and controls.

Variables	Non-Vegetarian food habit			P value
	High (Mean+SD)	No (Mean+SD)	Normal (Mean+SD)	
FPG	137.1+35.04	132.3+20.07	140.2+38.28	0.18
PPG	218.7+111.0	208.0+26.04	209.3+53.51	0.47
HbA1C	7.09+1.19	7.19+1.09	7.15+1.06	0.79
FPI	9.52+1.39	9.61+1.35	9.60+1.44	0.86
Cholesterol	244.5+15.74	245.9+15.10	245.9+14.89	0.73
HDL	46.05+10.13	47.26+12.76	46.74+11.26	0.75
LDL	193.0+28.29	190.5+31.55	192.8+28.34	0.79
Triglycerides	334.9+90.79	381.5+105.1	358.6+97.82	0.005

*note: data presented as means±sd; p-value<0.05 considered significant. analysis of variance (anova) - one-way anova from summary data.

diseases like T2D and CVD.(Hannou et al., 2015) In the current research work significant difference in distribution of CDKN2A/2B genotype among T2DM cases and controls was observed. An independent association of mutant TT and heterozygous genotype CT were found to be related with increased risk of T2DM. It was found that TT and CT genotype in patients showed 05 to 2-fold increase as compare to healthy control. The CDKN2A/2B rs10811661 "TT" genotype exerts significant effect on HbA1C, Cholesterol, HDL-C and LDL-C level in both patients and controls (*P* values = 0.02, 0.04, 0.02 and 0.03 respectively). HbA_{1c} is now approved in developed countries as a diagnostic as well as monitoring test for (type 2) diabetes although the debate regarding its applicability for diagnosis still prevails (d'Emden et al., 2012; "Diagnosis and Classification of Diabetes Mellitus," 2010; "WHO | Diabetes programme," n.d.; Long et al., 2017).

HbA1c has been used as a specific indication of glycaemic control in all type of diabetes. In T2DM patients, HbA1c emergence is a direct function of the average blood glucose concentration. Utilization of HbA1c as a diagnostic test has edge, international standardization (Committee, 2009). It provides an index of glycaemia over the entire 120-day lifespan of the red blood cell, (Tahara and Shima, 1995). In our study CDKN2A/2B genotype showed significant association with increasing level of HbA1C. In CDKN2A/2B genotype CT and TT showed 7.01±1.04 and 7.01±1.04 with p value of 0.02 showing their association with T2DM because the significant p value is less than 0.05

Table 8. Comparison of biochemical parameters with sedentary life style factor (Exercise) among T2Dcases and controls.

Variables	Exercise		P value
	Yes (Mean+SD)	No (Mean+SD)	
FPG	139.3+37.50	135.4+29.46	0.27
PPG	220.1+91.67	203.5+38.34	0.02
HbA1C	6.87+0.93	7.43+1.20	<0.0001
FPI	10.60+9.06	9.23+1.24	0.04
Cholesterol	244.3+16.01	246.7+14.24	0.13
HDL	48.17+11.36	45.27+11.19	0.01
LDL	184.5+34.48	199.7+20.36	<0.0001
Triglycerides	339.2+96.97	375.8+98.04	0.0003

*note: data presented as means±sd; p-value<0.05 considered significant. analysis of variance (anova) -one-way anova from summary data.

Cholesterol is one of the major biochemical parameters for risk of T2DM and cardiovascular diseases. Individual with diabetes commonly have same level of total cholesterol levels and the 'good' (HDL) cholesterol as the general population. However, on an average the level of 'bad (LDL) cholesterol and triglycerides is more in individual with diabetes as compare to individual without diabetes.This is primarily because diabetes can upset the equilibrium between 'good' (HDL) and 'bad' (LDL) cholesterol levels in several ways("Cholesterol," n.d.). In case of CDKN2A/2B genotype the cholesterol showed significant association with p value of 0.004 which was less than the significant p value of 0.05. Although plasma LDL cholesterol level is usually normal in type 2 diabetic patients, metabolism of LDL is significantly modified.(Vergès, 2005). In case of T2DM attribute important modification of both LDL and HDL which are likely to play an major role in the occurrence of atherosclerosis (Vergès, 2009). Type 2 diabetes is related with reduction of the HDL (Visser et al., 2017). In our study the LDL and HDL show significant association with CDKN2A/2B CT and TT genotype. Genotype CT have LDL 195.3±25.96, and TT have LDL value of 182.1±35.01 and significant p value of 0.03. In HDL CDKN2A/2B CT, TT genotypes have 44.72±10.18, 48.80±8.91 and p value is 0.02 which show significant association.

Other biochemical parameters also showed significant association with type 2 diabetes a comparative analysis between T2DM cases and control in the study showed that weight, BMI, FPG, PPG and blood pressure p value (0.0001). In our study we compare biochemical parameters with sedentary life style factors such as Smoking, Alcoholism, Non vegetarian food habits and physical Exercise, it was observed that PPG and FPI, HDL showed significant association with smoking with p values of 0.04, 0.02 and 0.07 respectively, Similarly alcoholism is associated with HDL cholesterol with significant p value of 0.05 found, Non-Vegetarian food habit is associated with Triglycerides, showing significant p value of 0.005 and exercise is associated with PPG, HbA1C, FPI, HDL, LDL and triglycerides with significant p values of 0.02, 0.0001, 0.04, 0.0001 and 0.0003 respectively. The patients with less physical activity habit have increased PPG, HbA1C, FPI, HDL, LDL and triglycerides level. Rest of the biochemical parameters showed no significant association with sedentary life style factor parameters because all the remaining parameters had p value more than the significant value of 0.05. Researchers confirmed that the *CDKN2A*-rs10811661 polymorphism was found to be major constituent related with prediabetes in the model adjusted for age, sex, obesity, blood pressure, dyslipidaemia, socio-economic status, and lifestyle factors, (Binh et al., 2015). Obesity, which is one of main factor responsible for insulin resistance and dysfunction of beta cell resulted in development of prediabetes, (Kahn and Flier, 2000).

Studies suggested that CDKN2A/2B genotype is also related with CVD and these risks related with unhealthy dietary pattern. High BMI showed that obese with a TT genotype had a higher level of TG, TG/HDL ratio, compared to individuals with a normal BMI. Moreover, the presence of a TT genotype was associated with increased risk of hypercholesterolemia, insulin resistance and CVD. These effects were more pronounced in the subgroup with low physical activity and a high dietary energy intake, (Mehramiz et al., 2018) .The study came to an end that CDKN2A/2B rs10811661 gene polymorphism was found to be related with risk of T2DM and risk was connected with heterozygosity and mutant homozygosity. HbA1C, Cholesterol, HDL-C and LDL-C are possible risk factor for developing T2DM and significantly altered biochemical parameter in T2DM patients. Comparison of biochemical parameters with sedentary life style factors such as Smoking, Alcoholism, non-vegetarian food habits and physical Exercise, was conducted and it was observed that some biochemical parameters showed significant association. These compelling results of the study require further validation on larger population. Carrying out a similar study on T2DM female patients to reveal combined gender/polymorphism effect. The Study signifying the association between sedentary life style and T2M also urges patients to maintain/control BMI, HbA1c, LDL-C, triglyceride levels, body weight and change in life style, food habits, reduce the consumption of alcohol and cigarette smoking meanwhile maintaining a proper healthy life style with routine exercise regime and healthy eating habits. in order to avoid the complications associated with T2DM.

ACKNOWLEDGEMENTS

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. We gratefully acknowledge the study subjects who were part of this study.

Disclosures: All authors report no conflict.

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Biological
Communication

Biosci. Biotech. Res. Comm. 12(2): 266-274 (2019)

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Isolation and identification of bacteria associated with red palm weevil, *Rhynchophorus ferrugineus* from Hail region, northern Saudi Arabia

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ABSTRACT

The red palm weevil (RPW), *Rhynchophorus ferrugineus* (Olivier) is an obtrusive, ruinous pest of date palms, (*Phoenix dactylifera)* causing critical economic losses in the Middle East including the Kingdom of Saudi Arabia. However, relying on insecticides alone for controlling the RPW (*R. ferrugineus*) can have negative effects on human health and the environment. Natural enemies are considered as the fundamental part of the biological control, which is safe for controlling the RPW. Therefore, knowledge of the natural enemies against the RPW (*R. ferrugineus*) is an important to create techniques for the integrated pest management (IPM). The present study aimed to isolate and identify the bacterial species associated the RPW (*R. ferrugineus*) in Hail region during. Adults of the RPW were monthly collected from infested date palm farms in various sites in Hail region. Several bacterial species were isolated from the investigated RPW and the obtained sequences were edited in MEGA7 software and compared to available sequences in the Gen Bank database. The 16S rDNA sequencing showed that bacteria isolated from the investigated RPW were mostly Gram positive and belonged to *Proteus mirabilis* (33.3%), *Klebsiella pneumonia* (25%), *Serratia marcescens* (25%), *Staphylococcus sciuri* (8.3%) and *Providencia rettgeri* (8.3%). Overall, the results of this study can be utilized a baseline data for applying the biological control program of the RPW.

KEY WORDS: RHYNCHOPHORUS FERRUGINEUS, BIOLOGICAL CONTROL, INTEGRATED PEST MANAGEMENT, NATURAL ENEMIES, BACTERIA, CHEMICAL CONTROL

ARTICLE INFORMATION:

Corresponding Author: n-almansor@hotmail.com
Received 17th April, 2019
Accepted after revision 20th June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/9

INTRODUCTION

The red palm weevil *Rhynchophorus ferrugineus* (Olivier) (RPW) (Coleoptera: Curculionidae) is considered as a dangerous insect pest of date palm trees and makes huge losses to date palm's farmers. Indeed, the Food and Agriculture Organization (FAO) has recorded the RPW as a class -1 pest of date palms in the Middle East (Al-Shawaf *et al.*, 2013). This, subsequently, has encouraged to utilized several strategies to control the *R. ferrugineus* including chemicals, biotechnological frameworks utilizing semi chemicals or the improvement of the Sterile Insect Technique, and biological control (Mazza *et al.*, 2014). Although chemical control has been broadly connected for controlling the R. ferrugineus, there is creating stress over the negative impacts of insecticides application on human health and the environment (Mazza *et al.*, 2014; Asiry, 2015; Nicolopoulou-Stamati *et al.*, 2016). *R. ferrugineus* collected from different typologies of prothoracic spots have been found in Malta, Sicily and Pakistan (Bannu, Khyber Pakhtunkhwa) which shows a degree of diversity in RPW population around the globe (Ul Haq *et al.*, 2018).

Biological control can be characterized as an ecosystem service which advances the concealment of pests by their common natural enemies including their parasites, parasitoids, predators and pathogens (DeBach and Rosen, 1974; Bale *et al.*, 2008). Biological control offers ecological and monetary favorable circumstances as yield loss might be diminished without undesirable natural outcomes coming with application of insecticides (Bianchi *et al.*, 2006). The improvement of a biological control segment for a productive IPM requires the distinguishing proof of the common adversaries of the RPW and its defensive mechanisms against its regular natural enemies.There have been a few endeavors to separate pathogens from the RPW (Gindin *et al.*, 2006; Güerri-Agulló *et al.*, 2008; Salama and Abd-Elgawad, 2002; Salama *et al.*, 2004). These reviews prompted to the disclosure of a cytoplasmatic polyhedrosis infection, and a yeast isolated from the RPW's haemolymph. Be that as it may, none of these can be classified as potential biocontrol agents, mostly because their application in normal conditions is restricted (Banerjee and Dangar, 1995; Salama *et al.*, 2004). Cytoplasmic polyhedrosis virus infected RPW larva showed 80-100 % mortality with a viral dose of 80×106 (Mahmoud *et al.*, 2018). However, the accomplishment of biological control agents is frequently lacking and any control of the RPW is by all accounts.

Pathogenic entobacteria generally belong to the families Bacillaceae, Pseudomonadaceae, Enterobacteriaceae, Streptococcaceae and Micrococaceae (Tanada and Kaya, 1993). Although numerous bacteria can infect insects, just members of two genera of the order Eubacteriales, *Bacillus* and *Serratia*, have been enrolled for the control of insects (Tanada and Kaya, 1993). For the genus *Rhynchophorus*, bacteria have only been isolated from the RPW: Dangar and Banerjee (1993) found some pathogenic entobacteria belonging to *Bacillus* sp., *Serratia* sp. and the coryneform group in larvae and adults in India, while Alfazairy *et al.* (2003) and Alfazariy (2004) isolated *Bacillus thuringiensis* Berliner and *Bacillus sphaericus* Meyer and Neide from larvae and adults in Egypt. Alfazariy (2004) reported successful control of the RPW in laboratory conditions by infection with *B. thuringiensis* subspecies kurstaki isolated from larvae in Egypt. Conversely, different authors demonstrated an alternate weakness of the RPW to *B. thuringiensis* (Manachini *et al.*, 2008a,b, 2009). *Pseudomonas aeruginosa* (Schroeter) was isolated from infected larvae collected in Kerala, India (Banerjee and Dangar, 1995). Research facility examines that this bacterium was pathogenic for weevils when ingested through force-feeding or when insects were forced to wade through a suspension of bacterial cells. Mortality occurred eight days after inoculation and small larvae were more susceptible than larger larvae (Banerjee and Dangar, 1995), probably most likely because of absence of antimicrobial cuticular compounds (Mazza *et al.*, 2011a).The current study was carried out to characterize bacterial flora associated with the RPW in Hail region. Also to identify and screen bio-control bacterial strains. As a result, this can be set as a baseline data on the screening of natural enemies of the RPW in this region.

MATERIALS AND METHODS

Collection of red palm weevils (RPW) samples: Monthly, 120 adults of the red palm weevils (RPW) were collected from five different infested farms in Hail region, namely: Al Gayed, Jubbah, Helala, Horir and Gutha Sharagiya. The study was conducted during the period October 2017 to December 2018. The pheromone traps were used for the RPW's collection. In each area, 40 adults of the RPW were separately placed in plastic boxes. The collected RPW were kept in a freezer at –20 °C at the laboratory at the Department of Biology, Faculty of Science at University of Hail until used for investigating the bacteria.

Identification of microorganism flora: Isolation of bacteria: For bacterial isolation, the collected RPW were surface-sterilized with 70% ethanol for 5 min (Poinar and Thomas, 1978) and washed 3 times in sterile distilled water. The bodies of the investigated RPW were homogenized in nutrient broth using a glass tissue grinder, and the homogenate was filtered. Then, 10, 25, and 50 µL of

sample extracts was plated on nutrient agar and incubated at 30 °C for 2–3 days. The remaining mixtures were incubated at 30°C for 3–4 h to enrich the number of bacteria that have low concentration. From these mixtures, 10, 25, and 50 µL were also plated on nutrient agar and incubated at 30°C for 2–3 days. Finally, the incubated RPW suspensions were heated in a water bath at 80°C for 10 min to eliminate nonspore-forming bacteria (Ohba and Aizawa, 1986). After heat inactivation, 10, 25, and 50 µL of the heated suspensions were plated on nutrient agar and incubated at 30 °C for 2–3 days. The bacterial Isolates were distinguished according to their colony color and morphology. Pure cultures of bacterial colonies were prepared and stocked in 20% glycerol at –80°C in the Microbiology Laboratory, Department of Biology, and Faculty of Sciences at University of Hail. Bacterial cultures were identified according to their morphology, nutritional features, and biochemical and molecular characteristics.

Molecular identification of the bacterial isolates: Inoculation of isolates: The bacterial isolates were grown on 5 ml tubes contained 2 ml of Luria broth (LB). The tubes containing isolates were incubated horizontally at 37°C for overnight with shaking at 200 rpm in an incubator shaker (Lab-line Instruments, Inc.). To precipitate bacterial pellets for extraction of DNA, 2 ml of LB media was centrifuged at 5000 rpm for 5 min.

DNA Extraction using modified Dellaporta procedure: DNA was extracted using modified method of Dellaporta *et al*., (1983) as the following protocol: Twenty mg of fresh harvested mycelium or bacterial pellet were ground with pestles in a 1.5 ml tube with 500 µl of Dellaporta buffer (100 mM Tris pH 8. 50 mM ethylenediamine-tetraacetate EDTA, 500 mM NaCl, 10 mM beta mercaptoethanol) (BME). Thirty three µl of 20% sodium dodecyl sulfate (SDS, w/v) were added, and the mixture was vortexed and incubated for 10 min at 65°C.One hundred and sixty µl of 5 M potassium acetate KoAc (Sigma chemicals) were added and vortexed. The mixture was then centrifuged for 10 min at 10,000 rpm, and 450 µl of supernatant was transferred to a new tube. Four hundred and fifty µl phenol, chloroform and isoamyl-alcohol (PCI) were added with a ratio of 25:24:1 and vortex for 5 min and then centrifuged for 5 min at 10,000 rpm. 400 µl of the upper phase were then removed to a clean microcent. The supernatant was removed and the total nucleic acid was precipitated in the bottom of the tube. The pellet was washed with 70% ethanol and spun 5 min at 10,000 rpm. Then, the pellet was re-suspended in 100 µl of Double-distilled water (ddH2O).

Polymerase Chain Reaction (PCR): Amplification of the 16S rRNA gene from bacterial isolates was carried out using the universal primers; 27F 5'-AGA GTT TGA TCM TGG CTC AG-3' and 1492R 5'-TACGGYTACCTTGTTACGACTT (Weisburg *et al*., 1991), in a total 50 µl of PCR reaction. The main PCR steps were programmed as follows: denaturation at 94 °C for 45 s, annealing at 55 °C for 60 s, and extension 72 °C for 60 s. in 30 amplification cycles, followed by a final extension step at 72 °C for 10 min. PCR was conducted in the ESCO Swift Maxi Thermal Cycler with initial denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 30 sec, 52°C for 30 sec, and 72°C for 30 sec, and the final cycle is a polymerization cycle performed at 72°C for 10 min. PCR Products were purified using QIAquick® PCR Purification Kit (Cat. No. 28106) according to manufacturing procedures.Macrogen Inc., (Korea), sequenced the purified PCR products and sequencing of the purified isolates was performed in both directions using ITS5 and ITS4 primer pairs. Sequence alignments were edited by MEGA7 (Kumar *et al*., 2016).

RESULTS AND DISCUSSION

Rhynchophorus palm weevils are large insects belonging to the family Dryophthoridae, subfamily Rhynchophorinae, and tribe Rhynchophorini (Bouchard *et al*., 2011). All *Rhynchophorus* species are polyphagous and have a comparable life history and some of them are significant pests due to the serious economic damage they cause, specifically to several species of the family Arecaceae. In excess of 50 characteristic adversaries have been accounted to attack *Rhynchophorus* species, among the considered organisms, bacteria is a critical to be considered for incorporation in the integrated pest management programs. In the present study, obtained sequences were edited in MEGA7 and compared to available sequences in GenBank database. The 16S rDNA sequencing showed that bacteria isolated from the investigated RPW were mostly Gram positive and belonged to *Proteus mirabilis* (33.3%), *Klebsiella pneumonia* (25%), *Serratia marcescens* (25%), *Staphylococcus sciuri* (8.3%) and *Providencia rettgeri* (8.3%) as shown in Fig. 1 and 2.

The dominated bacteria *P. mirabilis* was found to be on the bodies of the tested dead red palm weevil (RPW)

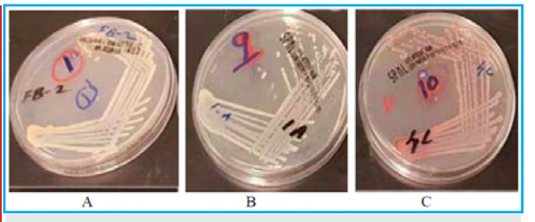

FIGURE 1. A: Proteus mirabilis: B: Klebsiella pneumonia; C: Serratia marcescens

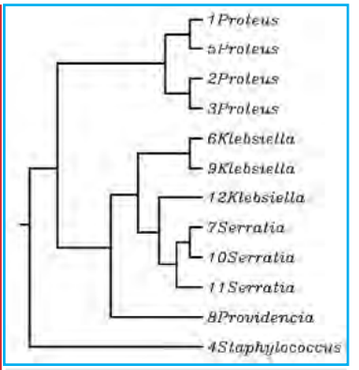

FIGURE 2. Phylogenetic analysis of 16S rDNA sequence by UPGMA software.

and it was accounted 33.33% of all bacterial isolates from RPW body. *Proteus* species are part of the Enterobacteriaceae family of gram-negative bacilli. *Proteus* organisms are embroiled as genuine causes of diseases in humans along with *Escherichia, Klebsiella, Enterobacter,* and *Serratia* species. *Proteus* species are the most regularly found in the human intestinal tract as a component of ordinary human intestinal flora, alongside *Escherichia coli* and *Klebsiella* species, of which *E. coli* is the dominating inhabitant. *Proteus* is also found in various natural environments, including long-term care facilities and medical clinics (https://emedicine.medscape.com/article/226434-overview).

The most characterizing normal for *Proteus* microscopic organisms is a swarming marvel, a multicellular differentiation procedure of short rods to extended swarmer cells. It allows population of bacteria to move on strong surface. The virulence of *Proteus* rods has been related to several factors including fimbriae, flagella, catalysts (urease - hydrolyzing urea to CO_2 and NH_3, proteases corrupting antibodies, tissue framework proteins and proteins of the supplement system), iron acqusition frameworks and poisons: hemolysins, *Proteus* poison agglutinin (Pta), as well as an endotoxin - lipopolysaccharide (LPS)

Proteus mirabilis, a Gram-negative rod-shaped bacterium most noted for its swarming motility and urease activity, frequently causes catheter-related urinary tract infections (CAUTI) that are frequently polymicrobial (Chelsie *et.al.*, 2017). *P. mirabilis* belongs to the class Gammaproteobacteria, and has long been perceived as a member of the order Enterobacteriales, family Enterobacteriaceae. However, one group recently created a reconstructed phylogenetic tree based on shared core proteins, ribosomal proteins, and four multilocus sequence analysis proteins, and has suggested that the order Enterobacteriales be renamed, putting *Proteus* within a new Morganellaceae family (Adeolu *et. al.*, 2016).

P. mirabilis can be found in a wide assortment of environments, including soil, water sources, and sewage, yet it is transcendently a commensal of the gastrointestinal tracts of humans and animals (Armbruster and Mobley, 2012). While the bacterium is fit for causing an assortment of human diseases, including those of wounds, the eye, the gastrointestinal tract, and the urinary tract, it is most noted for infections of the siphoned urinary tract, known as catheter-associated urinary tract infections (CAUTI) (Warren *et.al.* 1982; Mobley and Warren 1987; Breitenbucher 1984; Jacobsen 2008; Armbruster *et. al.*, 2016). These infections are common in long-term siphoned patients, for example, the individuals who dwell in nursing homes and chronic care facilities, and may be of particular danger to spinal cord injury patients. During infection, histological damage is brought about by cytotoxins including hemolysin and a assortment of proteases, some autotransported. The pathogenesis of infection including evaluation of individual genes or global screens for virulence or fitness factors has been evaluated in murine models of ascending UTI or CAUTI using both single-species and polymicrobial models. Global gene expression studies carried out in culture and in the murine model have revealed the remarkable metabolism of this bacterium (Chelsie *et.al.*, 2017). Vaccines, utilizing MR/P fimbria and its adhesin, MrpH, have been appeared to be strong in the murine model.

Klebsiella pneumoniae was also found in high numbers in the body of the tested RPW. It represented 25% of the total numbers of isolated bacteria. *Klebsiella* species are Gram-negative coliform bacteria that can cause mastitis, prompting noteworthy economic losses on dairy farms. *K. oxytoca* and *K. pneumoniae* are the species that are responsible for causing mastitis. Typical *K. pneumoniae* is an opportunistic pathogen, which for the most part influences those with debilitated immune systems and will in general reason for nosocomial infections. A subset of hypervirulent *K. pneumoniae* serotypes with elevated production of capsule polysaccharide can affect influence previously healthy persons and cause hazardous community acquired infections, such as pyogenic liver abscess, meningitis, necrotizing fasciitis, endo- phthalmitis and severe pneumonia. *K. pneumoniae* uses an assortment of virulence factors, particularly capsule polysaccharide, lipopolysaccharide, fimbriae, outer external membrane proteins and determinants for iron procurement and nitrogen source usage, for survival and immune avoidance during infection (Bei *et. al.*, 2014).

K. pneumoniae, a member of the family Enterobacteriaceae, is a rod-shaped, Gram-negative, lactose-fermenting *bacillus* with an unmistakable case. Normal *K.*

pneumoniae is an opportunistic pathogen that is widely found in the mouth, skin and intestines, as well as in hospital settings and medical devices. Opportunistic *K. pneumoniae* mostly influences those with compromised immune systems or who are weakened by other infections. Colonization of the GI tract by opportunistic K. pneumoniae generally occurs prior to the development of nosocomial infections, and *K. pneumoniae* colonization can be additionally found in the urinary tract, respiratory tract and blood (Podschun 1998). *K. pneumoniae* biofilms that structure on therapeutic gadgets (e.g., catheters and endotracheal tubes) provide a significant source of infection in catheterized patients (Schroll et. al., 2010). Nosocomial infections brought about by *K. pneumoniae* tends to be chronic due to the two following major reasons: *K. pneumoniae* biofilms formed in vivo protect the pathogen from attacks of the host immune responses and antibiotics (Jagnow and Clegg 2003); and nosocomial isolates of *K. pneumoniae* often display multidrug-resistance phenotypes that are commonly caused by the presence of extended-spectrum β-lactamases or carbapenemases, making it hard to choose appropriate antibiotics for treatment (Paterson et. al., 2004; Munoz-Price et. al. 2013).

Serratia marcescens was also found in high numbers in the body of tested RPW. It represented 25% of the total numbers of isolates. Analysis by the 16S rDNA sequences allotted the selected bacteria to the genus Serratia (family Enterobacteriaceae), with the most noteworthy similitude found for the species *Marcescens*. The genus *Serratia* includes, at least, 10 species (Grimont and Grimont 2006). Serratia is a bacterium found in the family Enterobacteriaceae that can cause opportunistic infections even though it is usually a weak pathogen. Analysis by the 16S rDNA sequences, classified our isolates into the species *marcescens*. The phylogenetic trees dependent on the 16S rDNA and the linked housekeeping gene sequences arranged our strains within the *S. marcescens* cluster (Figure -2).

This cluster was plainly particular from those of the other known red pigment-producing *Serratia* species (Grimont and Grimont 2006; de Araujo *et al*. 2010). *S. marcescens* has likewise been accounted for, at least for some red pigment-producing strains, to display an antimicrobial activity against some gram-positive and gram-negative bacteria (Ibrahim *et al*. 2014; Lapenda *et al*. 2015). I then verified if this was also the case for my isolates. *S. marcescens* is available as extracellular symbiont in various formative phases of the RPW. Additionally, the antimicrobial activity exhibited versus *Bacillus* spp., *Paenibacillus* spp., and *Lysinibacillus* spp., reported as insect pathogens and potential candidates for biocontrol agents, could attribute for *S. marcescens* a potential protective role (Maria Scrascia *et. al*. 2016).

Serratia marcescens is among the most widely recognized irresistible agents in infections related with *Serratia*. They cause infections with noteworthy mortality and morbidity in infants (Edmond *et. al*., 1999; Roy *et. al*., 1997; Ania et. al., 2008; Bayramoglu et. al., 2011). Moreover, *S. marcescens* is a significant irresistible agent that causes hospital-acquired respiratory and urinary tract infections in neonatal-adult intensive care unit and immunodeficient patients. The diffuse nearness of *S. marcescens* inside the infested palms highlighted the capacity of this bacterium to replicate and spread along the palm tissue.

The RPW is a singular insect with no or restricted contact between adult and developing individuals. Solitary insects, moreover to their own safeguards, can make utilize of symbionts to better protect themselves, offspring, or nutritional *assets* against pathogens, predateors, parasites, or parasitoids (Kellner 2002; Kaltenpoth *et al*. 2005; Brownlie and Johnson 2009*)*. This protection can be interceded by various mechanisms, which include the production of antimicrobials. The cooperation among microorganisms and hosts have dependably been the object of escalated studies. Specifically, studies on the mutualistic relationships among bacteria and insects have dynamically uncovered the pertinent pretended by the formers on the life cycle of their hosts. The identification of red pigment-producing *S. marcescens* as extracellular symbiont of the RPW will add to the knowledge on a mutualistic connection among bacteria and the RPW (Maria Scrascia *et. al*. 2016). It has been reported that populations of certain weevils are sometimes definitely decreased by naturally occurring pathogens, for the most part under conditions for example, delayed high humidity or dense pest populations. Banerjee and Dangar (1995) isolated the bacterium *Pseudomonas aeruginosa* from naturally infected adults of *R. ferrugineus*. The bacterium is observed to be pathogenic to adults forced to feed on a suspension of bacterial cells and mortality happened 8 days after ingestion.

Staphylococcus sciuri was identified in 8.3% of the bacterial isolates from the tested RPW body. *S. sciuri* belongs to the group of oxidase-positive, novobiocin-resistant coagulase negative *staphylococci* (CoNS) (Stepanovic *et. al.,* 2006). This bacterium is widespread in nature and can be isolated from an assortment of pets, wild and domestic animals, insects, environment (soil, sand, water, air samples, etc.), and foods (Stepanovic *et. al*., 2006, Stepanovic *et. al.* 2005).

It has additionally been recuperated from the hospital environment (Dakic *et. al*., 2005) and in spite of the fact that *S. sciuri* is just occasionally isolated from humans, it has been associated with a number of serious infections such as septicemia, endocarditis, peritonitis, pelvic inflammatory disease, urinary tract infections and

FIGURE 3. A: Staphylococcus sciuri ; B: Providencia rettger

wound infections (Severin *et. al*., 2010; Stepanovic *et. al*., 2005). However, there is a little information regarding the pathogenicity of *S. sciuri*. Members of the *S. sciuri* group are widely distributed in nature, and they can be isolated from a variety of animals and the products of animal origin (Takeuchi *et. al*., Gianneechini *et. al*., 2002; Waage *et. al*., 1999) as well as from human (Stepanovic *et. al*., 2001; Couto *et. al*., 2000), but most of them are a pathogenic to animals. It has been reported that numerous bacteria pathogens to insects are dynamic makers of secondary metabolites harmful to insects or other organisms that can be utilized as novel particles for controlling both plant pathogens and pests (Bode, 2009).

Providencia represents a genus of urease producing, gram negative bacilli which although rare, are very omnipresent in the environment. *Providencia* species intently take after *Proteus* and *Morganella* species. They are often isolated from wounds, respiratory tract and urinary tract (*P. alcalifaciens*, *P. rettgeri* and *P. stuartii*), stool of humans (*P. alcalifaciens*), poultry, faeces from reptiles (*P. rettgeri*), throat, perineum, axilla and blood of humans (O'Hara *et. al*., 2000). A report from Nepal in 2014, a cluster of surgical infections with regards to the isolation of *P. rettgeri*, demonstrated the presence and significance of this organism in the Asia-Pacific region (Tada *et al*., 2014). *P. rettgeri* has been involved in the etiology of gastrointestinal sickness in 1986, traveler's diarrhea in 2004, and ocular infection in 2006 (Muller 1986; Yoh *et. al*., 2005; Koreishi *et al*., 2006). *P. rettgeri* has additionally been involved as a causative agent of "purple bag syndrome", where the enzymatic activity gives rise to a purple tinged urine (Peters *et. al*., 2011).

There are not many records about the occurrence of natural enemies of *R. ferrugineus*, which may be ascribed to the mysterious living space of the eggs, larvae and pupae, which protect them from such common adversaries. Ordinarily, the natural enemies do not play an important part in controlling of *R. ferrugineus*. Reginald (1973) suggested that natural enemies don't have a significant impact in controlling world's worst pest of palm trees, *R. ferrugineus* (RPW) and few studies have been conducted on natural enemies of *Rhynchophorus* (Murphy and Briscoe, 1999; Faleiro, 2006a,b). In this study, bacteria associated with the red palm weevils have been investigated by considering their pitfalls and potentialities in order to pinpoint management techniques to be considered in the development and reconciliation of biological control procedures.

CONCLUSION

The study demonstrated the many pathogenic bacteria were associated with the red palm weevil, *Rhynchophorus ferrugineus* Olivier adults, however, the pathogenicity of these bacteria could be attributed to the production of secondary metabolites harmful to the RPW. The extraction and recognizable proof of secondary metabolites delivered by the entomopathogenic bacteria isolated in this study as well as the in vivo activity of bacterial cells against *R. ferrugineus* require further investigation.

ACKNOWLEDGEMENTS

I am very grateful to the Deanship of Scientific Research (DSR) in the University of Hail who funded this research project (Number: BA-1511).

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Technological Communication

Biosci. Biotech. Res. Comm. 12(2): 275-282 (2019)

BBRC Bioscience Biotechnology Research Communications

Semantic and sentiment analysis for Arabic texts using intelligent model

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ABSTRACT

This paper presents an intelligent model to analyze, understand and classify Arabic tweets. The proposed model includes four main phases; preprocessing, feature extraction, language model, and classification model phases. In the preprocessing phase, the corpora and the stop words will be employed. The language model includes morphological, lexical, syntax, and semantic analysis. Moreover, stem, root extraction and number indication will be involved. Consequently, we have different features that represent the analyzed Arabic tweets (meanings, word order, syntactic features, number features ...). Therefore, the classification phase is used to classify Arabic tweets model. The proposed solution uses tweets corpora written in Arabic, so the generated dictionary/lexicon has been made of Arabic words with their meaning. After getting the content data from the corpora, the language model analyzes and understands the content and stores it into deep structure or internal representation. Therefore, feature extraction extracts tweets features, and classification model classifies the new tweets. This study uses linguistic preprocessing tasks and similarity functions to outperform Arabic tweets clustering. Consequently, machine learning will generate the result of the analyzed tweets.

KEY WORDS: ARABIC TWEETS, MACHINE LEARNING, CORPUS, SENTIMENT ANALYSIS

ARTICLE INFORMATION:

Corresponding Author: Hassanin M. Al-Barhamtoshy
Received 5th April, 2019
Accepted after revision 20th June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/10

INTRODUCTION

Arabic is the main language in the Middle East; therefore, it is the language of a large number of posted messages in social media applications. Arabic is the most growing language over the web. Millions of messages and followers in Arabic are posted daily in social media. However, Arabic still suffers deficiency to analyze and extract valuable knowledge. Sometimes, dialectal and modern standard Arabic may be used in different Arabic countries. Recently, short text messages in social media, such as Facebook posts as well as Twitter tweets have been increasing. Therefore, opinions analysis, subject clustering, and messages classification are used to extract valuable knowledge such as sentiments, products, services and different events. Many researches handle tweets sentiment analysis, (Abuaiadah, et al., 2017) (El-Nagga, et al., 2017) (Nsouli, et al., 2018) (Samdi & Qawasmeh, 2018) (Badarneh, et al., 2018).

Preprocessing tasks such as rule-based stemming and light stemming are optimized to be used in clustering. Many classification and clustering algorithms have been used to retrieve information from applications. Therefore, natural language processing (NLP) has been used to support name entities, question answering, and sentiment analysis (El-Nagga, et al., 2017). In addition, hybrid approach between modern standard Arabic and dialectal Arabic (e.g. Egyptian dialectal) should be needed for sentiment analysis.

Real time traffic events and road conditions with congestion attentions based on twitter data analysis, is one of the hot applications that support intelligent transportation system (ITS) (Nsouli, et al., 2018). The 'ITS' helps drivers to know shortest roads and avoid cars' accidents based on (Abuaiadah, et al., 2017) traffic tweets post analysis.

A supervised machine learning method is announced to extract events for Arabic tweets. The proposed method in (Samdi & Qawasmeh, 2018) based on triggering event, time event and type of event. An Arabic tweets dataset is built and annotated by two human experts, in case of emotional analysis (Badarneh, et al., 2018); (Al-A'abed & Al-Ayyoub, 2016); (Hmeidi, et al., 2016). Emotional analysis focuses on emotion such as (joy, fear, happiness, sadness, surprise, anger, etc.). Also, lexicon-based approach is used to analyze Arabic children stories with their emotional expressions (El Gohary, et al., 2013). Accordingly, natural language processing (NLP), morphological, syntactic and semantic tasks with stemming task will be used in sentiment analysis.

The present paper has been divided into following. Section 2 introduces literature review with related works for sentiment analysis. Section 3 explains Arabic text analysis framework and describes the different design stages of all models. The classification model with machine model algorithms has been illustrated in section 4. Section 5 evaluates output results with accuracy indicators. Conclusion and future work is presented in section 6.

Sentiment and opinion mining of tweets are considered to be more interesting than documents mining. In addition, this mining is ambiguous and vague (Abuaiadah, et al., 2017); (Efron, 2011).Other challenging points to work with the sentiment analysis, opinion mining and the corresponding needed datasets are mentioned in many literatures (Oraby, et al., 2013). Therefore, it is significant to evaluate messages tweets on social networks as indicators of people opinions. An assistant opinion model with messages orientations are presented in (Nabil, et al., 2015); (Ibrahim, et al., 2015) to analyze and classify tweets in social media and documents analysis.

An improvement of aspect sentiment analysis has been presented in (Do, et al., 2019). Aspect extraction and tweets classifications of messages reviews for products are two objectives of this research. Therefore, machine learning approach with syntactic and semantic messages features are achieved.Word co-occurrence networks for microblogs have been presented to analyze and uncover hidden parameters (Garg & Kumar, 2018). Evaluation and experimental results have been done based on FSD dataset.

Ismail et al presented their work to analyze Arabic tweets dialected with Sudanese people to observe their opinions (Ismail, et al., 2018). Four classifiers (NB, SVM, K-Nearest and Multinomial logistic regression) were used in training with dataset includes 4712 tweets. The SVM classifier gave and revealed highest score with 72%. Alayba et al mentioned that the use of deep neural networks gauge to classify and analyze sentiment analysis and natural language processing applications (Alayba, et al., 2018). Therefore, Word2Vec model is described; and implemented by using a large Arabic corpus from ten newspapers. They reported that they obtained good accuracy of classification. Alharbi et al work introduced their paper to identify activities that led to Saudi citizens' happiness. Entertainment events, Saudi cities, and happiness activities are used events to measure the happiness base on Twitter in Saudi Arabia (Alharbi, et al., 2018).

In Arabic countries, we have many dialectical languages, rather than "modern standard Arabic (MSA)". In Saudi Arabia, six major dialectical regions exist (e.g. Hejazi, Najdi) (Alwakid, et al., 2017). Table 1 includes some examples to show differences between MSA and Saudi dialectical (Hejazi, and Najdi).

Some troubles and difficulties such as understanding the concept and the meaning of dialectal language and related definitions need additional elaboration. The idea

Table 1. Some Examples to Illustrates Differences Between MAS and Arabic Dialected Words (Alwakid, et al., 2017)

English	Modern Standard Arabic	Dialected in Saudi	
		Hejazi	Najdi
Window	نافذة	طاقه	شباك
Leave it	اتركها/دعها	سيبها	خلها
What	ماذا	ايش	وش

behind that is lexical units with similar meanings should appear in similar context. Therefore, official repository for semantic information for emotions' content and sentiment orientation will be involved.

Arabic Text Analysis Framework Materials

The natural language processing (NLP) operations will be employed to understand the Arabic text tweets. Then text analysis step will take place. Different NLP operations will be explored in detail such as preprocessing with standard linguistic notation. Therefore, the preprocessing phase includes tokenization, tagging, chunking, stemming and lemmatization. Based on these operations, we obtain words, phrases and sentences or any other tokens. Text cleaning is also needed to increase the accuracy of the classifiers (e.g. normalizing text). Other important aspects should be involved such as syntactic structure and POS, parsing and grammars. The architecture of the proposed framework is illustrated in Figure 1.

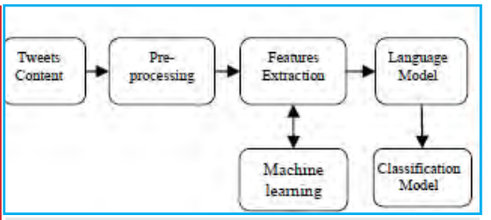

FIGURE 1. Evaluation method phases and steps

Many preprocessing tasks and related models are needed to analyze messages in the following description. The pre-processing phase will be described in the following algorithm.

Step 1. Given dataset as stream of tweets
Step 2. Splitting each tweet alone (Splitting algorithm)
Step 3. Clean each tweet from

HTTP link, and URLs for each tweet using regular expressing algorithm.

Non-Arabic words
Unnecessary and special characters
Diacritics and punctuation marks
Repetition of characters
Using Cleaning algorithm
Step 4. Tokenize each tweet by split algorithm.
Step 5. Apply Stemming process
Step 6. Apply the stop word algorithm.
Step 7. Return by the tweet

A. Language model

The objective of language model is to analyze and understand the content text in order to extract relevant information and create internal representation of the analyzed text. This internal representation is very important to understand the context meaning of the text. Consequently, the task of the language model includes tokenizer, morphological, syntactic and semantic analysis phases. Therefore, the language model uses a well-defined dictionary, lexicon and grammatical rules. The output results of language model is used to achieve the internal meaning (internal representation).

B. Tweets Corpus Tokenization Phase

This task splits Arabic tweets (text corpus) into pieces called tokens. The splitting task of tweets sentences is performed by using specific delimiters such as (".", ",", ";", and "\n"). Therefore, many of Arabic tweets are needed to create our corpus to work with the natural language to work with the Arabic natural language toolkit. Next, tokenizer task tokenizes the Arabic text stream into separate words (tokens) using the pre-trained Arabic tokenizer and the NLTK interface.

The first step includes two sub-steps: sentence/phrase splitting, tokens (words) and word derivations with affixes processing. Any Arabic text content will be segmented into separate sentences/ phrases (chunks). After that, each sentence/phrase will be segmented into separate words (tokens). So, the morphological analyzer takes place to analyze and make derivations of the current word and remove affixes from it, and therefore, find its root. The affixes can be processed to find out additional parameters or indictors to support the word part of speech (verb, noun, character, etc.), gender, tense, number (singular or plural). Details of the preprocessing phase and the details of tokenizer module are shown in Figure 2.

C. Words Tokenization

This phase receives its input from tweets stream (previous task) and splits the segmented sentence into their component list of words (tokens). This process is important to clean and normalize the generated words (tokens) and extract root for each individual word. Any proposed word tokenizer includes the following steps:

Step 1. While there is a stream for tweets text Do
Step 1.1. Separate and split the input text stream out periods that exist at the end of each tweet.

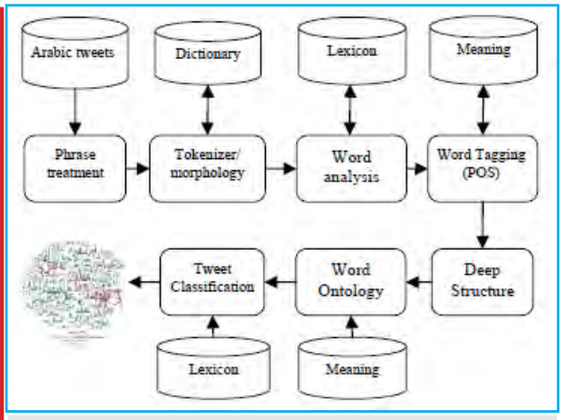

FIGURE 2. Arabic Tweets Sentiment Analysis

Step 1.2. Separate and split the sentence stream when find out any delimiters followed by white spaces.
Step 1.3. Split words using standard root using contractions method.
Step 1.4. Employ the word tagging (POS) for the whole tweet.
Step 4. Stop.

This task will be applied before and after cleaning task. The root and/or stem will be recognized with different lexical indicators.

Next section describes the normalization tasks to get clean textual data that understand its meaning. The only thing we need is to maximize the Arabic tweets data to be very large. However, depending on how we plan to use our model, we need to be more or less satisfied with the quality of the dictionary and lexicon we use. Whenever there is doubt, the general rule is the more data we have, the better the case is. Moreover, depending on the corpus size (tweets content), training can take several hours or even days, but fortunately, and we can store the analyzed data and extracted features on a storage disk. Thus we do not have to perform the analyzed tasks of model training every time we use it.

D. Normalize Tweets

As illustrated in (Alwakid, et al., 2017), many challenges have been encountered to analyze Arabic tweets due to unstructured written language: orthographic errors, dialected words, ironic-sentences, contractions, idiomatic terminologies/ expressions, or abbreviations. Moreover, repetition of Arabic characters needs to be normalized (unified). Therefore, normalization is used to change some of special characters into one form (shape). Sometimes, Arabic letters (آ , أ, إ) can be changed into one form Arabic letter (ا), see table 2.

This process includes series of steps to clean, tokenize, standardize and wrangle tweets stream into a suitable

Table 2. Some Examples to Illustrates Differences Between MAS and Arabic Dialected Words

Letter process	Normalized letters	Examples	
ا أ إ ء آ	ا	إذا => اذا	إذا => اذا
ه ة	ه	جميلة => جميله	حلوة => حلوه
ى ي	ى	جري => جرى	علي => على

form that could be processed by NLP various analytic methods.

Clean Tweets

Sometimes many of extraneous and unnecessary characters and tokens are not needed or not required, such as symbol tags and repeated data. Therefore, such unnecessary tags and repeated data can be removed. Removing unnecessary and special characters will be done before and after cleaning task.

Contraction Tweets

This task is important to avoid and recover the standard text of Arabic. Some contraction types can be classified into normal (auxiliary verb), negated, and colloquial. According to the Arabic tweets nature, the colloquial contraction with an acronyms or short end description (abbreviated), such as "د" Means as "دكتور" and "أد" means "أستاذ دكتور" are two type of such contractions. Other types such as euphemism taboo word (restricted), vulgar and accepted will be discussed in other literatures.

Stem

Morpheme is the smallest unit in morphological analysis, it consists of stem and affixes. The stem is known as a base form of a word, and we can generate new words by attaching affixes to this base form. Therefore, the stem can be extracted from that inflection/derivation; a process known as stemming.

Al-Barhamtoshy et al. (Al-Barhamtoshy, et al., 2007) remarked that Arabic is a derivational and morpho-

Table 3. Some examples to explain different surface forms

Arabic Word	Forms	English meaning
ذهب	ذهب ذُهِب أذهب أذهب؟	Gold Painted Go Is this gold?
حب	حب حُب أحب تحب يحب ...	Grain Love I love You love He loves ...

logical language. The reason that allows several different patterns or surface forms or stem for single words. Table 3 describes examples of some cases.

Lemmatization

Lemmatization is similar to stemming after removing word affixes. It is the task of finding base form. Sometimes this base form needs to make extra inflection or derivational and lexicographical process to find correct word in the dictionary (root stem).

Ontology Phase

We are now moving from corpus data (unstructured data that include tweets' messages) to deep structure or internal representation (semi structured data). If we know the tweets sender name, we also know in which country the sender lives; sender write tweets that are published in specific dates and written in a particular language, etc.

There is a whole series of information to be drawn from this tweets story - this is the goal of the ontology. In addition, phonology helps us understand missing data. If the NLP analyst has recognized the sender and title, what has not been recognized? It seems that the tweet is published in group domain. So let's look for history - it's there. Moreover, it seems that the language is also involved - we can find it too.

The classification process is based on ontology within the Interlingua language model approach. The syntactic/semantic generation module with a very limited domain of classification (forecast ontology could support classification). Therefore, the ontological representation determines which route (meaning) to track and so, classify the words correctly.

Classification of Model Architecture

Tweets Dataset

Arabic twitter can be used to create tweets dataset (dictionary and lexicon) of the proposed solution with classification model. Table 4 illustrates some examples that are selected from the created corpus. Some of this dataset is constructed and released, annotated for morphological and syntactic analysis, and others are used in clustering (Nabil, et al., 2015).

Many tweets representing judgement directions can be assigned to several categories of sentimentality. Primarily, these tweets are used to represent the tweets corpus. Tweets classification can be classified into two types: content-based and request-based classification.

To automate and create "tweets classification system", we will classify tweets corpus to domain categories or classes.

Usually, supervised and unsupervised machine learning algorithms are relevant to classify such input tweets. In addition, reinforcement and semi-supervised learning may be used.

To describe the classification process of tweets in scientific notation. The text tweets (TT) is a set of combined texts and labels. TT = { (T_1, c_1), (T_2, $c_{2)}$ ... ,(T_n, c_n)} where, T_1, T_2,..., T_n, are a list of tweets, and their paired labels are classes: c_1, c_2, ... , $c_{n.}$.

Table 4. Tweets Dataset Description

Dataset Title	Original Content	Words/Tokens	Size
Arabic-Violence-Twitter	Voilence Keywords	Words	237
	Emotion Icons	Icons	800
	Stop words	Words	126
Arabic Sentiment Tweets Dataset	Train.txt	Tweets	2002
	Test.txt	Tweets	218
TripAdvisor.com	Attraction ATT.csv	Tweets (2154)	893 KB
Qaym.com	Hotel HTL.csv	Tweets (15572)	14.5 MB
Elcinemas.com	Movie MOV.csv	Tweets (1524)	5.11 MB
Souq.com	Product PROD.csv	Tweets (4272)	515 KB
TripAdvisor and Qaym	Resturant RES.csv	Tweets (10970)	4.24 MB
MPQA-Ar	Training (CSV) Testing (CSV)	Tokens	20430823153
Astd-artwitter	Training (CSV) Testing (CSV)	Tokens	42076 4519
LABR-book-reviews	Training (CSV) Testing (CSV)	Tokens	937765 99947

Assuming that the learning algorithm L is trained with the training dataset TT, the classifier φ such that F(TT)=φ is used. The workflow of the proposed automated tweets classification system is illustrated in Figure 3.

Usually, the dataset is divided into two datasets, training and testing datasets. The preprocessing phase and the features extraction phase are overlapped to be used in both training and testing. In the training phase, each tweet has its own equivalent type (category or class) that was labeled before. The cleaning tweets will be forwarded to the feature extraction phase to extract significant features (numeric arrays or vectors).

These significant features (vectors) are feeding with the corresponding related labels to the machine learning algorithm to learn various tweets patterns related to each category and combine classification model. This gained knowledge will be used to predict categories for new tweets. Once we have the classification model (working model), we can test such model using accuracy metrics.

Feature Extraction (Feature Engineering)

Features are measurable properties for every data element in a dataset. The feature extraction (engineering) is the process to transform the input stream of dataset into a set of measurable value (numerical values). These features can be extracted using machine learning algorithm in order to differentiate and recognize the tweet's dataset.

"Vector Space Model" or "Term Vector Model" is used as mathematical model to represent tweets text as numeric vector. We have a tweet T in a tweet vector space VS. The dimension of each tweet is the number of distinct words for all tweets in the vector space.

$$VS = \{W_1, W_2, ..., W_n\} \quad (1)$$

Where n represents distinct words in the whole tweets. We can represent tweet (T) in such vector space by:

$$T = \{W_{T1}, W_{T2}, ..., W_{Tn}\} \quad (2)$$

Where W_{Tn} describes the weight of word (n) in tweet (T). This weight represents frequency (or average frequency) as numeric value, or term frequency (TF) weight.

Evaluation of The Proposed Model

To evaluate the proposed work (Arabic sentiment analysis) through word embedding, precision, recall, accuracy and F_1-score of tweets, classification will be used. Also, confusion matrix with detailed of the classification result will be used for performance measurement. Table 5 describes the analyzed results of our dataset. These analyzed data include the tokenized training dataset, and tokenized testing dataset. Then the vectorization

Table 5. Analysis Results of Tokenization and Vectorization

Tweets Dataset	Method Approach	Number of Tokens	
		Training	Testing
Mpqa-ar	Tokenization	204561	22203
	Vectorization	8996	1000
astd-artwitter-ar	Tokenization	41420	5175
	Vectorization	3187	355
LABR-book	Tokenization	930364	107348
	Vectorization	14803	1645
Products	Tokenization	48859	5894
	Vectorization	3841	427
Restaurant	Tokenization	325170	37352
	Vectorization	7527	837

Table 6. (a) Mpqa-ar Dataset Evaluation Results

Classification Approach	Average	Prec.	Recall	F1
Random-Forest	74.69	75.00	65.90	70.16
SGD-Classifier	76.49	76.86	68.42	72.40
Linear-SVC	74.54	76.46	69.11	72.60
Nu-SVC	74.74	71.17	72.31	71.74
Logistic-Regression	76.79	76.31	70.02	73.03
Gaussian-NB	69.95	72.62	55.84	63.13

Table 6. (b) Astd-arTwitter Dataset Evaluation Results

Classification Approach	Average	Prec.	Recall	F1
Random-Forest	74.69	85.80	73.23	79.02
SGD-Classifier	82.71	87.03	81.31	84.07
Linear-SVC	80.24	86.78	76.26	81.18
Nu-SVC	83.29	87.98	81.31	84.51
Logistic-Regression	81.94	88.95	77.27	82.70
Gaussian-NB	74.01	80.11	71.21	75.40

Table 6. (c) Astd-artwitter Dataset Evaluation Results

Classification Approach	Average	Prec.	Recall	F1
Random-Forest	78.29	85.80	73.23	79.02
SGD-Classifier	82.71	87.03	81.31	84.07
Linear-SVC	80.24	86.78	76.26	81.18
Nu-SVC	83.29	87.98	81.31	84.51
Logistic-Regression	81.94	88.95	77.27	82.70
Gaussian-NB	74.01	80.11	71.21	75.40

Table 6. (d) Restaurant Dataset Evaluation Results				
Classification Approach	**Previous Results**	**Prec.**	**Recall**	**F1**
Random-Forest	60.31	78.27	94.52	85.63
SGD-Classifier	65.48	80.36	92.91	86.18
Linear-SVC	71.92	84.16	89.86	86.92
Nu-SVC	69.08	82.09	92.27	86.88
Logistic-Regression	73.27	85.12	89.37	87.20
Gaussian-NB	51.30	86.78	41.22	55.90

Table 6. (e) Products Dataset Evaluation Results				
Classification Approach	**Previous Results**	**Prec.**	**Recall**	**F1**
Random-Forest	63.80	76.53	95.35	84.91
SGD-Classifier	75.48	82.13	94.68	87.96
Linear-SVC	76.92	83.99	92.36	87.98
Nu-SVC	71.08	79.89	95.02	86.80
Logistic-Regression	76.27	83.53	92.69	87.87
Gaussian-NB	63.30	88.27	57.48	69.62

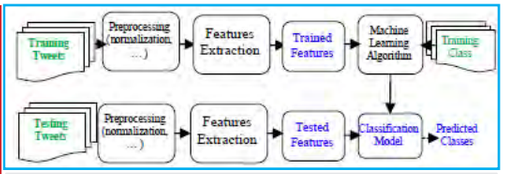

FIGURE 3. Architecture of Automated Tweets Classification System

process is employed with vectorized training tokens with 14803, and vectorized testing tokens with 1645.

We used six classifiers, *Random-Forest*, *Stochastic Gradient Descent* (*SGD*), *Support Vector Machine* (*Linear-SVC)*, *Nu-SVC*, *Logistic-Regression*, and *Gaussian-NB*. Classification results of these methods are demonstrated in table 6.

Figure 4 illustrates the receiver operating characteristic (ROC) as a relation between sensitivity and specificity (true positive and false positive rates) for all cut-off points. So, if we have true positive 0.85, we have a probability of 0.15 of being wrong.

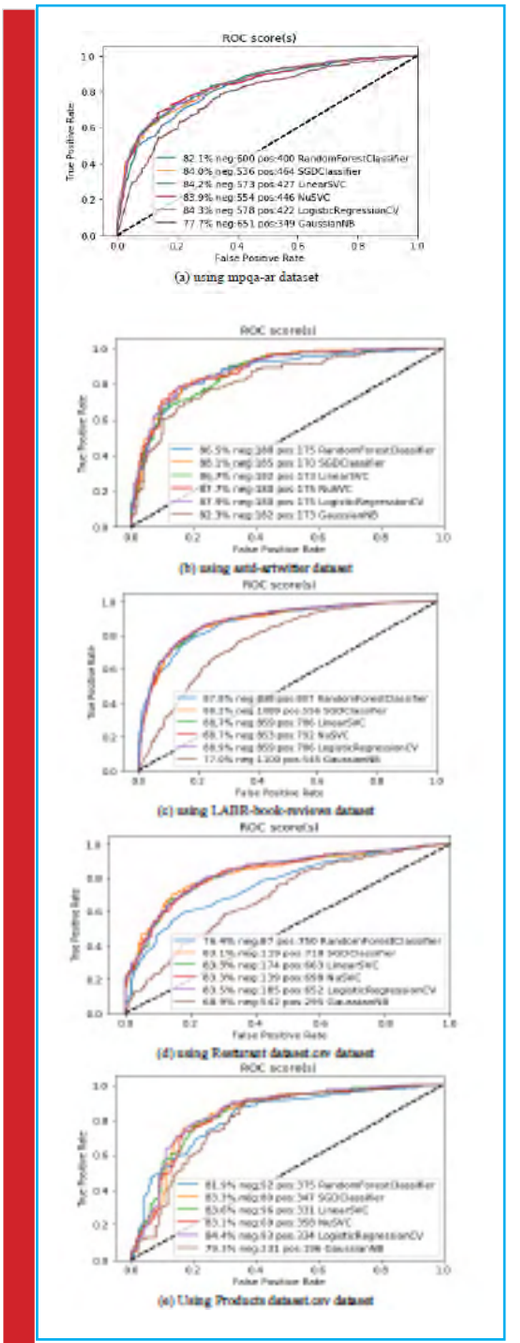

FIGURE 4. Realtion between sensitivity and specificity

CONCLUSION

In this paper, we have presented a proposed model to analyze and classify Arabic tweets. The real application domain includes many of dataset in different domains. The proposed solution is tested with five corpora for Arabic tweets. The proposed model has been tested according to different six classifiers; the output results indicate that our model increased the accuracy of the sentiment classifications tasks in all the used datasets based on the language model.

Some difficulties such as understanding the concept and the meaning of dialects and related definitions need additional elaboration; the reason is that the lexical units that have similar meanings should appear in similar context. Therefore, official repository for semantic information for domains' contents and terminologies definitions should be involved using ontological additional works.

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Technological Communication

Biosci. Biotech. Res. Comm. 12(2): 283-296 (2019)

BBRC Bioscience Biotechnology Research Communications

Arabic calligraphy, typewritten and handwritten using optical character recognition (OCR) system

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ABSTRACT

This paper describes an Omni OCR system for recognizing typewritten and handwritten Arabic texts documents. The proposed system of the Arabic OCR system can be classified into four main phases. The first phase is the pre-processing phase; it focuses on binarizing, skewing treatment, framing, and noise removing from the prepared documents (dataset). The second phase aims to segment the preprocessed documents into lines and words. Two main tasks are pointed during this phase: language model with the used Arabic dictionary, and the detection of segmented lines and segmented words. The third phase is features extraction phase; it is used to extract features for each segmented line/word according to the used language model. Finally, the classifier or the recognizer will be used to recognize each word/line into a text stream. Therefore, scientific evaluation of the four phases will be applied to measure the accuracy of the Arabic OCR system. The recognition approachis based on Hidden Markov Models (HMM) with the prepared datasets and software development tool are discussed and introduced. State of the art OCR's recognition systems are now capable to perform accuracy of 70% for unconstrained Arabic texts. However, this outline is still far away from what is required in a lot of useful applications.In other words, this paper describes a proposed approach based on language model with ligature and overlap characters for the pro-posed Arabic OCR. Therefore, a posterior word-based approach is used with tri-gram model to recognize the Arabic text. Features are extracted from images of words and generated pattern using the proposed solution. We test our proposed OCR system in different categories of Arabic documents: early printed or typewritten, printed, historical and calligraphy documents. The test bed of our system gives 12.5%-character error rate compared to the best OCR of other systems.

KEY WORDS: ARABIC OCR, SEGMENTATION, FEATURE EXTRACTION, CALLIGRAPHY, TYPEWRITTEN, HANDWRITTEN, HMM

ARTICLE INFORMATION:

Corresponding Author: Hassanin M. Al-Barhamtoshy
Received 6th April, 2019
Accepted after revision 29th June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
Crossref Clarivate Analytics
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/11

INTRODUCTION

Currently, OCR systems achieve important role in document analysis and content retrieving with high accuracy. Though, Arabic document analysis and Arabic retrieving systems have more challenges (Stahlberg & Vogel, 2016). The document outline analysis is the method of classifying and labeling the zones of the image documents into a segments of text documents. Somewhat OCR system involves the segmentation of text regions from non-textual ones. Nevertheless, Arabic text regions play diverse its logical parts inside the manuscript (title, subtitle, notes, cross-references, etc.) and this kind of semantic labeling is the opportunity of the layout analysis. Figure 1 shows sample of the challenges in Arabic scripts. Due to these properties the current performance of state of art Arabic OCR systems is much lagging compared with the performance of other Latin-based OCR systems.

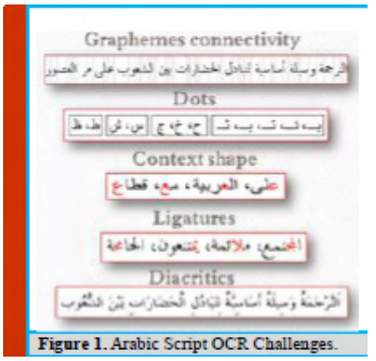


Figure 1. Arabic Script OCR Challenges.

Recent submissions that used Arabic text segmentation and Arabic OCR systems are discussed in (Stahlberg & Vogel, 2016). Other published applications to recognize Arabic handwritten is illustrated in (Cao & Natarajan, 2014). A wide range of document analysis, layouts processing to locate text blocks and none text blocks and separating between them have been presented in (Nobile & Suen, 2014). Script identification has been a real challenge in OCR and information retrieval systems (Pal & Dash, 2014). The most state of the art papers are published into the OCR machine printed character domain.Moreover, segmentation process leads to errors more than the other processes in document analysis and processing (Cao & Natarajan, 2014).

Text classification addresses the problem of document analysis into "classifying modern machine printed text, handwritten text and historical typewritten text from degraded noisy document" (Zha, et al., 2014). Therefore, text classification approach based on iVector is proposed in (Zha, et al., 2014). The text line is classified using SVM in iVector space. An OCR for multilingual documents (Amazigh-Frensh) has been proposed in (El-Gajoui, et al., 2015). Hence, Amazigh writing transcription methods are employed using Latin or Arabic alphabet. Acomprehensive survey on Arabic cursive scene text recognition, and the text having variations infont styles, size, alignment, orientation, reflection, illumination change, blurriness and complexbackground had been illustrated in (Bin Ahmed, et al., 2019). Arabic text recognition system is presented using deep learning architecture, and text localization and feature extraction are also presented. Then, it injects such feature vectors to the HMM. Holistic Arabic printed word recognizer is introduced along with discrete Markov classifier, HMM toolkit (HTK), and discrete cosine transform (DCT). Five fonts are used, having size of 14 points with plain style. Additional details, technical points and paper analysis are presented in (Nashwan, et al., 2017).

The documents to be studied within the datasets are composed of different fields under different formats styles (document styles, sizes, colors ...). Figure 2 shows samples of documents with different fonts, styles, colors ...). Accordingly, different manipulation with respect to syntax, styles, and colors during the analytic processes are needed of the proposed system.This paper is organized as the following description. Section 2 introduces an overview of multi classification of OCR system and related definitions. Section 3 gives the proposed solution of the Arabic OCR system with preprocessing operations (binarization, skewing, frame removing, and segmentation modules). Section 4 provides a language model and a lexicon building with Arabic OCR dataset description. Section 5 introduces features extraction and HMM decoding description. Performance evaluation results will be discussed in section 6. Section 7 summarizes conclusion and future works.

Figure 2. Samples of Arabic documents

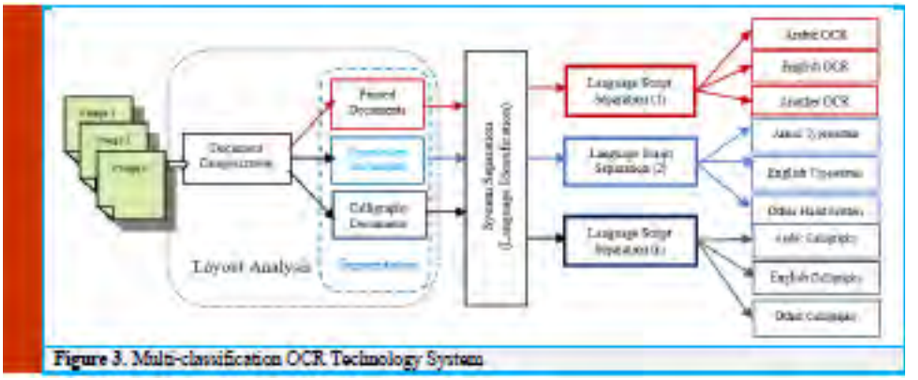


Figure 3. Multi-classification OCR Technology System

Analyzing the layout of documents is difficult, particularly in typewritten, handwritten, historical images, and manuscript notations. Any documents can be classified based on its category; either printed, typewritten, handwritten, or scripts and manuscripts documents. The documents require dedicated preprocessing modules to deal with some common properties, structure, clearing irrelevant objects or noises, language direction...etc. Therefore, the documents require pre-processing procedures because of some familiar properties such identifying unrelated objects or noises, language direction ...etc. An OCR system takes into consideration the different categories of such documents. Figure 3 illustrates a flow diagram of multi-classification that used in OCR technology.

Generally developing an OCR system framework for multi-script language documents is more difficult than a single-script OCR, due to features associated with the language models, structures, properties, styles, and the nature of writing. The first module in the layout analysis is to organize text and non-text regions. Therefore, given a document image, then converts it into a decomposition of smaller regions. Thus, these regions are classified as text or non-text elements. Such text regions are fed to the second module of the OCR system, in order to declare category of the document (Printed, calligraphy ... or typewritten). In this proposal, we are dealing with heterogeneous large-scale documents with wide varying structured category. Furthermore, there could be multi-page document with different languages. Accordingly, the language domain will be identified within the language script specification module.

The goal is to improve the OCR accuracy by creating auto selection of the OCR system. To enable this goal, the layout module aims to:

1. Identifying the document image category.
2. Detecting and segmenting text and non-text regions.

A script is a visual representation or an organized arrangement of distinct graphic characters in precise patterns known as the alphabet of a language Grapheme, allograph and glyph are needed to study the script of a language, and they contribute to the script formation, as well as designing fonts Any OCR can be carried out using one of the following steps:

- Building a general OCR that recognizes all words and characters of the alphabets in all possible languages.
- Building language separation module to identify each single script with related OCR engine.

Arabic historical documents are classified as one of the most important documents that includes historical, political, and ancient information over the world archives. Figure 4 shows two examples of Arabic historical documents and one multi script (Arabic/English) with different kinds of orientation.

(b) Mono Oriented (c) Multi Oriented (d) Multi Scripts

Figure 4. Examples of Calligraphy and Handwritten Ancient Arabic Documents

1. Proposed Methods and Preprocessing Block Diagram

Several commercial systems have been developed based on images analysis, segments determination and features extraction techniques. Most of such documents contains a lot of challenges; such as documents layout forms, physical structure, low quality due to aging, etc. this in addition to; the Arabic documents challenges; such as language writing orientation, Arabic dots, and Arabic diacritics. The data flow diagram (DFD) of the preprocessing stage of document analysis is shown in Figure 5.

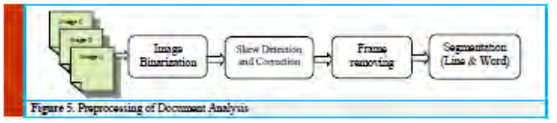


Figure 5. Preprocessing of Document Analysis

It starts with documents binarization (Al-Barhamtoshy, et al., 2019).

2.1 Binarization

The main goal of binarization is separating the foreground text from the background to enable and identify useful feature for character recognition (Al-Barhamtoshy, et al., 2019). According to document degradation, low contrast, shadows, background intensity, smear, etc.; we need further processing for these challenging tasks. Several algorithms are used in binarization for modern documents, the most commonly used algorithmsare the Otsu algorithm, the Niblack algorithm, and the Sauvola algorithm(Hadjadj, et al., 2016). In the binarization development process, Sauvola algorithm will be used with adaptation method to work with the Arabic documents. The proposed solution gives good results for the documents, as shown in figure 6.

Figure 6. Original Colored Documents and Binarized Documents (after binarization)

Local binarization computes a threshold t(i,j) for each pixel according to the following formula:

$$F2 = \frac{\sum_{i=1}^{i=H} i.r(i)}{\sum_{i=1}^{H} r(i)}$$

The threshold t(i , j) is calculated after getting the mean value of m(i , j) and standard deviation S(i , j) of the pixel intensity in window (w , w) centered around the pixel (i , j).

$$Fi = \frac{100 * Fi}{H} \quad i = 1,2,4,5,8$$

where R =128 for grayscale, and the default value of k= 0.34 gives best results.

2.2 Skew Detection and Correction

There are many methods used in documents skew detection. For example, projection profiles (El-Gajoui, et al., 2015), Hough transform, nearest-neighboring, segmentation (Al-Barhamtoshy & Rashwan, 2014), and coloring documents are used. In the projection profiles, horizontal and objective functions are computed to detect the skew rotating angle in the image. Such horizontal profile has consecutive local maxima and minima as the document is rotated to the computed angle. The objective function is used to minimize the searching process. Another technique is used by dividing the document into vertical slices, and then compute the horizontal projection profile for each slice. Also, wavelet decomposition can be used with inaccurate results. Hough transform can detect skew angle and estimate the lines in the documents and obtain their angles within time complexity (slow speed and it takes lot of memory).

In (Subrahmanyam, et al., 2018) a skew estimation is obtained based on image borderlines by using a run-length algorithm with large connected components in the whole documents. The proposed de-skewing algorithm presented by, first it segments the imaged document, then it detects the skewing angle using the segmented objects, but when it tries to obtain the skewing angle from page borders, it faced a problem that some pages does not contain any borders and also some borders are not continuous therefore, it will be hard to be extracted. This leads us to try another technique in skew detection, first we assume that the skewing angle ranges from -5 to 5, all borders and small components are removed, then the page is segmented into lines using a histogram technique, curve fitting is then used to obtain the skew angle for each line, and finally the average skew angle is calculated and rotate the whole page by this skew angle. The main advantage of this method is its ability to detect the skewing angle even if it is very small. Figure 7 shows the block diagram of the de-skewing algorithm.In our case, we use connected component to remove the marginal noise to perform documents cleaning. Then vertical histogram profile is used to obtain a rough estimation for text lines, and then we use a curve fitting to obtain the skew angle.

Figure 7. DFD of the Skew/de-skewing Algorithm

Figure 8 display two documents before and after the skewing algorithm. A straight forward approach to correct skewing angle, we use the following formula:X = x cos θ + y sin θ, and Y = y sin θ – x cos θ. Where X and Y are the new point from the original (x , y) point.

2.3 Frame Detection and Removing

This section focuses on removing frames or border lines; especially in old documents. A rule lines are removed, and broken characters are reconstructed. In (Arvind, et al., 2008) line detection is done by examining the peak of connected component to obtain the page borders which is the most common and easy method to be implemented. Eliminating borderline noise is done by

Figure 8. documents before and after skewing

cleaningavailable connected components based on their size and aspect ratio. Projection profile is used to detect the location of border objects, analyze them and then remove them.In the current algorithm;the connected components methodis used to remove the page frame, the average width and height of all components are computed, then searching for components with width greater than *x**average width, and height greater than *x**average height, then exclude those components from the original image, where *x* is a constant value multiplied by the average width or average height, the value of *x* is obtained experimentally (threshold), and the best result was when x equal to 6 as shown in figure 9.

Figure 9. Frame Detection and Removing Layout Example

In Arabic typewritten and handwritten texts, usually lot of overlapped components can exist, and it is causing segmentation problems. Figure 10 includes samples of these overlapped components of Figure 9(a). The following algorithm is used to separate the overlapped components:

1. Obtain image's connected components.
2. Calculate average height, and width of all components.
3. Obtain components that are greater than (thresh1 *average height), and components that are greater than (thresh 2*average width), by trying different values thresh1 = 5, and thresh 2 = 10.

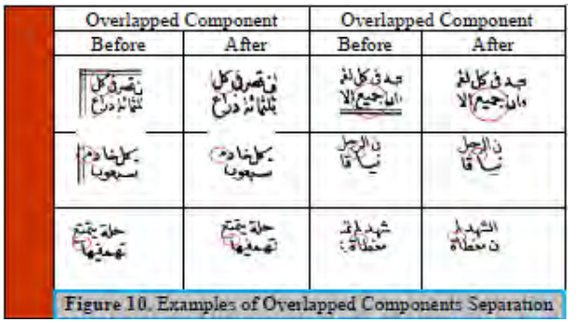


Figure 10. Examples of Overlapped Components Separation

4. Obtain the histogram for each component, and if peak exceeds a certain threshold (experimentally 0.7) remove the region around this peak.
5. IF there are no peaks in a component with a large height, split the component into two components from the middle.

2.4 Segmentation

Line segmentation is a technique to extract lines from a scanned document (Gatos, et al., 2006). Line segmentation techniques basicallycategorized into fourdifferent categories: Hough Transform (Gatos, et al., 2006), projection profiles, smearing, and segmentation based on connected components. In our case, we first use vertical projection profile but if there were some overlapped characters between lines, we try to segment lines using connected components techniques and this leads to segment lines with overlapped characters.

In the segmentation algorithm, three methodsof smearing, projection profiles, and connected component methods are used. The problem in smearing method was the overlapping between lines and each other's, in early printed Arabic books. Therefore, when smearing is done two lines may be connected to each other, and this will lead to the segmentation of two lines as one line. Projection profile methods give us better results than smearing methods, but this leaves us with the problem of having very small components in extracted lines as results from upper or lower components in other lines. That is the reason we move to these connected component methods. In the proposed segmented case; first vertical projection profile is invoked; but there were some overlapped characters between lines, thereforethe segmented lines using connected components techniques are invokedand this leads to segment lines with less overlapped characters, as shown in Figure 11. The output of the line segmentation are shown in figure 12 after frame removing in figure 10.

The connected components of the document are proposed to obtain a sequence of words to realize word indexing. Features victors of the words will be computed and stored in the query indexed database.

Figure 11. DFD of the Segmentation Process

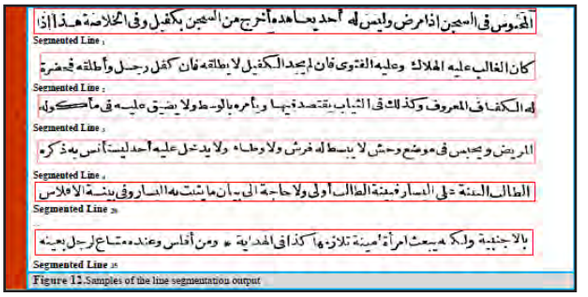


Figure 12. Samples of the line segmentation output

Line segmentation is done by following Algorithm steps (Figure 13):

1. Use horizontal projection profile to obtain an initial position for the separated lines, and baselines.
2. Divide the image into smaller regions, by taking the region between each two successive base lines, for each region do the following steps:

a. Obtain energy map.
b. Remove small regions from energy map (like removing dots and diacritics).
c. Fill the horizontal edges of the image with high values in order not to be included in path.
d. Find seam with minimum energy following the given steps

i. Set initial point in a path by using the approximated baseline.
ii. Obtaining the next step in the path, by searching the row with minimum value in a window with size equal to 2/5 of the image height.
iii. Translate the previous step to be as far as possible from the two components that lie above and below it.

The segmentation module is the most important phase in documents analysis and OCR recognition systems. Three types of segmentation are described, two are existing before: (1) Global and local approches, and the third is new: line segmentation approach.

Figure 13. Arabic documents (Before and After Segmentation process)

A. Global or Word Approach

It aims to segment the whole words (or Piece of A Word: PAWs) of the entire document, and keep track of the index/references of their location in the text.

B. Local or Letter Approach

It allows to segment words' images into letters, graphemes, and/or legitures. This kind of methodology may derive enhanced recognition percentage with slow in implemntition task.

C. Line or (n-grams) Approach

This approach is based on the whole line segments (number of words). It trys to use linguistic knowledge such as dictionary, lexicon, and/or language model with word probabilities. Such dictionary, lexicon and language model are generated before or during training phase of the OCR systems. Therefore, it trys to select and identify the correct segment hypothesis.

3. The Language Model and Lexicon Building Results

A statistical language model will be used based on probability distribution and function of sequences of likelihood word of different phrases. Consequently, an N-gram can be defined as an overlappingsequence of N Arabic words. For the line$_2$ (in Figure 12), the following sets of N-grams can be attained.

1- Uni- Grams (unigrams): قحضرة ,واطلقه ,رجل ,كفل ,فإن ,يطلقه ,لا ,التكفيل ,يجد ,لم ,فإن ,القنوى ,و عليه ,الهلاك ,عليه ,الغالب ,كان
2- Two-Grams (bigrams): التكفيل ,يجد ,التكفيل ,لم يجد ,فإن لم ,القنوى فإن ,و عليه القنوى ,الهلاك و عليه ,عليه الهلاك ,الغالب عليه ,كان الغالب
واطلقه قحضرة ,رجل واطلقه ,كفل رجل ,فإن كفل ,يطلقه فإن ,لا يطلقه ,لا
3- Tri-Grams (trigrams): القنوى فإن لم ,و عليه القنوى فإن ,الهلاك و عليه القنوى ,عليه الهلاك و عليه ,الغالب عليه الهلاك ,كان الغالب الهلاك
رجل واطلقه قحضرة ,كفل رجل واطلقه ,فإن كفل رجل ,يطلقه فإن كفل ,لا يطلقه فإن التكفيل لا يطلقه ,يجد التكفيل لا ,لم يجد التكفيل ,فإن لم يجد
...
4- N-grams: The complete line:
كان الغالب عليه الهلاك و عليه القنوى فإن لم يجد التكفيل يطلقه فإن كفل رجل واطلقه قحضرة

Therefore, n-grams is said to include order of n by applying a sequence task of probability to arrange the desired probability of a line. The rule of the segmented line2 can be calculated according to the following:

$$P(\text{Linei}) = P(W1) * P(W2|W1) * P(W3|W2W1) * ..... P(Wn|Wn-1 ....W1)$$

Accordingly, Arabic corpora and lexicon are used to support the proposed language model.The proposed model is based on features extraction for each line/word during training or classification module. This module focuses on HMM based classifier with full language model data. Figure 14 displays the DFD of the proposed system for our syntactic classifier.

Once the Arabic imaged – documents is scanned, the pre-processing stage takes place (as discussed before). Therefore, the segmented lines/words are extracted from the imaged– document using appropriate algorithm. From each segmented lines/words, many features will be selected and extracted by utilizing the vocabulary of syntactic language model data base. The language model can reduce the computational overheadand enhance the recognition rate.

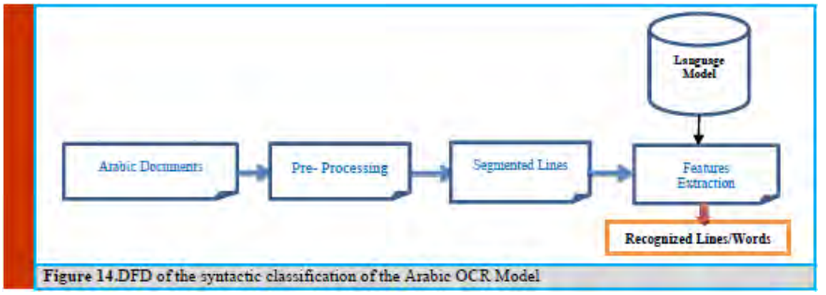


Figure 14.DFD of the syntactic classification of the Arabic OCR Model

3.1 Lexicon building

A dictionary of a unique 800k words has been built from very large corpus. The data of corpus has been collected from different resources to guarantee occurrence of words in different fields of life. The same corpus has been used to build our language model. Our implemented language model contains up to 3-words (tri-gram).

The character shape in Arabic is context sensitive, that is, depending on its position within a word (isolated, start, middle, or end). Also, Arabic characters are rich in diacritic marks and delayed strokes (dots, Shadda, Hamza, etc.). By tracing our training data, a total of 341 character models has been generated by taking into consideration the fact that an Arabic character may have different shapes according to its position in a word. Another model for space has been added to solve the problem of intra and inter spaces in the line. A total of 342 models have been generated and trained with the embedded training data.

A dictionary of all the different unique words in our database has been constructed. Each word in the dictionary is described by its related ligatures and characters as shown in table 1.

Table 1Dictionary Description with different forms and ligatures

Word-ID	Image of Word	Segments of Characters	Ligature
1	محمد	م ح م د	
1	محمد	مح م د	مح
1	محمد	محم د	محم
2	عوم	ع و م	
2	عوم	عو م	عو
3	...	...	...
...	...	...	...

3.2 Arabic OCR Dataset Description

In this section, we present Arabic OCR dataset that is used in this research. The proposed Arabic OCR dataset was developed by RDI and the Arabic OCR team project, Faculty of Computing and Information Technology at KAU in Saudi Arabia. It is made by helping staff of RDI and Arabic Language Technology Center (ALTEC) dataset. The creation of this dataset is a result of cooperation to the three team works; Arabic OCR system team, RDI team and ALTEC team.

The dataset is required to consist mainly of images (one page), and the corresponding formal description of that image (XML transcription file). The number of images to be produced is anticipated to be in the order of same number of images, with the corresponding transcription files. These are mainly using two streams; the first will be generated using word lists and the second will be generated using a collection of files and theses documents.

The production of the required dataset is carried out according to the following descriptions:

1. Fonts include: (a) Simplified Arabic, (b) Arabic Transparent, and (c) Traditional Arabic.
2. Sizes: Each font is required and produced for sizes of: 10, 12, 14, 16, 18, 20, and 22.
3. Books and Thesis Documents:

   It is required to select 1500 pages from different scanned documents (average of 10 pages from each book for copyright constraints which gives approximately 150 titles). The titlesmust be chosen to cover uniformly the past 50 years.
   In addition, 1000 pages from theses (in Arabic) have been selected as well which is also covered uniformly the past 50 years. Books came from at least 15 different categories based on the fonts and sizes used. The used books are classified manually and approved by ALTEC. Theses come from 10 different categories based on the used fonts and sizes. The typo thesisis classified manually and approved by the team members.
4. Documents Production:
   The documents production stage has two steps: the first is the printing step and the second is the scanning step. As for the books and theses, we go directly to the scanning step.
   In printing step, the produced output files are printed then undergo different processes to add noise to the produced documents. At the end of this step, the following document versions have been produced:

a. A Clean Version: the clean version is the first print out from the created files. Printing is done using a

different printer for every document set. In addition, the original document produced by typewriter is to be considered as a clean version.

b. A Copy Version: the clean version is photocopied using different photocopying machines.

c. Take a shot of the clean version using Digital Cameras and Mobile Cameras. (10 digital cameras and 10 mobile cameras are used). In this case, no scanning is required since we get the ".tif" images directly. All cameras have at least 5M-pixel of resolution, and the distance to the documents is 50cm. There is a 50% of the imaging with separate cameras, and 50% with mobile cameras. The produced (.tif) images of this step is not undergo any further processing.

In the scanning and digitizing step, the documents produced by the printing step (a and b above) are scanned using a different scanner for every set of documents and saved in (.tif) format. The scanning is done using the following resolutions: 200, 300 and 600 dpi. As for the books and theses, a Book Digitizer is preferably used to produce three resolution versions of each page: 200, 300, and 600 dpi. As well, 300 pages of the books and 300 pages of the thesis's pages are also captured by digital or mobile cameras.

3.3 Calligraphy, Typewritten and Handwritten DatasetsPreparation

Designing general OCR engine for multiple documents is very difficult than a single script OCR engine. This is because existence of many of different features, properties, fonts, styles and nature of language writing for each language script are needed.

Definition 1. (Multi-Dimensional Language Documents) A multi-dimensional documents set D is a set of documents D_1, D_2 ... D_n, such that each language script L_i contains a set of f features denoted by d_i^f.

Definition 2. (Language Script L_i) An OCR of language length n documents contains w words (w_{i1} ... w_{in}). Each word contains numeric features at each font (F_1... F_f), together with a set of m sizes (S_1 ... S_m), such that the word W_{ij} is associated with font F_i and size S_i.

Definition 3. (Document Data Clustering). Given a dataset DS, then decompose its words into sets W_1 ... W_k, such that W_i can be represented as rows with set of features d_i^f and each feature is "similar" to one another, according to their fonts and sizes.

Definition 4. (Document Data Classification). Given an n x d training data set DS, and a class label value in {1...k} associated with each of the n in DS. Create a training model M, which is evolved to predict the class label of dimensional record Y & D.

The Arabic calligraphy, typewritten and handwritten texts have many of documents' varieties, as follows:

Height. Measured as the letter zones; the distance between the upper, the middle and the lower zones.

Width. Defined as the length of the connecting strokes and therefore it examines the breadth of the letters, the distances between letters, words, lines, and the slant of the writing.

Spacing. It examines the margins and the spacing between lines, between words and the balance between the height and the width of the letters.

Depth. Ittries to determine the direction of the stroke from the width of the upstrokes and the downstrokes.

Curved writing. In Arabic typewritten and handwriting, some of letters composed from oval shapes(loops: formations of circle), or other Arabic letters are comprised of parts of a circle.

Overly Embellished Writing. It likes all extremes in handwriting, a facade or compensation for inner weakness.

Pieces of Arabic Word (PAW) is defined as sub-words, ligatures, and diacritics are two issues should be handled.

Writing Direction. Arabic printed, typewritten or handwritten writing directions are from right to left direction. To determine the size of Arabic typewritten andhandwritten text, the system initially looks at the middle zone letters. If the height was within 1/8" (3mm) then the size will be normal, if it was greater than (or less than) 1/8" then the word should be normalized.

The proposed dataset contains images of calligraphy, handwritten Arabic text which can be used to train and test calligraphy, printed and handwritten text recognizers and to perform OCR recognition and verification experiments.

The dataset was first prepared at the ICDAR. Using this dataset an HMM based recognition system for printed documents was developed and published at the ICPR 2000. The segmentation scheme used in the second version of the dataset is documented in (Nobile & Suen, 2014) and has been published in the ICPR 2002. The Altec- is described in (Pal & Dash, 2014). We use the dataset extensively in our own research, see publications for further details.

3.4 Training Documents

Training documents preparation is the process of segmenting and extracting boxes of objected-words from a large set of Arabic documents and storingeach objected-

word as a separate labeled image. This label identifies the text it contains, and this makes the usability of this dataset much easier.Extracting training documents can be automated through a smart computer tool, in addition toa manual segment selection usingthe developed tool.

The manual technique of the segment selection is done using a developed computer tool,with the helping of an expert user that produces objects of the lines/words with its coordinates. These coordinates are defined as (x_i , y_i , x_j , y_j) where (x_i , y_i) are the coordinates of the upper left corner in the bound box of the objected-word (segmented word), and (x_j , y_j) are the coordinates of the lower right corner in the bounding box of the objected-lines/word. This proposed development tool makes the manual efforts to be more efficient in time and accuracy than the ordinary methods. In the first training dataset, there are 28 used typewritten books, the overall of the selected objected-words from these typewritten books includes 84,000 words.

Therefore, the automated tool generates two types of outcomes: (1) Extracting segment regions; and (2) Generating text file description. The resulting segmented regions and text labeling with its segmented coordination are used to build a classification model. The automated tool generates object-segments or word-segments coordinates using x_i, y_i and x_j, y_j points as a region of word/line picture. Then, the automated tool stores such pictures in the name of pictures' words. Each segment is manually labeled for its text by annotator peoples.

Many processing algorithms need considerable tuning to address the special features of Arabic documents. The following section describes these modifications.

Now, we have two streams of picture files (automated segmented regions: asr) and labeled text files (txt). The learning algorithm uses such two streams to extract visual features from the two input streams. Accordingly, for each documentfile, the output of this step creates two generated files as illustrated in Fig 13.

1. ASR file: Extract the segmented region with its coordinates; that includes all keyword box locations (x_i, y_i, width, height).
2. TXT file: Generate textile labels of these segments that represent the segmented region itself (keywords text; it may be a single word or compound words).

Accordingly, an overview of indexing and querying data files for each documentdataset are formulated. The first step of the indexing or querying is the computation of the word representation in this section. The documents indexing is done in advance to allow a quick examination.

Also, each lines file is segmented and described with each document and contains the segmented line coordinates and its labeled text description, see Figure 16.

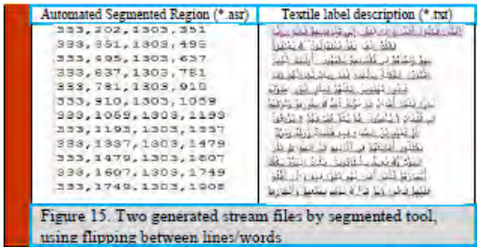


Figure 15. Two generated stream files by segmented tool, using flipping between lines/words


Figure 16. Two generated pages by segmented tool, using flipping between lines/words

The next process; each word file is related with each document and includes the coordinates of such words in the page, the positions (left upper and lower right corners) and their labeled text, as the <pawposition = "x_1, y_1 , x_2, y_2">Arabic Word</paw>". Each segmented Arabic word or sub-word (PAW) is fully described using XML standard format. The labeling of the segmented word, or sub-word contains tags that represent ground truth information about sequence of words/PAWs. Figure 17 gives generated example about information of document, word text with PAW segment locations.

4. Proposed Model Discussion

In this section, we introduce our extracted features technique. Most of these features are well known and used. The input image in binary after the pre-processing stage is used to extract a feature vector. The background pixels of the image are labeled by logic "0" and the foreground pixels by logic "1".A sliding window from right to left is used. The window is of one column width "W" -without any overlap- and the image's height "H".The

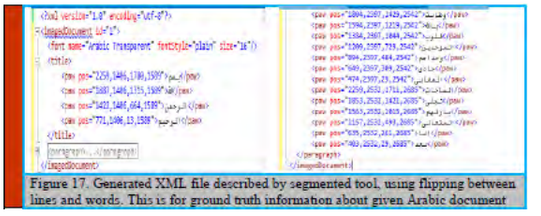


Figure 17. Generated XML file described by segmented tool, using flipping between lines and words. This is for ground truth information about given Arabic document

following three features represent the distribution of pixels per column in the given image.

a) Number of "1" pixels in each column were counted to from the first feature (F1)
b) The gravitational center of each column was calculated to form the second feature (F2), as shown in the following formula:

$$F2 = \frac{\sum_{i=1}^{i=H} i.r(i)}{\sum_{i=1}^{H} r(i)}$$

Where, r (i) is the number of "1" pixels in the i^{th} row of a column.

a) The second order of moments was used to form the third features (F3). The second order of moments was used to represents the variance of each column in the image.
b) The following four features represent the style of characters and ligatures.
c) Position of the upper contour in the column per image (F4).
d) Position of the lower contour in the column per image (F5).
e) Orientation of the upper contour in the column per image (F6).
f) Orientation of the lower contour in the column per image (F7).
g) Number of "1" Pixels between the lower and upper contours (F8).

The previous eight features used before with Latin and gave a promising accuracy. To make this feature suitable for Arabic, we normalized some of them to be independent of the height of the image. (F1, F2, F4, F5, F8) were normalized by the following formula.

$$Fi = \frac{100 * Fi}{H} \quad i = 1,2,4,5,8$$

Delta and acceleration were calculated and then concatenated with the extracted features to form feature vector of length 24. A short script is used to read the previous output files that contain the text and the locations' points of the bounding boxes of each objected-wordand output the objected-word as a separate labeled image file.

Multi-match process is proposed to retrieve similar matched features of the words, based on word spotting. The objective of the matching is to filter number of words to be measured with the searchable word query. Accordingly, a set of bound features is measured as adequate for matching, such as ratio of their words' segments sizes. If such a ratio does not lie within a specific threshold related to the size of the searchable word query, therefore, don't consider this word as candidate. Then, compute the similarity between searchable word queriesstored features of the candidate set of the comfortable content. Therefore, word spotting with vector of features was computed and used in a word holistic approach.

The over mentioned features' vectors are used to compare words with the given in word query, in case of retrieving the relevant words and documents.

Figure 18 illustrates the DFD of the proposed matching module, consideredthe distance that is used to compare two words.

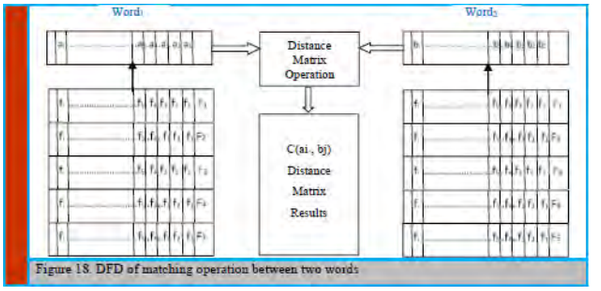


Figure 18. DFD of matching operation between two words

4.1. HMM Classifier/Decoding Module

In this section, we present the decoding stage in a general way and describe what happens to the test image from the moment it enters the system until the output text is resulted from the system. First, the test image goes through pre-processing stages that binarize the image, remove the noise, remove frame (if exist), resolve skewing, and segment the image into lines (Figure 19).

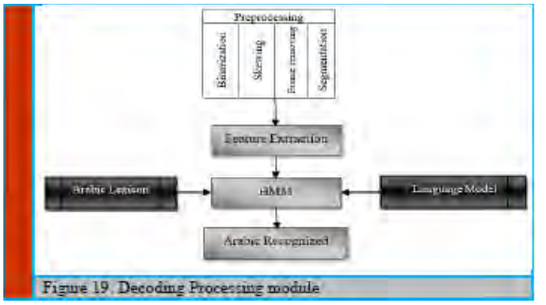


Figure 19. Decoding Processing module

Then, the pre-processed lines of the given image go through the feature extraction stage to extract a set of robust features by using frames from right to left. The different blocks of the architecture are going to be explained in detail in the following sections of this paper.

4.1 Hidden Markov Model

A hidden Markov model (HMM) represents stochastic process to generate sequence of output over period. Officially, an HMM can be modeled by 4 parameters (λ = {S, A, Π, B}), where S = {S_1, S_2, ..., S_n} represents set of states, A = (a_{ij}) is the state transition probability matrix,

Π signify the start probability vector; $(\pi_i \equiv P(x_0 = i))$, and B = $\{b_1, b_2, ..., b_n\}$ stand for emission probabilities of states (Figure 20).

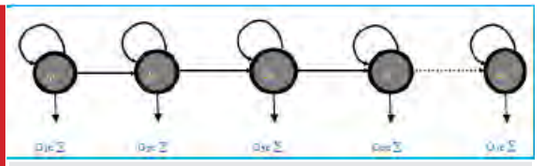


FIGURE 20. HMM State topology for Arabic OCR System

Where Π is the initial state probability vector with the following descriptions: $0 \le \pi_i \le 1 \quad \sum_{i=1}^{N} \pi_i = 1$; The transition probability matrix A is described as:

$$a_{ij} \equiv P(x_{t+1} = j \mid x_t = i) \quad , \quad 0 \le a_{ij} \le 1 \quad , \text{and} \quad \sum_{j=1}^{N} a_{ij} = 1$$

The Baum-Welch algorithm is used as a training method. It considers the probability P(O| λ) that a known sequence O is produced by the HMM λ and calculates the latest model λ which likely to have produced the given sequence. A detailed description of the Baum-Welch algorithm is given in ([1]). A modified algorithm is applied during training phase. Hence, the text line HMMS are accumulated using character of HMMs or whole-word HMMs. The HMMs characters are coupled to form word HMMs according to the language model dictionary (Figure 21). The recognizer is restricted to those words are existing in the language model dictionary. Therefore, thus, a model for word sequences is attained by the word models concatenating.

In Arabic OCR system, a segmented textual line object is handled from right-to-left signal and modeled as a sequence of extracted feature represented in vectors sampled at a fixed slice rate.Each segment is divided into several slides, and at each slide, object features are computed. Accordingly, histogram features are obtained by disintegrating the object signal at the pixel into gradient, structural and concavity features.

A character object is divided into patches; three features (gradient, structure, and concavity) are employed and calculated. The gradient features are calculated by using Sobel gradient operators to the whole character object. Then, the gradient at each pixel is quantized and transformed to 12 directions. Therefore, 12 features are calculated according to the following equation:

Feature $_{\text{Gradient(i)}}$ = (No of pixels in the patch with gradient degree i) / (Total no. in the patch)

The second step is related to derive structure features from gradient features. Therefore, the 12 assignments of directions are clustered in 12 classes.

1 http://www.lasorsa.com/wp-content/uploads/2015/02/Handwriting-Analysis.pdf

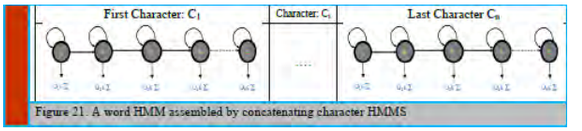


Figure 21. A word HMM assembled by concatenating character HMMS

This clustering represents the local structure central pixel. Therefore, every structure feature is defined for each patch as following equation:

Feature Structure$_{(i)}$ = (No of black pixels of class i in the patch) / (Total number in the patch)

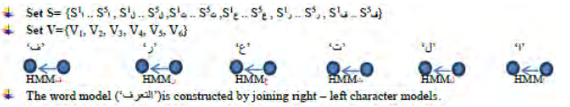
The next step computes the concavity features by using two directions scanning (from one side to the opposite side). The scanning is employed row by row (from right to left), then column by column (from up to down). During the scanning, preserve path of all the scanned white pixels until the first black one is reached and end. Figure 3 displays labeled white pixels during scanning from right to left and from top to down. At this stage, histogram features are computed in the same way, using the following equation:

Feature$_{\text{concavity(i)}}$ = (No. of scanning white pixels in the patch at i) / (Total no. in the patch)

Also, the hole feature is calculated as:

Feature $_{\text{hole(i)}}$ = (Number of scanned white pixels in the patch at i) / (Total no in the patch)

Example of imaged - word (التعرف) generation of a 6-characteralphabets ('ا', 'ل', 'ت', 'ع', 'ر', 'ف')



The hidden Markov model is a double stochastic process which can efficiently model the generation of sequential data. The HMM used in this paper is a continuous HMM with one HMM for each ligature or character. In this paper, we use the same HMM classifier without modification as simple mentioned in HTK Speech Recognition Toolkit (Rabiner, 1989)]. However, we implement our own parameters of the HMM. We allowed transition to the current and to the next state only. HTK models the feature vector with a mixture of Gaussians. It uses the Viterbi algorithm in the recognition phase, which searches for the most likely sequence of a character given the input feature vector.

In the training phase, an iterative optimization of the model with respect to the training data is performed, and we used Baum–Welch algorithm, a variant of the expectation maximization (EM) algorithm, for optimization of the HMM model depending on the training data.

To achieve high recognition rates, the character HMMs have to be fitto the problem. In particular, the

number of states, the possible transitions, and the type of the output probability distributions have to be carefully chosen.

HTK is principally concerned with continuous density models in which each observation probability distribution is represented by a mixture Gaussian density. In this case, for state j, the probability $b_j(O_t)$ of generating observation O_t is given by:

$$b_j(O_t) = \prod_{s=1}^{S} [\sum_{m=1}^{M_s} C_{jsm} \psi(o_{st}; \mu_{jsm}, \Sigma_{jsm})]^{\gamma_s}$$

where M_{js} is the number of mixture components in state j for stream s (1-stream used), the exponent γ s is a stream weight and its default value is one, C_{jsm} is the weight of the mth component, and $\psi(o;\mu,\Sigma)$ is a multivariate Gaussian with mean vector μ and covariance Σ that is:

$$\psi(o;\mu,\Sigma) = \frac{1}{\sqrt{(2\pi)^n|\Sigma|}} e^{-\frac{1}{2}(o-\mu)^T\Sigma^{-1}(o-\mu)}$$

where n is the dimensionality of 0.64 mixtures have been chosen to give a robust model for each character and high recognition rate.

4.3 Testing and Evaluation

To evaluate the preprocessing module, RDI and ALTEC-dataset are used. The used dataset consists from documents of Arabic typewritten books, and documents of Arabic handwritten documents. Such experimental test validates each individual module in the preprocessing phase, as well as for testing the Arabic OCR project. We need 2 subsets:

(A) 200 selected documents taken from Arabic typewritten books.
(B) 200 documents gathered from Arabic handwritten documents.

Datasets of any documents are very important part to measure the accuracy of system. The accuracy achievement is very important to measure the performance of the recovery model. Table 2 illustrates the present visibly and available documents datasets that will be used in the current paper.

Table 2. Datasetsfor Current Research

Dataset Name	Nature / Type Style	Content /Size	Classifier
RDI	Calligraphy documents	200 images	HMM
ALTEC	Printed documents	200 images	HMM

The collected dataset includes 200 Arabic typewritten and 200 handwritten documents. Each document from typewritten dataset contains 35-40 Arabic text lines. For training, we select 15 documents from each book, and for testing we select 5 documents from each book.

The percentage value of the accuracy of the proposed Arabic OCR for typewritten text can be calculated by the following equation:

$$PercentAccuracy = \frac{N - D - S - I}{N} x\ 100$$

N: represents the total number of words in the reference file.
D: stands for the deleted words in the resulted file.
S: represents the substituted words in the resulted file.
I: is the inserted words in the resulted file.

The proposed line segmentation algorithm takes its input as typewritten and handwritten documents, and produces segmented text waved line images, as illustrated in Figure 11. The proposed algorithm is tested on 40 different documents with 1480 lines (35-40 text lines for each image). Table 3 summarizes detail results of lines segmentation.

Table 3. The segmentation accuracy of the calligraphy books.

Book	No. of segmented lines	Accuracy
1	37	99.94
2	35	99.95
3	38	99.96
...	...	...
39	35	99.93
40	36	99.92
Total	1480	99.94 %

The following table (Table 4) summarizes the percent accuracy of the recognition processof the used typewritten books.

Table 4. The accuracy percentage relative to the calligraphy books.

Book	Accuracy	Book	Accuracy	Book	Accuracy	Book	Accuracy
1	82%	11	78%	21	68%	31	67%
2	79%	12	77%	22	70%	32	67%
3	78%	13	76%	23	70%	33	65%
4	80%	14	74%	24	70%	34	63%
5	78%	15	76%	25	64%	35	65%
6	79%	16	74%	26	64%	36	66%
7	79%	17	70%	27	69%	37	65%
8	76%	18	73%	28	60%	38	65%
9	75%	19	74%	29	68%	39	78%
10	71%	20	72%	30	67%	40	81%
Average	72.00 %						

During *calligraphy* testing Dynamic Time Warping (DTW) is a good solution to be used in image matching. A flexible dynamic matching is performed when the two feature vectors are compared using DTW. Consider the two words A and B of widths i and j. The two vector sequences A={a_1 ... a_i} and B= {b_1 ... b_j} are representing the two words, where a_i and b_j are two vectors in a distance matrix vector space with 5 dimensional space features. In addition, a matrix C of dimension i x j is created where every element C(i,j) represents matching cost between sequence a_i and b_j according to the Euclidean distance:

$$C(a_i, b_j) = \sqrt{\sum_{n=1}^{k} (a_{i,k} - b_{j,k})^2}$$; where k is the number of features.

Due to Arabic text peculiarities: font, style andsize variations, writing direction in Arabic handwritten text,

an adopted routine is used to handle these variations. The proposed routine uses set of thresholds of distance normalization to adapt the well-known variations. Therefore, divide the C(i, j) by average width of the two words:

$$\text{Distance}(\text{word}_a, \text{word}_b) = \{C(i, j)/(i+j)/2\}$$

Another factor of normalization should be taken into consideration is the size of the words to be compared and can be defined as the number of processes concerned in the best possible matching. In this case, the normalized matching cost of the two words is given by the normalized word distance = W ($Word_{Query}$, $Word_{testing}$) / N. Table 5 illustrates an example of matching words "السماء" and "أبواب السماء" with respect to the pre trained dataset.

Table 5. Fetching Similarity between Query and Pre-trained imaged words

Query Word	Pre-Trained Stored Dataset				Final Cost
	Word	Word	Word	Word	
السماء	0.94	0.89	...	0. 17	1
أبواب السماء	0.52	0.45	0.92	0.16	1
..	..	..	..	..	n

When searching about word, it will be fed into the Arabic OCR system, the stored features of the dataset words are compared with the given word (query word). We can perform word by example via selecting ona word in a page of the displayed documents.

CONCLUSION

This paper introduced an Omni Arabic OCR system to analyze, segment and recognize the Arabic calligraphy and typewritten documents. Consequently, the paper described the preprocessing, the segmentation, and the dataset and language model preparations modules. The line and word segmentations are the most challenging algorithms, and they are essential requirement in OCR system. In this paper, typewritten and handwritten documents are segmented with accuracy 99.4% and 98.1 respectively in line segmentation, and with 95.3% and 90.2% in word segmentation.

The system used the modified HMM classifier to extract features and therefore recognize the input documents into Arabic text. The proposed Arabic OCR system is tested and achieved accuracy not less than 72 % for typewritten and handwritten.

The experimental test with the classification algorithm uses a dictionary of 800,000 classical Arabic words without redundancy, which has been built from many Arabic corpora. Two types of datasets are used during testing the OCR system, the first test includes the typewritten imaged documents; it includes 40 typewritten documents, the classification accuracy is in average equal72%.The second dataset includes 30 documents from historical handwritten books; the recognition accuracy was 63.52%.

Accordingly, enoughgreat of training dataset is prepared to be used in the proposed system.Dueto HMMs drawbacks (Cao & Natarajan, 2014), Artificial Neural Network (ANN) will be used specially in early printed and handwritten retrieval documents. Especially Convolutional Neural Network (CNN) accepts feature vector and makes useful information to estimate and expect the output label. Therefore, to overcome some limitation pre-segmented objected-word is needed for each position in the input stream sequence of segmented-words. Additional designed output layer for the CNN also, will beimplemented to map the input word sequence to related label text sequence without need of pre-segmented objected-words.

In future work, extended work may include other approaches and algorithms to segment and recognize other categories of Arabic documents such as handwritten and historical. In addition, the language model and the statistical lexicon/corpus will be extended with probabilistic and statistics rules. The lexicon/corpus of the language model will include different types, categories and domains of Arabic documents and scripts. Therefore, this extension may improve recognition results significantly. Also, we plan to implement our Arabic OCR prototype system as a final product. This are, in addition to, Arabic OCR web services that will be implemented.

ACKNOWLEDGMENT

The teamwork of the *"Arabic Printed OCR System"* project was funded and supported; by the NSTIP strategic technologies program in the Kingdom of Saudi Arabia-project no. (11-INF-1997-03). In addition, the authors acknowledge with thanks Science and Technology Unit, King Abdulaziz University for technical support.

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Computer Sciences
Communication

Biosci. Biotech. Res. Comm. 12(2): 297-308 (2019)

BBRC
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Research Communications

On the development of a skin cancer computer aided diagnosis system using support vector machine

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ABSTRACT

Melanoma is the most dangerous of skin cancer types and causes the most deaths. This paper aimed to provide a usable Computer Aided Diagnosis (CAD) system that helps dermatologist in the diagnosis of skin cancer. The proposed CAD system called Skin Cancer Computer Aided Diagnosis support system (SCCAD) consisted of six components, namely; image acquisition, image pre-processing, segmentation, features extraction, image classification and viewing result. Image pre-processing is achieved by various pre-processing approaches. Image segmentation is based on Otsu's threshold method. The extracted features were texture, color, and shape. These features became the input to the Support Vector Machine (SVM) classifier to classify the lesions as melanoma or non-melanoma. We obtained the dermoscopic images from the PH^2 and the digital image archive of the Department of Dermatology of the University Medical Center Groningen (UMCG) databases. We evaluated the performance of the classification model by using 10-Fold cross-validation and the confusion matrix. In addition, we compared between the SVM and ensemble classifier. The accuracy values of SVM and ensemble are 92.6%, 91.1% respectively. In addition, we evaluated the usability of the CAD system by informal study with Human Computer Interface (HCI) experts.

KEY WORDS: COMPUTER AIDED DIAGNOSIS (CAD) SYSTEM; FEATURES EXTRACTION; MACHINE LEARNING; SUPPORT VECTOR MACHINE (SVM)

ARTICLE INFORMATION:

Corresponding Author: DuaaAlsaeeddalsaeed@ksu.edu.sa
Received 15th April, 2019
Accepted after revision 25th June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/12

INTRODUCTION

Skin cancer is "the uncontrolled growth of abnormal skin cells". It's classified into melanoma and non-melanoma. Non- melanoma is more common than melanoma and is divided into Basal Cell Cancer (BCC) and Squamous Cell cancer (SCC). Melanoma is the most dangerous type of skin cancer (SkinCancerFoundation). Most skin cancers are caused by exposure to ultraviolet radiations because ultraviolet light damages the DNA in the human skin (NationalCancerInstitute, 2010). According to the World Health Organization (WHO), 132,000 new cases of melanoma and between 2 and 3 million non-melanoma are diagnosed globally each year (WorldHealthOrganization). Skin cancer is the most prevalent form of cancer in the US. Recent estimates are that 9,730 of American's (6,380 male, 3,350 female) will die from skin cancer over the course of 2017, and the number of cases has doubled in the past 30 years. Statistically, every hour, one person in the US dies from melanoma (American Cancer Society, 2017).

Studies showed that if the skin cancer was detected early it is almost a curable disease (World Health Organization). It can be diagnosed physically by examining the area, size, shape, color, and texture of suspicious spot, or performing a biopsy and examine it under the microscope (Cancer Treatment Cancers Of America). The application of computational intelligence methods provides better and more reliable diagnoses. Developing CAD system helps in the diagnosis of skin cancer from images by finding the location of a lesion and estimating the probability of disease (Masood and Al-Jumaily, 2013).The usage of ML in the medical imaging field has rapidly increased, using Computer Aided diagnosis (CAD), due to the complexity of representing objects accurately; such as lesions, and organs by using simple equations or models (Suzuki, 2017). Furthermore, one of the most important usages of ML in medical imaging is to classify objects (e.g., cancer or non-cancers), it plays an important role as an aid in diagnosing skin cancer. In this paper we propose a CAD system that helps dermatologist in the diagnosis of skin cancer using image processing and ML classification algorithms.

RELATED WORK

Previous studies show that there are several classifiers used to classify skin cancer images. Some of the most used classifiers are: Support Vector Machines (SVM), Neural Networks (NN), K Nearest Neighbors (KNN), AdaBoost, and decision trees (C4.5 and CART). The features extracted from skin cancer images are divided into three categories: texture, color, and shape. Each of these can be extracted using different algorithms. Combinations of features offers greater advantages (Thomas 2014). These extracted features are used as the input to the classifier. Moreover, the classifier will obtain more accurate results if the features are well extracted (Antony et al., 2016). In the following subsections we will discuss some previous studies based on the type of classifier.

Support Vector Machines (SVM): The SVM classifier uses hyper-planes to determine boundaries of separation between data of different classes (RAY, 2015). There are several studies (Maurya et al., 2015, Almaraz-Damian et al., 2016, Ansari and Sarode 2017, Filali et al., 2017) in which texture features were extracted by using Gray Level Co-occurrence Matrix (GLCM) as an input to the classifier. Maurya et al. (2015) provided an automated system for the detection and classification of skin cancer of four classes: Melanoma, Basal cell carcinoma, Actinic Keratosis, and Squamous cell carcinoma. The features they extracted with using GLCM method are autocorrelation, contrast, energy, entropy, and homogeneity. Thus, these features were used as the inputs to the multi-SVM classifier. The proposed system obtained result with an accuracy of 81.43 %. Almaraz-Damian et al. (2016)proposed another method based on the features of shape, color, and texture. "Shape" was based on the ABCD rule, "color" obtained by applying Non-Linear Diffusion and k-means methods, and "texture" feature by GLCM. Researchers classified images as malignant (cancerous) or benign (noncancerous) by using the SVM and discovered that the performance of the proposed system yielded a 75.1 % accuracy. Ansari and Sarode (2017) proposed a skin cancer detection system based on SVM and using GLCM methodology to extract texture features. They concluded that the usage of SVM and GLCM was easy and yielded a high degree of accuracy. Filali et al. (2017) suggested a new approach for the automatic segmentation and classification of skin lesions. K-means was one such algorithm they used in the segmentation process. They extracted texture features by using GLCM and noted them as energy, contrast, correlation, homogeneity, and entropy. The researchers also used SVM as a classifier and found that the proposed approach provided a good segmentation and an average accuracy of 83 %.

Some other studies (Almansour and Jaffar, 2016, Manerkar et al., 2016) have extracted the texture features using GLCM with some different algorithms. Almansour and Jaffar (2016) used SVM to classify images of skin cancer as "melanoma" and "non-melanoma". They extracted the texture features in two ways: Local Binary Pattern (LBP) on different scales and GLCM. In addition, they extracted color features using four statistics called "color moments": Mean, standard deviation, variation and skewness. They measured the performance of the classifier by its accuracy, sensitivity and specificity.

They learned that their proposed method was better than the other compared methods. Another system proposed by Manerkar et al. (Manerkar et al., 2016), applied multi SVM classifiers to the classification of multiple classes of skin cancer. This study employed GLCM and Image Quality Assessment (IQA) methods to extract texture features. As result, the overall accuracy of this proposed system ranged from 96 % to 98 %.

Furthermore, the ABCD rule of melanoma has been favored by some researchers (Gautam and Ahmed, 2015, Mete et al., 2016) to represent features of images. Gautam and Ahmed (2015) proposed a Decision Support System to analyze the degree of risk exhibited in a sample. The system classified images as "malignant" or "benign" by using SVM and they optimized these results by using Sequential Minimal Optimization (SOM). They applied the ABCD rule to extract the features and concluded that SVM is an effective method of classifying skin cancer images. Mete et al. (2016) proposed a novel system to classify skin cancer images in three classes: Melanoma, Dysplastic Nevus, and Benign. The proposed system contained two layers of scrutiny. They first used three SVM Binary classifiers: Melanoma or Benign, Melanoma or Dysplastic Nevus, Dysplastic Nevus or Benign. The second layer worked in a decision-maker role to map lesions to classes based on the outcomes that were obtained from the first layer. They extracted features from the images according to the ABCD rule and which were optimal for SVM. They discovered that their system provided an F-measure accuracy of 85 %.

Neural Network (NN): Neural networks have the ability to solve highly complex problems (Jaleel et al., 2013). There are several types of neural network that used by researchers with featuring different extraction techniques; such as back propagation neural network (BPNN), feed forward back-propagation neural network, radial basis function neural network (RBFNN), feed forward multi-layer ANN, auto-associative neural network (AANN), and adaptive-network-based fuzzy inference system (ANFIS). In some of the studies (Antony et al., 2016, Jaleel et al., 2013, Suryapraba et al., 2015) the extraction of texture features was done using GLCM and feed as input to different neural network classifiers. Jaleel et al. (2013) gave a system to aid the diagnosis of skin images as "cancerous" or "non-cancerous" based on BPNN. The features extracted using GLCM are of contrast, correlation, energy, mean, and homogeneity. The proposed system yielded an accuracy of 82 % and the researchers suggested the use of optimizing techniques such as Particle Swarm Optimization to enhance accuracy still further. Suryapraba et al. (2015) proposed an algorithm to enhance diagnosis of melanoma using ANN as classifier. They too extracted texture features from images using GLCM and the proposed algorithm presented highly accurate results. Antony et al. (2016) suggested a method based on ANN. They also used the GLCM method to extract texture features of contrast, correlation, energy, entropy, and homogeneity. They stated that their proposed method classified an image as "cancerous" or "non-cancerous" with an accuracy of 86.66 %.

In some other studies (Singhal and Tiwari, 2015, Sharma and Srivastava, 2016, Arasi et al., 2017) the features were extracted via Discrete Wavelet Transform (DWT). Singhal and Tiwari (2015) proposed a method of detection based on ANN. They used a neural network BPNN and RBFNN to extract features using a multi-level 2-D Wavelet Transform. The features extracted included mean, maximum, minimum, median, standard deviation, and variance. They concluded that BPNN was more accurate, simple and effective than RBFNN but that RBFNN was able to train data faster. Another work presented by Sharma and Srivastava (Sharma and Srivastava, 2016) classified skin cancer images as "cancerous" and "non-cancerous" by also applying two types of neural network: BPNN and AANN. They extracted unique features from images using a 2-D Wavelet Transform and discovered that the BPNN achieved an accuracy of 91 % with three hidden layers, while the overall accuracy of AANN was 82.6 %. Recently, Arasi et al. (2017) proposed a computational intelligence approaches for melanoma diagnostics using BPNN and ANFIS. ANFIS is the combination of ANN with "fuzzy" systems and has the advantages of both approaches. In the features-extraction process, researchers used DWT before applying Principle Component Analysis (PCA) thereby minimizing the dimensionality of the wavelet transformation data to give a more accurate classification (the feature being represented by the variance of Principal Components (eigenvalue)). Researchers found the BPNN obtained higher accuracy of 98.8% compared with ANFIS (95.18 %).

Other classification techniques*:* In some other studies, researchers have tried using hybrid classification techniques, combining two classifiers together to gain more accurate results. Others, in their studies, have tested and compared the results of several classifiers. Sumithra et al. (2015), proposed a system for segmentation and classification purposes. In their experiments, they compared the classification results of using SVM, KNN, and a hybrid method using SVM combined with KNN. They found that classifiers in combination achieved better results than those used singularly. Furthermore, using KNN alone provided less accuracy than using SVM. Another work proposed by Farooq et al. (Farooq et al., 2016) developed a framework using both SVM and Neural Classifiers. The results of SVM were shown in three cases: "high risk" (i.e. melanoma), "low risk" (i.e. non-melanoma), or "medium risk" (i.e. indeterminate). The

ANN classifier was used to implement further classification of the results obtained from SVM or biopsy tests. Nizar and Kumar (Nizar and Kumar, 2016) proposed a system based on SVM and KNN techniques in order to classify several types of skin cancer. They concluded that SVM achieved greater accuracy than KNN. Another comparative analysis was undertaken by Amirjahan and Sujatha (2016).

They compared three types of classifiers: SVM, C4.5 and Classification and Regression Trees (CART) to predicate the efficiency of each. Their results showed that SVM provided a higher level of accuracy than others. Elgamal (2013), used both Feed Forward Back-propagation ANN and K-NN to classify images as "normal" or "abnormal" skin cancer images. He discovered the two proposed classifiers showed robust and effective results. The work done by Barata et al. (2014) had two objectives, one of which was to apply two systems for the classification of skin lesion and compare their results. The systems were global and local. The global system extracted global features (e.g. color moments) and applied SVM, AdaBoost, and KNN classifiers. While the local system was applied to some features that cannot be obtained (e.g. unknown boundary) by selecting a small region of image (Philbin et al., 2007). The other objective of this study was to determine which set of features was more discriminative by comparing color and texture. The study concluded that both systems provided good results in their respective datasets, and that color features performed better than texture when used in isolation. Mhaske and Phalke (2013) compared the results of three types of classifiers: KNN, ANN, and SVM. They found that the result obtained from SVM was more accurate than ANN and K-NN, followed by ANN. The least accurate results were obtained from K-NN. Similar work was undertaken by Victor and Ghalib (2017) to detect skin cancer by using KNN, SVM, Decision Tree (DT), and Boosted Tree (BT). Their experiments showed that; SVM again yielded the most accurate results, followed by KNN. The DT classifier was the third most accurate and the BT the least accurate.

MATERIAL AND METHODS

Proposed SCCAD System: The proposed SCCAD system is a computer aided diagnostic support system for segmentation and classification of skin lesions. It also extracts a set of discriminating features from skin lesions for efficient classification. The proposed system allows the dermatologist to upload a skin image for a patient and provides the diagnosis (melanoma or normal). It also provides some extra features for the dermatologist such as, saving images and some patient data if needed. The overview of the proposed SCCAD system is shown in Figure 1 below.

As shown in Figure 1, the classifier uses a pre-trained skin cancer classification model, and up to our knowledge there is no such classification model on hand and ready to use, so we developed, trained and tested our own model. In the following subsections we will discuss the developed skin cancer classification model and the design of proposed SCCAD system.

A. Proposed Skin Cancer Classification Model

The classification model consists of six main components: image acquisition, image preprocessing, segmentation, features extraction, image classification and view result. A general block diagram of the proposed model is illustrated in Figure 2.

a. **Image acquisitions:** This is the first step, reading the input RGB image. We obtained from images PH^2 dataset and from MED-NODE .

b. **Image preprocessing:** a preprocessing step is important to reduce unwanted distortions and enhance images to increase image quality. In our model, we used the preprocessing techniques proposed by Hoshyar et al. (Philbin et al., 2007), they showed that the most beneficial techniques used in the preprocessing step for skin cancer detection are as follows:

i. Image enhancement: image enhancement aims to improve the visual appearance of an image. We applied two algorithms: image scaling to resize

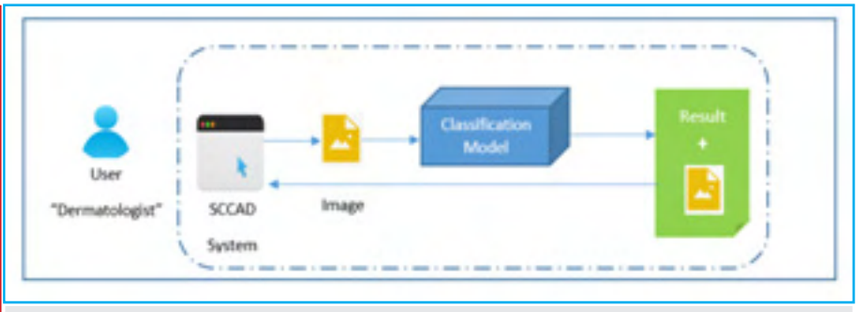

FIGURE 1. The proposed SCCAD system

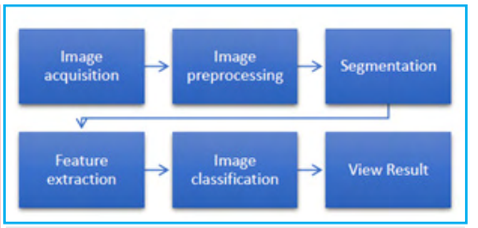

FIGURE 2. Proposed classification model general block diagram

image to 575*765 pixels and a symmetric image filter to enhance or highlight details of image as showing in the following equations:

Resize Image = imresize (RGB Image, [575,765]);

Where imresize is a MATLAB function that used to specify the size of the output image by passing a vector that contains the number of rows and columns in the output image.

Filter Image = imfilter (Resized Image, 'symmetric');

Where imfilter is a MATLAB function that used to apply symmetric filter on the image.

ii. Grayscale conversion: a grayscale image consists of brightness information. Grayscale images are faster and easier to process when compared to color images. Moreover, the images processing techniques are applied on the grayscale image (Jayaraman et al., 2009). We converted skin images into grayscale images to use in the segmentation component as showing in the following equation:

Convert Image = rgb2gray (Filtered Image);

Where rgb2gray is a MATLAB function that used to convert RGB image to gray image by removing the hue and saturation information while keeping the luminance.

iii. Image restoration: image restoration defines the process of retrieving a degraded image which has suffered noise or blurring. In the proposed model the wiener filter is used as showing in the following equation. It is a powerful technique used in medical applications to reduce the image noise and blur.

Wiener Filtered Image=wiener2(Gray Image);

Where wiener 2 is a MATLAB function that used to apply wiener filter on 2-dimensional image.

c. Segmentation: This component aims to divide images into multiple segments. This process helps to make the image's Region of Interest (ROI) easier to evaluate. In the proposed segmentation was done using Otsu thresholding method (Vala and Baxi, 2013). Otsu is one of the best thresholding methods and is widely used due to its effectiveness. We used global image threshold that using Otsu's method as showing in the following equation:

Level of Thresholding=graythresh (Gray Image);

Where graythresh is a MATLAB function that used to computes a global threshold to separate the background from ROI. The basic idea is to find the threshold that minimizing the weighted within-class variance and maximizing between-class variance for separating lesion in an image from the background based on their gray-level distribution. Threshold computed with the following equation:

 (1)

Where 1 means that the pixels corresponding to lesion or object while 0 to background. The weighted within-class variance is computed with the following equation:

$$\sigma_w^2(t) = w_1(t)\sigma_1^2(t) + w_2(t)\sigma_2^2(t) \quad (2)$$

Weights w_i are the probabilities of the two classes separated by the threshold t, and σ_i^2 are variances of these classes. Otsu shows that minimizing the within-class variance is the same as maximizing between-class variance σ_b^2:

$$\sigma_b^2(t) = \sigma^2 - \sigma_w^2(t) = w_1(t) + w_2(t)[\mu_1(t) - \mu_2(t)]^2 \quad (3)$$

Where σ^2 is the image pixels variance, μ_i are the class means. By computing the threshold, the lesion pixels correspond to the pixels with the values lower than t (Celebi and Schaefer, 2012).

d. Features extraction: extract relative features from a segmented image to be used by the classifier to identify melanoma. In the proposed model, three types of feature were extracted:

i. Texture: it describes it describes local brightness variation from pixel to pixel in a small neighborhood of the image (Russ, 1999). It also refers to the attributes or information representing the spatial arrangement of the gray levels of the pixels in regions of a digital image (IEEE, 1990). The Gray Level Co-occurrence Matrix (GLCM) was used as studies showed that it is the most effective and most cited method for feature extraction. GLCM represent how often different combinations of pixel brightness values (grey-levels) occur in an image. It considers the relationship between two neighboring pixels, the first pixel is

known as a reference and the second is known as a neighbor pixel (Almansour and Jaffar, 2016). The four statistical measures extracted from GLCM matrix are: contrast, energy, correlation, and homogeneity. Statistical measures are computed with the following equation:

GLCM=graycomatrix(Gray Image);

Where graycomatrix is MATLAB function that used to create gray-level co-occurrence matrix from image by calculating how often a pixel with the intensity (gray-level) value I occurs in a specific spatial relationship to a pixel with the value j. The co-occurrence matrix P of an image I of size N*N is can be defined as:

$$P(i,j) = \sum_{x=1}^{N}\sum_{y=1}^{N}\begin{cases}1, if\ I(x,y) = I\ and\ I(x+\Delta x, y+\Delta y) = j\\ 0, otherwise\end{cases} \quad (4)$$

Where the offset (Δr, Δy) represent the distance between the interested pixel and its neighbor.
The statistics information about the texture of an image are given in the below:

$$Contrast = \sum_i \sum_j (i-j)^2\ P[i,j] \quad (5)$$

$$Energy = \sum_i \sum_j P^2\ P[i,j] \quad (6)$$

$$Correlation = \sum_i \sum_j \frac{(i \times j) \times P(i,j) - (\mu_x \times \mu_y)}{\sigma_x \times \sigma_y} \quad (7)$$

Where μ and σ are the mean and standard deviations of probability matrix P.

$$Homogeneity = \sum_i \sum_j \frac{P[i,j]}{1+|i-j|} \quad (8)$$

ii. Shape: we used the ABCD rule (Asymmetry, Border, Color and Diameter) as it is the main geometric feature that best describes a melanoma lesion (Jain et al., 2015), the ABCD features are as follows:

- Asymmetry: to determine the area of the image of the mole. We calculated the asymmetry. Asymmetry Index (in pixels) is computed with the following equation (Hanon AlAsadi and M. Alsafy, 2015):

$$AI = \Delta A A * 100 \quad (9)$$

Where μ is the area of the total image, Δσ is the area of difference between total image and lesion area. For asymmetry increases, the ratio approaches closer to 0, otherwise reach to 1.

- Border: the irregularity of the mole border is a feature for melanoma. Irregularity index is a function of area (A) and perimeter (P). it is calculated (in pixels) using the following equation (Hanon AlAsadi and M.Alsafy, 2015):

$$IR = (4\pi A)/P2 \quad (10)$$

For the border to be irregular, the value of IR must reaches 0, otherwise 1.

- Color: to determine if the mole is composed of a non-uniform color, we evaluated the color distribution in the skin lesions. The skin lesion contains combination of three or more such as red, dark brown, and light brown; we declared it as melanoma.
- Diameter: the diameter of the mole if greater than 6mm (22.6772 pixels) then it will be considered as melanoma. We calculated the diameter as the distance between each pair of points.

iii. Color: color is one of the important features used as a descriptor to identify melanoma. We extracted four statistics (usually called color moments or color feature) to identify the colors in the segmented lesion regions. These are mean, standard deviation, variation and skewness. We converted RGB image into HSV (Hue, Saturation, Value) and extracted color moments as showing in the following equation:

HSV Image = rgb2hsv (RGB Image);

Where rgb2hsv is a MATLAB function that used to convert RGB image to HSV image.

e. Image classification: Image classification refers to the process of classifying the input image into one of the two groups: melanoma or non-melanoma based on the extracted features from the image. For building the classification model, two classifiers (SVM and ensemble) were tested and evaluated based on their performance (more details in section 4 Results and Discussion). Recent work on melanoma classification have proven that SVM is one of the most effective classifiers with a high degree of accuracy (Thamilselvan and Sathiaseelan, 2015). Ensemble is also a good classifier and can improve predictive accuracy if used in a hybrid model (Rahman and Tasnim, 2014). Results showed that SVM gave better performance than ensemble, according to that it was used in building the classification model. Based on this result, SVM was also selected as the classifiers to be used in the SCCAD system.

f. View Result: in this step the result of classifying each image. The two classifiers return results by predictFcn(X) function either 1 for melanoma or 0 for non-melanoma. The features are the input, while the output either 0 or 1.

The proposed methodology of discrimination between melanoma or not is shown in Figure 3.

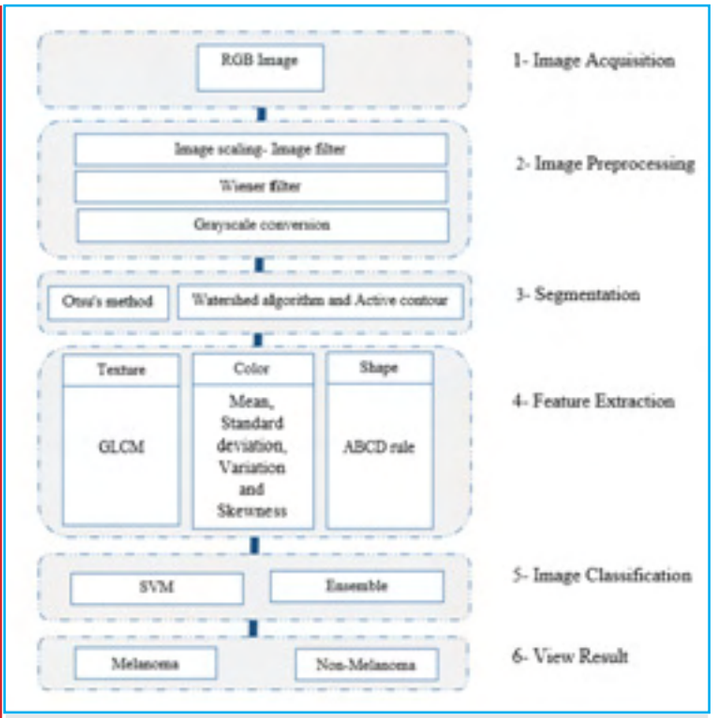

FIGURE 3. Techniques and algorithms that used in the classification model

B. Design and structure of proposed SCCAD system

The main components of the proposed SCCAD system are: image acquisition, image preprocessing, segmentation, features extraction, and image classification. The classification component uses the proposed classification model and the extracted features to return the prediction result. Figure 4 shows the architectures of classification model and the SCCAD system. The same process

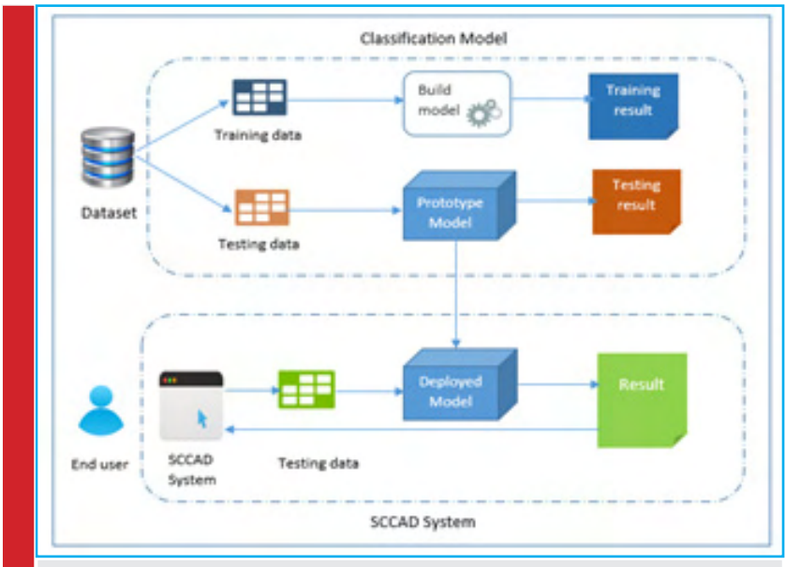

FIGURE 4. at the top: Classification model architecture and at the bottom is the SCCAD system architecture

used in building the classification model (Figure 2) is used in the proposed SCCAD system in its components apply the same algorithms used in the proposed classification model which are explained above.

C. Implementation

To develop both the classification model and the SCCAD system, we used MATLAB R2018a, an image processing toolbox, as well as statistics and ML toolboxes.

For developing and testing our proposed classification model and SCCAD system, we used 151 images. The images have been collected from the PH^2 dataset and from dermatology database used in MED-NODE . PH^2 dermoscopic images database were obtained from the Dermatology Service of Hospital Pedro Hispano (Matosinhos, Portugal). The PH^2 database consist of 200 images of melanocytic lesions. 40 of them are melanoma, 80 are common nevi, and 80 are atypical nevi. These are 8-bit RGB color images with a resolution of 768*560 pixels. Furthermore, MED-NODE's dataset consists of 70 melanoma and 100 nevus images from the digital image archive of the Department of Dermatology of the University Medical Center Groningen (UMCG) used for the development and testing of the MED-NODE system for skin cancer detection from macroscopic images.

In order to have a good accuracy, we tried to balance the number of melanoma and non-melanoma images to be 51% (76) and 49% (75) respectively, equaling (151) as a total. We used 90% (136) of images for training and testing process, and 10% (15) for testing by the end user who uses SCCAD system. To increase the learnability and enhance the accuracy of the model, a preprocessing step for training images is important. One of the preprocessing operations was cropping, (for the images with the black corners) because the segmentation algorithm segment it as lesion based on its darker color and it appeared as a lesion to the segmentation algorithm instead of the correct lesion. Figure 5 shows the segmented image before and after crop with the black corners. For hair removal, we used software called Dull Razor to remove thick hairs manually. Figure 6 shows the image before and after Dull Razor usage.

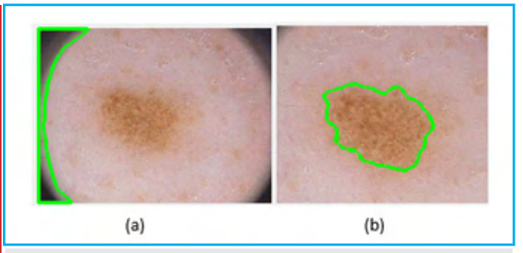

FIGURE 5. (a) Segmented image before and (b) after crop the black corners

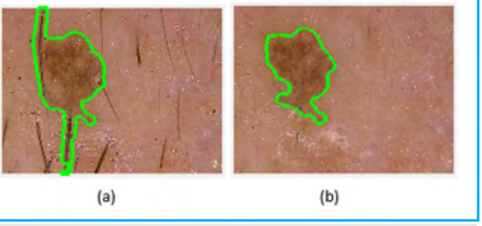

FIGURE 6. (a) Image before and (b) after Dull Razor usage

RESULTS AND DISCUSSION

The proposed system combines two main sub systems: the skin cancer classification model and the SCCAD system itself. To evaluate the performance of the proposed model, we used the K-Fold cross validation and confusion matrix, while for evaluation our proposed SCCAD system by performing informal study with Human Computer Interface (HCI) experts. In the following subsection we will discuss these evaluation processes.

A. Evaluating the performance of proposed classification model

To measure the quality of our classification model, we used the K-fold cross validation as the evaluation method. We set it to 10-fold cross-validation. In the 10-fold, the dataset randomly partitioned into 10 equal size sub-dataset, one sub-dataset used for testing the model and the remaining 9 sub-datasets used for train the model. The cross-validation process is then repeated 10 times (the folds), with each of the 10 sub-datasets used exactly once as the validation data. The 10 results from the folds can then be averaged (or otherwise combined) to produce a single accuracy. We used cross-validation instead of conventional validation (70% training, 30% testing) because the use of a single dataset for testing may not reflect the true accuracy our model .

After training the model and obtaining the average accuracy, we obtained the confusion matrix, sensitivity, and specificity. The accuracy is best when using all 20 features: texture, color, and shape.

i. Confusion matrix

The confusion matrix (Agolytics) describes the performance of the classification model and shows the way in which the model is confused when it makes a prediction. The matrix is an N*N matrix, where N=2 is the number of classes that being to predicate. We used the following terminologies:

- True positive (TP): correct classification of melanoma 1.

- True negative (TN): correct classification of non-melanoma 0.
- False positive (FP): incorrect classification as melanoma.
- False negative (FN): incorrect classification as non-melanoma.

Figure 7 shows the confusion matrix of SVM, while Figure 8 shows the confusion matrix of ensemble.

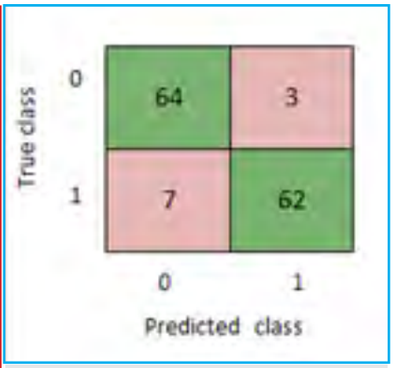

FIGURE 7. Confusion matrix of SVM

FIGURE 8. Confusion matrix of ensemble

ii. Accuracy

The overall accuracy is the measure of effectiveness of classification algorithms. It is defined as the overall correct classification of melanoma and non-melanoma images to the total number of images as follows:

$$\text{Accuracy} = (TP+TN)/(TP+TN+FP+FN)$$

In other words, the classification accuracy is the ratio of correct predictions to total predictions made.

$$\text{Accuracy} = \text{Correct predication} / \text{total predication} * 100$$

The accuracy values of SVM and ensemble are 92.6% and 91.1% respectively.

iii. Sensitivity

The sensitivity (also called the true positive rate, recall) is defined as the correct classification number of melanoma divided by total number of melanoma. The sensitivity of the classification model is defined as follows:

$$\text{Sensitivity} = (TP)/(TP+FN)$$

The sensitivity values of SVM and ensemble are 90%, 87% respectively. In the SVM classifier, it means 90% of the images were classified correctly as melanoma and 10% of images were wrongly kept as non-melanoma. Furthermore, in the ensemble, it means 87% of the images were classified correctly as melanoma and 13% of images were wrongly kept as non-melanoma.

iv Specificity

The specificity (also called the true negative rate) is defined as the correct classification number of non-melanoma divided by total number of non-melanoma. The specificity of the classification model is defined as follows:

$$\text{Specificity} = (TN)/(TN+FP)$$

The specificity values of SVM and ensemble are 96% and 96% respectively. In the SVM classifier, it means 96% of the images were classified correctly as non-melanoma and 4% of images were wrongly kept as melanoma. Furthermore, in the ensemble, it means 96% of the images were classified correctly non-melanoma and 4% of images were wrongly kept as melanoma. Figure 9 shows the sensitivity and specificity of SVM, while Figure 10 shows the sensitivity and specificity of ensemble.

As a performance comparison between SVM and ensemble, the SVM is better than the ensemble. So, we applied the SVM classifier in our SCCAD system. Table 1 summarize the differences between them.

B. Evaluating the usability of proposed SCCAD

To evaluate the SCCAD system, two HCI experts have inspected and examined the SCCAD system to identify possible usability problems. Their evaluation leads to important findings and recommendations which ultimately beneficial to the improvement and enhancement of the UI. This study with HCI experts was directed by the main study research questions in Table 2. The main recommendations for the UI were as follows:

- No need to show the values of the extracted features and displaying only the information that

FIGURE 9. Sensitivity and specificity of SVM

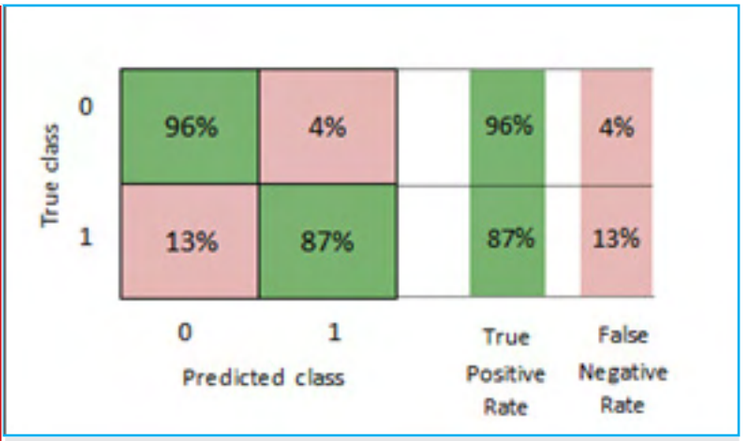

FIGURE 10. Sensitivity and specificity of ensemble

Table 1. Comparison between SVM and ensemble

	Accuracy	Senstivity	Specificity
SVM	92.6%	90%	96%
Ensemble	91.1%	87%	96%

beneficial to the dermatologist without unnecessary technical terminology.

- Add at the re-upload image task, they suggest using the same button for upload and re-upload.

Table 2. Main objectives and usability research questions

No.	Objectives	Research questions
1	To determine how satisfactory, efficient, and effective the SCCAD system	- Do experts understand our system interface components and purpose? - Is the information on the help page helpful and readable? - Do the experts satisfy the system of the UI? - Do the experts find the system unnecessarily complex?
2	Identify any usability problems	- What could prevent the users from completing the tasks based on experts options? - What problems might users have with system based on experts options?
3	Finding ways of improving the usability of our system	- Do experts have suh suggetions for improvement?

- Add an option for saving the system's status in case of failure or undesirable action.
- These recommendations helped in enhancing the usability of the proposed SCCAD system.

CONCLUSION AND FUTURE WORK

Skin cancer is a very serious type of cancer diseases and Melanoma is the most dangerous type of skin cancer that may cause death. If early diagnosed, skin cancer can almost be curable. In this paper we proposed a CAD system that helps dermatologist in the diagnosis of skin cancer (melanoma or non-melanoma) using image processing and ML classification algorithms. For this purpose, we developed a classification model and a SCCAD system. We started by building and evaluating the classification model by training and testing two classifiers, SVM and ensemble, and using 20 features of texture, color, and shape. SVM gave better results than ensemble based on the K-fold cross validation with k-10. The next step after developing the classification model was to design, implement and evaluate the SCCAD system which is the main goal in this work. The SCCAD system provided the dermatologists with many features that help the dermatologist in the diagnosis process. The classifier used in SCCAD was SVM according to the results found in the phase of developing classification model. An evaluation by HCI experts was performed to enhance the usability of the SCCAD system and their recommendation were implemented. For futurework, hair removal and image cropping techniques can be integrated in the SCCAD system as part of the preprocessing steps. A hybrid model of classification by used to enhance the accuracy of prediction. Improving the classification model is possible by using another dataset to increase the learnability of classification model.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the support by the "Research Center of the Female Scientific and Medical Colleges", Deanship of Scientific Research, King Saud University. They also would like to acknowledge the HCI experts, Dr. Shiroq Al-Megren and Dr. Balsam Al-Sugair from King Saud University Riyadh, for their contribution in evaluating the usability of the proposed SCCAD system.

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Medical
Communication

Biosci. Biotech. Res. Comm. 12(2): 309-316 (2019)

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Preliminary analysis of perception, knowledge and attitude of home health patients using tele rehabilitation in Riyadh, Saudi Arabia

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ABSTRACT

Telerehabilitation is defined as delivery of rehabilitation services over telecommunication networks and the internet, which comprise of clinical assessment (the patientɔs functional abilities in his or her environment) and clinical therapy.This new area of medical advancement, using state of the art technology is developing at a great speed and is definitely going to be the next milestone in health care revolution.The objective of this study wasto explore the awareness, knowledge and perception of the patients for using telerehabilitation as a medium to provide physiotherapy services as a part of home healthcare services.A pretest-post test design was used where thehome healthcare patients (n = 90) aged between 50 -75 years were asked to express views by given a validated modified TUQ questionnaire followed byan indepth interviewing to develop a key understanding regarding the themes. Interviews were transcribed and a qualitative thematic analysis was conducted. The awareness level regarding the telerehabilitation changed significantly from 57% to 96% post session($p<0.05$). Similarly, the knowledge of the participants regarding online consultation, followup and online therapy changed significantly from 50%, 47% and 57% to 96%, 76% and 96% respectively post session of rehabilitation($p<0.05$). The perception level regarding the key benefits including its usage in emergency(83%), convenience of no travel(84%), ease of getting treated at home(97%) and availability of specialist consultation (84%) were the prime ideas for excellent rating among 95% participants ($p<0.05$) post session.Findings are helpful to health practitioners in designing their intervention programsacross the kingdom. However the actual impact could be only derived from future studies which has to conducted based on different clinical conditions.

KEY WORDS: HOME HEALTHCARE, TELEREHABILITATION, PHYSIOTHERAPY

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Received 12th Jan, 2019
Accepted after revision 12th March, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/13

INTRODUCTION

Telerehabilitation is defined as the provision and delivery of rehabilitation health services at a distance using information and communication technologies and tools (Tan 2005; Russell 2007). Throughout the world, the health care practices is going through major transformation as it is driven through sea change because of the increased use of technology. The kingdom of Saudi Arabia too is witnessing a massive change with significant restructuring of healthcare systems with some major high-end technology driven development solutions. The increased demand is created on account of rapidly increasing saudi population including the growing elderly community, changing disease patterns, global climatic changes and financial inequity (Mahmood 2018). According to a United nations report the elderly population of Saudi Arabia thoseaged 60 and above is projected to increase from 3% in 2010 to 9.5%and 18.4% in 2035 and 2050, respectively (UN Report, 2018).

Similarly, comparing this phenomenon to an average life expectancy of the population in Saudi Arabia, the latest WHO data published in 2018, suggests that Saudi male and female have an average of 73.5 and female 76.5 life years with an average life expectancy of 74.8 years as against an average world life expectancy of 84 years. The increased demand in kingdom also raised because of immense economic pressure with steep fall in global oil prices in 2015-16 affecting the GDP significantlythereby been one of the key stimulus for the government to take timely corrective actions and diversify the economy from heavily oil dependent to develop other verticals for revenue generation (MoH Report, 2018).

Brianchild of Crown Prince HH Mohammad Bin Salman, Vision 2030 was adopted in April 2016 and has identified its priorities across all economic sectors and serves as a roadmap for the economic development of the KSA with development of health services been one of the most important key themes. Therefore, as a part of realization of this vision the government strongly supports the partnership of private and public sectors and been seen as a strong indication of the Government's commitment for making healthcare accessible to its citizens irrespective of the disparities available in the Saudi society (Vision 2030 Report, 2016). Access to healthcare generally relates to people's ability to use health services when and where they are needed. Determinants of healthcare access are the types and quality of services, including the costs, time, distance (ease of travel) as well as regular interface between service users and healthcare providers. Saudi Arabia is the largest and fastest growing health care market in the region and is estimated to reach $40 billion by 2020 (NTP 2020 Report, 2016).

Moreover, the steep increase in the number of hospitals across all major cities of KSA are run by both government and private organizations which use corporate business strategies and technology driven specializations, which aim to create demand as well as attract high number patients as the facilities in majority of these hospitals are world class.

Among the various strategies listed in the NTP Report 2020, one of the key components of making healthcare accessible across the kingdom is the enhanced use of telemedicine (NTP 2020 Report, 2016). In the last one decade the health services across the kingdom have taken gigantic leap jumps with private healthcare taking lead and using innovations in delivering healthcare. One of such innovations is using Home Healthcare for delivering physiotherapy and other rehabilitation based services for the patients at home (Pulse Report 2018).

Rehabilitation is a very important component in medical care and helps in propelling patient to preinjury level. It is a well known fact that in all long term cases which requires follow-ups such as in surgical cases and other debilitating disorders including Stroke, Cancer, Multiple Sclerosios, rehabilitation is time consuming and financially constraining. To add to this, patients travelling long distances for treatment, it is not only physically challenging but emotionally draining too and especially in case of geriatric patients.Therefore home tele rehabilitation programs, are winding up progressively as an elective method of service delivery. In the western countries, quite a number of research studies has been proved that the Telerehabilitation for the delivery of health services is quite effective, however the scope of using such services in the kingdom is still novice and requires a detailed study, (Hailey et al., 2010, Johansson and Wild 2011, Chang et al 2019).

There are scant studies to prove its efficacy in the developing countries as its successful willdepends on a number of factors (Clemens et al 2018) . However, among all the variables, the two most important are the technological component and second been its implementation in real terms (Jackson and McClean 2012, Clemens et al 2018). Accordingly,these both are of extremecritical importance from the patient satisfaction point of view. The perceptions of the stakeholders, i.e. the patient and the members of the Rehabilitation team are of utmost importance for its use and wide spread application. The home healthcare services in Saudi Arabia is still in infancy stages with few delivery partners across the kingdom. The usage of telerehabilitation is even more nascent, as the perception of patients in using such a technology for delivering healthcare would be quite critical and important to understand the phenomenon which would be quite useful in framing the guidelines for its applications at a mass level, (Alaboudi et al 2016).

Therefore, this study is an attempt to study the awareness, knowledge and perceptions of the home healthcare patients in using physiotherapy services delivered via cloud based telerehabilitation. This study, to our knowledge is the first of its kind in the kingdom especially from the perspective of home healthcarepatients. It aims to explore the key ideas which might work in favour or against the successful implementation of telerehabilitation used for the home healthcare delivery.

MATERIALS AND METHODS

The pretest-post test study designwas conducted on home healthcare patientsso as to obtain an in-depth understanding of the patients' perception abouttelerehabilitationservices whichthey will receive as a part of home health services. While a few studies conducted earlier emphasized abouttelemedicine to be a key part in delivery of health services, however none of the studies emphasized on perception of patients to implement telerehabilitation as part of home healthcare (Clemens et al 2018, Khalil et al 2018).

Due necessary approval were taken from the ethical clearance committee of the respective organization, which is a reputed home healthcare organization based in Riyadh. In order to recruit participants for the study,sample population were selected from a pool of home healthcare patients who were undergoing treatment under one of the most prominent home healthcare organizations in the kingdom, which incidentally was the only first licensed stand-alone home healthcare services company in Riyadh province.

The study was conducted from Jan15 to May30, 2019. In this context, non-probability sampling method was used. Out of 113 home healthcare patients who underwent treatment for different ailments, 90 were randomly selectedwho also gave their consent to participate in the study out of which 57 were males and 33 were females. Those patients who suffered from orthopedic problems such as Knee pain, low back ache, disc prolapse etc. or underwent orthopedic surgeries such as knee replacement or meniscectomyetc. participated in the study. The study mainly included common geriatric patients for the study who were willing to participate but excluded the pediatric and the critical care, neurological and cardiac patients as they underwent major surgeries such as for stroke or CABG and also were unable to respond directly to answer the questions. The patients who were able respond in English or Arabic were recruited for the study.

Based on literature review and discussion with key stakeholders, aquestionnaire and an the interview guide was prepared, modified from Telehealth Usability Questionnaire (TUQ) based on key themes of perceived usefulness, ease of use and learnability, Interaction quality, Reliability and Satisfaction and future use (Langbecker et al 2017) . The questionnaire was converted to Arabic version adapted from the original English version and pilot tested for the home healthcare patients using both forward and backward translation methods and achieved very acceptable score of confirmatory factor analysis of 0.78 using SPSS. It was also pilot tested for the members of the rehabilitation team. The questionnaires as given in Appendix 1 were responded by the patients and the members of the rehabilitation team followed by a semi-structured individual interview from the patient as well as from the team membersinvolved in providing home health services. The interviews were audio recorded and transcribed verbatimusing Text Analysis Markup System (TAMS) Analyzer as suggested by Yin (Yin 2013).

The Tele-rehabilitation Technological solutions were a part of home health services which were delivered by the company. As a part of cloud based HIPAA compliant network, the telemedicine unit consists of a portal to track health metrics and rehabilitation treatment plan and progress by the PT specialists as well as the Case Managers. The system included case briefing, consultation by specialists as well as providing physiotherapy sessions both by Home health therapists or via health workers such as PTAs within the vicinity of home environment at patient's ease as schematically represented in Fig. no. 1.

The participants were given a pre and post session modified TUQand asked to reflect on their entire reha-

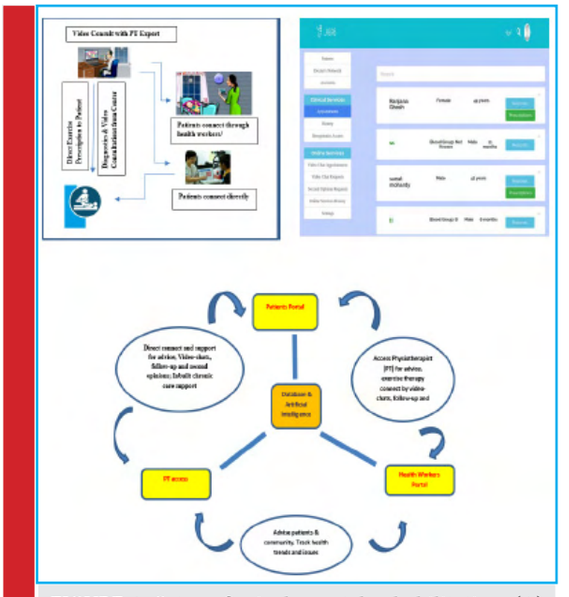

FIGURE 1. Set-up for in-home telerehabilitation: (A) Framework system; (B) dashboard Screen (C) Integrated loop with benefits

bilitation experience using the Telerehabilitation platform so as to get relevant information about telemedicine services including key events such as finding out they would receive services at home by videoconference, having the internet and videoconferencing equipment installed at home and receiving services by videoconference including dealing with technical issues. Following the same detailed interview was taken using the TAMS so as to identify key ideas which can affect usage of telerehabilitation.. Statistical tests was conducted using SPSS for Pre-post differences evaluation. using paired *t*-tests to assess factors associated with awareness, knowledge and perception. Significance was set a *priori* at $p < 0.05$.

RESULTS

The characteristics of the patients who participated in the study that could impact on the patient's experience of telemedicine (e.g., gender, age, educational qualification, Marital status and their medical condition) were presented in Table 1.

Table 1. showing demographic characteristics of home healthcare patients

Patient Characteristics	N =90
Gender	Male = 57 Female =33
Age	65.8 ± 9.4 (M) 58.7 ± 7.8 (F)
Educational Qualification	Illiterate = 3 Upto Primary Education = 9 Upto Secondary education = 36 Upto Higher secondary = 21 Graduate & above = 21
Marital Status	Married = 81 Unmarried = 3 Widowed = 6
Medical Condition	Orthopedics = 36 Neurology = 24 Sports = 15 Others = 15

The modified TUQ was given to the participants to measure their awareness, knowledge and perceptionfor using Telemedicine services for Home healthcare physiotherapy services They were asked a set of questions to evaluate their understanding about the novel concept, followed by which according to their comfort were given 1 session of telemedicine to get them acquainted to the concept post which again were evaluated for their understanding and perception about the technology and its usage.

On evaluating their awareness level about the conceptpretraining 70% (n = 63) of the participants confirmed about having heard about the concept. However out of which only 57% (n = 51) could actually knew about the concept correctly. However post session, this number was 100% percent, however 7 % still made mistake and could not answer it correctly. Similarly, been asked about the whereabouts of using telerehabilitation in Riyadh, only 57% knew or have heard about it however only 4% confirmed it of having it used earlier. The awareness level about the telerehabilitation and its nature changed significantly post session as the number swelled to 93% (n = 84) having stated the definition correctly and again 96% have known it been used by an organization called Labas, delivering home healthcare using such a technology (Table No. 2)

The second part of the questionnaire measured the knowledge. While 50% and 47% had an idea of using telerehabilitation for online consultation and online therapy respectively, 57 % had an idea of using it for post-surgery follow-ups. This perception changed significantly after training the participants with 96% been confident of using it for online physiotherapy consultation. The session also saw significant change in knowledge of participants of using the same for using it for online physiotherapy sessions as well with 76% been confident about its usage (Fig. 2). Similarly, the usage of tele-rehabilitation for followup consultations saw a significant change in patients knowledge with number reaching to a whopping 96% from 57% seen earlier ($p^{***} = .0001$) The participants were interviewed further to also seek about their knowledge and understanding about key benefits of using such a technology for imparting services. It wasfound that the perception of the majority of participants changed from (50%) pre session to 97% post session who felt, the ease of

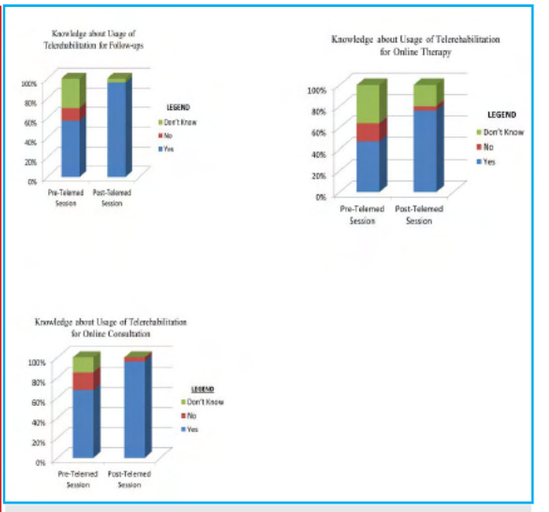

FIGURE 2. Depicting the knowledge about usage of Telerehabilitation

Table 2. Showing the assessment criteria for measuring awareness, knowledge, perception and satisfaction level of home healthcare patients with relation to the usage of pre and post telerehabilitation session

Patients (n =90)			Significance
Assessment Criteria	Pre-Telemed session	Post Telemed session	
Awareness of Telerehabilitation	Yes = 63(70%) No = 27 (30%)	Yes = 90 (100%) No = 0	
Awareness about definition of Telerehabilitation	Right = 51 (57%) Wrong = 20 (23%) Don't Know = 19 (20%)	Right = 84 (93%) Wrong = 6 (7%) Don't know = 0	p = 0.007***
Awareness about usage of telerehabilitation in Riyadh	Yes = 51 (57%) No = 30 (33%) Don't Know = 9 (10%)	Yes = 86 (96%) No = 0 Don't Know = 4 (4%)	
Knowledge about telerehabilitation usage for online consultation	Yes = 45 (50%) No = 12 (13%) Don't Know = 33 (37%)	Yes = 86 (96%) No = 4 (4%) Don't Know = 0	
Knowledge about telerehabilitation usage for follow-ups	Yes = 42 (47%) No = 16 (17%) Don't Know = 32 (36%)	Yes = 68 (76%) No = 4 (4%) Don't Know = 18 (20%)	P= 0.0001***
Knowledge about telerehabilitation usage for online therapy	Yes = 51 (57%) No = 12 (13%) Don't Know = 27 (30%)	Yes = 86 (96%) No = 0 Don't Know =4 (4%)	
Perception about telerehabilitation usability in imparting home health services	Yes = 45 (50%) No = 15(17%) Don't Know = 30 (33%)	Yes = 86 (96%) No = 4 (4%) Don't Know = 0	
Perception about key benefits of using telerehabilitation	Need not to Travel = 39 (43%) Ease of Home = 45 (50%) Availability of Specialist advice anytime = (41%)	Need not to Travel = 76 (84%) Ease of Home = 87 (97%) Availability of Specialist advice anytime = 75 (84%)	P = 0.006***
Perception about telerehabilitation usage in emergency	Yes = 48 (53%) No = 9 (10%) Don't know = 33 (37%)	Yes = 75 (83%) No = 3 (3%) Don't know = 12 (13%)	
Satisfaction regarding usage	NA	Yes = 81 (90%) No = 9 (10%)	
Rating about tele-rehabilitation as a modality for home health services	Excellent = 58 (64%) Good = 12 (13%) Poor = 20 (23%)	Excellent = 85 (95%) Good = 3 (3%) Poor = 2 (2%)	P = 0.0004***

getting treatment at home at your convenience is the best among all the benefits, with the comfort of not been having to travelfor treatment been responded by (84%) which was earlier found to be 50% (n = 45). The additional benefit of seeking any specialist advice anytime (84%) also added to the advantages of using telerehabilitation services for delivering physiotherapy treatment.

On evaluating the satisfaction level the participants were asked whether they would use the telerehabilitation services in general and in emergency condition. It was found that the participant number changed significantly from 64% (pre-session) to 94% (post session) in case of general usage and 53% (pre-session) to 83% (post session) in case of emergency respectively. Seeking Satisfaction level, it was found that a significant 90% were satisfied with the overall experience. Similarly, 90% rated it as good to excellent as a servicepost session. The hallmark finding about this aspect was that the 77% among 90% labelled the services as excellent post session as compared to 33% who termed it as excellent (pre-session) (Table 2).

DISCUSSION

Telemedicine innovations is one of the major developments of the present century. It has immense potential to change the phase of the healthcare industry and will prove to be a game changer in the coming times. Saudi Arabia is going through major reforms, as apart of Vision 2030, under National Transformation Program, Healthcare is one of the key areas of non-oil sources of revenue generation. Under the health reforms, the MoH has suggested 70 reform areas under which telemedicine is one of the focus areas (NTP 2020 Report, 2016). In the current study, we have focused on understanding the

market and its potential through evaluating the awareness, knowledge and perception of the Saudi citizens regarding the usage of telemedicine. Telemedicine is a wide area and we have restricted our focus on the use of telerehabilitation, which is one of the branches of telemedicine to substantiate our claim.

From the study, majority of the participants agreed that the telerehabilitation treatment as apart of home healthcare treatment serves as a good adjunct and have some great benefits. Upon analysis, based on the the patients awareness level, knowledge base and perceptions regarding the use of this this new technology could be clearly categorized in four main themes. The in detailed interview of the patients and using the verbatim quotes illustrate the results through which we have categorized the themes.

It was clearly evident from the results that once the participants underwent the training regarding the telerehabilitation usage almost all the participants had developed an understanding regarding its usage and benefits as well as its accessibility in Riyadh area. While discussing the benefits quotient, it was evident that improved access to the service in terms of not been having to travel long distances or far away places for treatment is the foremost benefit. On evaluating this phenomena with respect to the age group of the patients it was found that since majority of the patients (83%) were of geriatric category, proved to be statistically significant ($p < 0.05$) and definitely this advantage would be of immense value and will be one of the key USP of using this technology in the long run. This correlates to the finding of Tousignant et al., who conducted a study in Canada for the usage of telemedicine on post surgery rehabilitation among elderly patients and found that all participants gave a thumbs up to use this technology (Tousignant 2009). The total number though were only 5, where as in our study the number is 30. On interviewing deeper on further benefits regarding the elimination oftransportation time for the patient, it was suggested that it is not only the transportation which matters but the family had to make lot of arrangements to facilitate the transportation. Majority of the participants (80%) echoed the sentiment and said that they liked the idea of (telerehabilitation) and found it great to use. Since every time earlier I wanted to go, they felt bad since one of their family members, be it son or daughter had to make lot of preparations in terms of taking leave from his office, meeting and visiting the doctor, following up with the progress to name a few. All this could be prevented now I feel." Since majority of them were elderly, been too much dependent was one of the most critical factors that they were concerned about.Another significant observation that evolved from the discussion was that it was not just the travel that caused problem, the pain and the hassle of getting prepared especially after knee arthroplasty or disc surgery was of immense cause of discomfort for the patients. This phenomenon was observed not just the older population but also of concern for the middle aged as well.

In the present study, contrary to the previous quantitative study conducted the key factors which could affect the outcomes related to the satisfaction of patients concerning tele-rehabilitation (Russell 2007) were identified not only in terms of using a standard questionnaire but also taking indepth interviews which a covered a larger spectrum of the patient's experience.Majority of the respondents regarded the telerehabilitation videoconferencing technology would not be a deterrent to receiving quality rehabilitation services. However, two participants did express their reservations and gave preference for combining telerehabilitation with more traditional in-person services. These findings differed from that a study conducted by Hailey *et al*. (Hailey et al 2010), where the patients reported their perception regarding telerehabilitation based on a conversation where the participants were given an introduction regarding in-home telerehabilitation using various examples of telerehabilitation services, without actually giving them demonstration or getting them feel the experience.

In yet another studies reported (Johansson and Wild 2011, Jackson and McClean 2012) patients who were randomly allocated and made to use aweb-based exercise program so as to get the feel of telerehabilitation, the experience was expressed as positive as far as the ease-of-use andusefulness was concerned in comparison to those who were explained verbally and not experience it. Therefore the results found in the study might be one of the critical factors to explain the positive responses regardingpatient's perception with respect to telerehabilitation.In some of the studies conducted in western countries the perception was expressed after going through actual hands on training. It was reported by Johansson and Wild (Johansson and Wild 2011) that tele-rehabilitation used for treating stroke patientsreceived positive feedback and felt that adequate guidance and appropriate exercises could be conducted via this medium from a distance without actually compromising on the quality.

Even though this type of arrangement was outside the scope of the present study, however future researches could be conducted using the platform for providing rehabilitation for various clinical conditions and could be evaluated in comparison to the face to face therapy sessions. While interviewing another key component expressed was regarding the relationship of trust, comfort and easeregarding the patient and therapist. It was expressed by few participants that as far as the therapist who provided the treatment sessions in hospital or at home would take telerehabilitation sessions there would be absolutely no issues and would increase the satisfac-

tion of the patient. However, whether this criteria would effect the outcome is another debate. A study by Alaboudi et al. had discussed about some of the these types of barriers, which has to be tackled effectively so as to make telerehabilitation quite a success as far as home healthcare is concerned (Alaboudi et al 2016).

Some of participants felt that telerehabilitation should be used in adjunct with face to face therapy with on & off treatment sessions across a time period and would serve better in causing more satisfaction among the patients regarding the outcome. They expressed that differences between perceptions could actually depending on the medical condition and combination of in-person and distance services through telerehabilitation would be a better choice as compared to telerehabilitation alone. The study undertaken is a step forward to improve accessibility and availability of health services and henceforth in consistence with the emerging trends for the future medical care that is looking for immense growth as also reported by Khalil et al (Khalil et al 2018).

Therefore, in general, patients consistently reported more positive views of telerehabilitation which can have immense impact on the rehabilitation services in the manner they are executed and perceived at present. The present study therefore has helped in understanding some of the key aspects of theses perceptions and could serve as an effective platform to develop better understanding in framing the scope of services, effective utilization, delivery and execution of telerehabilitation based physiotherapy and other rehabilitation services across the kingdom especially with context to home healthcare. The study will help in understanding the usage, scope and perception of telerehabilitation based services in home health care. Though the study was based in Riyadh with limited number of patient base, future studies could be conducted using perceptions of patients from various cities across the kingdom including both urban and rural and could be used to understand whether the perceptions vary depending on geographical locations, gender, age groups etc. The future studies could be conducted by exploring the perception and satisfaction level of the patients with reference to actual home therapy been given across a wide range of medical conditions and been followed for a longer time duration. Similarly, other studies could be conducted comparing the perception of patients receiving different treatment modalities which could help explore further relationships between clinical outcome and patient perceptions.

CONCLUSION

This present study showed that participants were satisfied with most of the aspects of telerehabilitation while given as a part of home health treatment and gave valuable insights so as to develop the therapy further and help in leveraging its scope of practice with clear instructions, guidelines and delivery codes. As a part of vision 2030, this method of delivering therapy could be used as a very important component for not only facilitating the services but also have immense potential which can be leveraged at various degrees in different forms to reap economic benefits.

ACKNOWLEDGEMENTS

We thank all the participants who agreed to be part in this study as well the entire home health care team of Labas Home Healthcare which helped in conducting this study.

CONFLICT OF INTEREST

The authors report no conflicts of interest.

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Medical
Communication

Biosci. Biotech. Res. Comm. 12(2): 317-323 (2019)

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A cumulative physiotherapy education program assessment in Jazan university: Need for a healthy society in Saudi Arabia. A retrospective study

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ABSTRACT

The morbidity, co-morbidity and mortality rate is horrendously high in Saudi Arabia where road traffic accident (RTA) being the major cause. In the last 2 decades 7% population results in permanent disabilities. Due to this towering rate of RTAs, they suffer with physical, mental, socio-economic stress. For respective community needs focused Physical Therapy educational programs are constructed to reduce physical and economic impact on them also purpose of freedom from disability and improving the quality of life became of prior importance. This study is a retrospective study conducted collectively of students in the Physical Therapy department, Jazan University from 2009 to 2019. Data was collected and filtered on various criteria using Microsoft Excel to make it appropriate for the study Measure of Central Tendency (Mean Score) was used to calculate Highest and Lowest GPA for male and female students. Ratio analysis was done at the end to check the male and female faculty staff ratio and to analyze any significant differences between them. The Physical therapy graduate students from Jazan university the society by providing service at various hospitals, health care institutions, rehabilitation centers for the handicapped, defense establishment, special need & physically disabled children and community health centers

KEY WORDS: DISABILITIES, HEALTH CARE, PROFESSIONAL DEVELOPMENT, E-REGISTER

ARTICLE INFORMATION:

Corresponding Author: mshaphe@jazanu.edu.sa
Received 21st April, 2019
Accepted after revision 25th June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/14

INTRODUCTION

Over the last 2 decades, the road traffic accidents (RTAs) have rang the alarm bell for procuring a high number of casualties approaching around 86,000 and more than 611,000 injured victims with 7% of them sustaining permanent disabilities (Barrimah Iet al., 2012, Ghaffar et al., 2015). Following these, individuals suffer from various disabilities (physical, mental, socio-economic) which plant unwarranted burden on personal and Kingdom's economy. To reduce these impacts and improve the overall quality of life Physical therapy (PT) being a cost and efficient mode of treatment became a need of time for which PT teaching program became necessary which ascended in different universities all over Saudi Arabia. King Saud University, Riyadh commenced PT program around 33 years ago (Threlkeld 2007). The PT program was started late in Saudi Arabia when compared to other nations (Alghadir 2015, Threlkeld , 2007 and Hager-Ross 2007)

Since then, over the years many universities (mainly governmental) are running successfully PT program across the kingdom. Among universities in Saudi Arabia, lays a key university in the southern part named "Jazan University" which started PT program in the year 2009-2010, which has been operational since its commencement and running successfully in terms of theoretical teaching, hands on practical, mode of examinations, student development and research activities. The study was commenced with the aim to draw attention to the existing and future needs for a professional educational program in the southern part of Saudi Arabia to reduce sufferings from various physical, mental, social and economic disabilities by means of Physical therapy (PT). So, an education program to serve this purpose of freedom from disability and improving the quality of life became of prior importance, (Chahal et al., 2018).

The study was commenced with the aim to draw attention to the existing and future needs for a professional education program in the southern part of Saudi Arabia to reduce sufferings from various physical, mental, socio-economic disabilities occurring following RTAs etc. by means of Physical therapy (PT)

MATERIALS AND METHODS

This study was conducted in the Physical Therapy department, Jazan University as a retrospective study since the actual commencement of the program in 2009 till 2019. Students were not included in the study as individuals but rather as students' batches enrolled in the program, hence the study included 20 batches of male and female students collectively. Mean number of students enrolled in each level per semester was 47.23±20.44 for male students and 62.79±24.53 for female students. Different parameters of students' performance have been collected, scrutinized, analyzed and narratively presented in the form of tables and paragraph description. The data was obtained from e-register of Jazan University. E-register is username and password accessible webpage, through which many academic processes and/or information are applied or available. Teaching timetable must be registered through this website which show list of courses and sections (i.e., if a single course have two separate theoretical groups as well as three separate practical groups, each of which is called section) offered during this semester, with reference to the course name, code, maximum number allowed to be enrolled in each section, actual number enrolled at the commencement of the semester, time of the lectures (i.e., day, time of start, time of end of each lecture and/or lab), staff member assigned to teach this session and venue.

After registering the timetable on this website, multitude of information and reports could be obtained from this website, like, student's information (e.g., personal information, study plan, courses cleared, courses being currently studied, remaining courses to be cleared, courses studied in other university that match and equalize one of our courses, his marks obtained in previous semesters, percentage of attendance during the current and previous semesters, semester he joined the university, total period of study in the university, warnings conferred, Grade Point Average (GPA), transcript). Information about the classrooms and labs also can be obtained through this website, i.e., course being allocated every hour, course code and staff member assigned, percentage of classroom utilization, etc. In addition to the above, information about staff members, their teaching load, as well as their schedules can be obtained from this website. List of students enrolled in the current as well as previous semesters along with their GPA can be obtained from this website. Few days before commencement of the semester, the timetable and available course (and different sections of the course) are published by this website to all concerned students who can register the courses they are interested in and or choose their suitable practical session in terms of day and time allocation. The staff members as well can see the list of students enrolled in their course and record their attendance through this website.

RESULTS AND DISCUSSION

Data was collected and filtered on various criteria using Microsoft Excel to make it appropriate for the study. Data was exported into SPSS version 20.0 to make further analysis. Initially descriptive statistics (frequency distribution) was used to analyze but on later stages for

Table 1. Number of seats allocated in PT program per year.

Year	Number of seats allocated
2009-2010	60 (male section) 40 (female section)
2010-2011	100 (male section) 100 (female section)
2011-2012	100 (male section) 100 (female section)
2012-2013	100 (male section) 100 (female section)
2013-2014	100 (male section) 100 (female section)
2014-2015	100 (male section) 100 (female section)
2015-2016	100 (male section) 100 (female section)
2016-2017	100 (male section) 100 (female section)
2017-2018	100 (male section) 100 (female section)
2018-2019	100 (male section) 100 (female section)

detail enquiry cross tabulation was used to categorize the data under various categories and Chi square test have been applied to check the significant differences between categories of data. Measure of Central Tendency (Mean Score) was used to calculate Highest and Lowest GPA for male and female students. Ratio analysis was done at the end to check the male and female faculty staff ratio and to analyze any significant differences between them.

Table 1 shows evidence for total number of seats allocated in PT program from the year 2009-2010 till 2018-2019. The year 2009-2010 consist of fewer seats when compared to later years as the program was started in the second half of 2009-2010 year with only 1 semester in action. For over the years, the number of seats has been kept consistent due to higher enrollment of students in the program to meet the growing demand of PT professionals.

The above table depicts evidence for total number of students enrolled in each semester and mean of students enrolled in each level per semester. During the first 6 levels of program conduction, the number of levels being taught had gradually increased till reaching the maximum number (six levels per semester) in the first semester of the academic year 2012-2013.

The above table shows the number of male and female expatriates working in PT program. Initially, the program started with few teachers, which increased to nearly double fold as the strength of students increased in following years. In current, the PT program has sufficient man force to run the program in a smooth and efficient manner.

The above table represents the number of Saudi faculties (male and female) incorporated in the PT program.

Table 2. Number of students enrolled in each semester, and mean number of students enrolled in each level per semester.

Semester	Male		Female	
	Per semester	Mean no. in each level/semester	Per semester	Mean no. in each level/semester
First Semester 2009-2010	80	80	113	113
Second Semester 2009-2010	83	41.5	120	60
First Semester 2010-2011	107	35.66	169	56.33
Second Semester 2010-2011	123	30.75	218	54.5
First Semester 2011-2012	169	33.8	277	55.4
Second Semester 2011-2012	189	37.8	312	62.4
First Semester 2012-2013	223	37.16	311	51.83
Second Semester 2012-2013	252	42	348	58
First Semester 2013-2014	276	46	369	61.5
Second Semester 2013-2014	307	51.16	397	66.16
First Semester 2014-2015	362	60.33	427	71.16
Second Semester 2014-2015	386	64.33	456	76
First Semester 2015-2016	384	64	477	79.5
Second Semester 2015-2016	404	67.33	488	81.33
First Semester 2016-2017	413	68.83	485	80.83
Second Semester 2016-2017	402	67	491	81.83
First Semester 2017-2018	386	64.33	474	79
Second Semester 2017-2018	338	56.33	429	71.5
First Semester 2018-2019	277	46.16	365	60.83
Second Semester 2018-2019	268	44.66	362	60.33

Table 3. Total number of expatriate as faculties.

Year	Male faculties	Female faculties	Total
2009-2010	7	6	13
2010-2011	7	8	15
2011-2012	13	11	24
2012-2013	18	10	28
2013-2014	18	10	28
2014-2015	18	10	28
2015-2016	17	11	28
2016-2017	18	12	30
2017-2018	16	9	25
2018-2019	16	7	24

Table 4. Total number of Saudi faculties.

Year	Male faculties	Female faculties	Total
2009-2010	NIL	NIL	NIL
2010-2011	1	NIL	1
2011-2012	2	NIL	2
2012-2013	NIL	NIL	NIL
2013-2014	3	5	8
2014-2015	3	2	5
2015-2016	4	2	6
2016-2017	3	3	6
2017-2018	2	4	6
2018-2019	3	7	10

As universities in Saudi Arabia follow international standards for recruiting faculties, a minimum of master degree is required for appointment of lecturer while bachelor degree in PT for a demonstrator. As time passed by, the department saw an increase in recruitment of Saudi faculties after completion of their master program. "VISION 2030" is a plan aiming to the achievement of an ever advancing health care, educational, social, cultural and economic investments and improvement.

The above table portrays changes and improvements in terms of curriculum implementation and student developments. The major changes performed were drafting of the program, addition of prerequisites, modifications in the course specification for Electrotherapy I and Exercise therapy II for level II and III. A major transformation was made in the mode of examinations, wherein method of 2 midterm exam (theory and practical) and 1 final (theory and practical) was modified to 2 quiz (theory only), 1 midterm (theory and practical) and 1 final exam (theory and practical). In 2016, we were enthralled to update the study plan in an attempt to upgrade the quality of our outcomes (i.e., students' capabilities and skills) to match the international standards and benchmarks. The department received suggestions to update the program study plan (i.e., sequence of courses) as well as courses' contents and structure from an expert from one of the highly esteemed universities in USA, (Plack 2014).

Table 5. Significant changes in curriculum and student development.

Year	Significant changes in curriculum and student developments
2009	Drafting of PT program
2010	Addition of prerequisites: As all Saudi Universities entertain an American credit hour system, the whole program is arranged into semesters or levels (level 1 to level 8), each of which include number of courses. Each course has a specific name with individual code that represents its sub-specialty affiliation and its sequence in this sub-specialty. Each course (e.g., course B) requires the clearance of a pre-requisite (e.g., course A), which means that the student must clear course A before he/she registers for course B. He/she may register a course from any level provided that the student clears all pre-requisites of that course, but within the maximum credit hours allowed for him/her to register based on the attained GPA.
2011	Changes in course specification for Electrotherapy I and Exercise therapy II
2012	NIL
2013	Changes in mode of examination from (2 mid-term+1 final = 3 exams) to (2 quiz+1 mid-term +1 final = 4 exams)
2014	NIL
2015	NIL
2016	a) Updating course specification performa in subjects for all levels. b) Received suggestions to update the program study plan (i.e., sequence of courses) as well as courses' contents and structure from an expert from one of the highly esteemed universities in USA.
2017	NIL
2018	NIL

Table 6. Highest and lowest grade point average (GPA) scores among male and female students.

Year	Highest GPA score among male students	Highest GPA score among female students
2009-2010	3.82	4.65
2010-2011	4.55	4.68
2011-2012	4.58	4.74
2012-2013	4.51	4.77
2013-2014	4.53	4.64
2014-2015	4.65	4.81
2015-2016	4.84	4.83
2016-2017	4.87	4.90
2017-2018	4.87	4.93
2018-2019	4.88	5.00

Table 6 denominates the highest GPA scores among male and female students. By looking at the table it can be said that the GPA score has been on a gradual increase among both male and female students respectively from 2009-2010 till 2018-2019, except in 2012-2013 for male and 2013-2014 for the female students where there was a narrow decrease in GPA when compared to earlier years. Interestingly, female students have scored higher GPA score in all years from 2009-2010 till 2018-2019 in contrast to their male counterpart except in 2015-2016 where males score higher GPA than females, wherein the difference in score just being 00.01 which can be considered negligible. According the authors, this can be attributed to few factors. Firstly, it's a general trend seen all over the world that females devote more sitting hours when it comes to academics. Secondly, female students had higher attendance in college which reflects their keenness towards studies. Thirdly, as per the middle eastern culture, males are requested to attend to more outdoor responsibilities than females during the afternoon time that might affect their concentration and attention during their study, which give female students more privilege over male students and provide them with extra time for studying at home. Finally, female students are putting great efforts in learning the subject to get a job hence, to make them financially independent.

Table 7. No. of scholars (male and female) pursuing their postgraduate degree (Masters or Ph.D.).

Year	No. of male scholars	No. of female scholars	Total no.
2009-2010	4	NIL	4
2010-2011	5	NIL	5
2011-2012	5	NIL	5
2012-2013	5	NIL	5
2013-2014	4	NIL	4
2014-2015	4	3	7
2015-2016	4	6	10
2016-2017	6	5	11
2017-2018	7	4	11
2018-2019	6	1	7

Table 8. Number of students participated in foreign exchange program.

Year	No. of male	No. of female	Total
2009-2010	NIL	NIL	NIL
2010-2011	NIL	NIL	NIL
2011-2012	NIL	NI	NIL
2012-2013	10	NIL	10
2013-2014	NIL	10	10
2014-2015	NIL	10	10
2015-2016	NIL	NL	NIL
2016-2017	NIL	NIL	NIL
2017-2018	NIL	NIL	NIL
2018-2019	NIL	NIL	NIL

Jazan University has been a motivator and backbone for meritorious students who have performed excellence in their bachelor's programs after being recruited as demonstrators in the department. The university grants scholarship to these meritorious demonstrators to fulfill their dreams of pursuing master's program and Ph.D. provided that they acquire an approval for joining a well-structured program that is fulfilling the needs and goals of the department as well as the university and falls within the top 500 Universities all over the world as per Shanghai Ranking and being approved as an equivalent university by the Saudi ministry of education.

The above table displays number of students participated in foreign exchange program over the years in PT. As, Jazan University believes in quality education, to fulfill this approach it sent selected (based on their overall GPA performance), 10 male and 10 female students to Zuyd University, Heerlen, Netherlands under an exchange programs for 1 month every year. Both male and female students were accompanied with 1 male and 1 female faculty. The main objective of the exchange program was to upgrade their knowledge and acquaintance of new information and expose the students in terms of innovation and research technology to the field of PT. The exchange program was conducted for 3 consecutive years, but stopped due to some issues that falls beyond the capabilities of the department to solve or to sort out.

Current staff ratio:

Designation	Male	Female
Associate Professor	1	0
Assistant Professor	5	1
Lecturer	11	8
Demonstrator	1	4
Technicians	2	0

Saudi Arabia is one of the fastest growing economies in the world (Alghadir et al 2015). With time its requirement of manpower for PT has ascended (Awad Al-Omari et al., 2015) As, Saudi Arabia is a large country by size, the government has setup universities instituted in all major cities. Jazan University is situated in the southern part of Saudi Arabia. As per today it is working at its best by creating employment and serving the society. But reaching to the current scenario was at a snail pace as there were few key issues involved in the early days while starting the PT program, like all necessary equipment needed for the conduction of practical sessions were not available, during the first year only one classroom was allocated for each section (male and female). Although being enough during the first year, more classrooms were needed during the following years. Only two labs were available for each section. The male laboratories had some deficiencies in electric supply for the first few months of conduction with lack of projectors or cupboards to hold tools. Cupboards were installed later during the first year. With increasing number of levels and students enrolled in each level, more laboratories were needed which took some time for delivery. Some allocated laboratories were undersized in area that did not suffice for the number of students supposedly allocated in this lab. Laboratories were allocated in different building other than that of the classrooms, which greatly affected the conduction of midterm exams that required the utilization of all available facilities. But, with the active support from the university's administrative, all these shortcomings were resolved. Hence as per today, the PT program at Jazan University is working in multifactorial dimensions and has been a shining star in the kingdom since it commenced in the year 2009-2010. The program has provided a 360 degrees competency with other programs running under Jazan University (Al Maghraby et al., 2013 and Bithell 2007). The department as of today has sparkling staff members with degrees in doctorate and master's with specialties in Orthopedics, Cardio-respiratory, Neurology, Sports, Pediatrics and Basic sciences who impart both basic and advanced knowledge to students, (Ahmad et al., 2015).

CONCLUSION

Students graduated from PT department are working in different setups like private clinics, rehabilitation centers, military hospitals, government universities, hospitals and sport teams in different locations of Saudi Arabia. This accomplishment of young saudi male and females has developed a work force which is working for its citizens, enabling the kingdom to be self-efficient in terms of delivering health care and rehabilitation thus, achieving the target of 100 percent Saudization which is the main goal of "VISION: 2030".The take home message for others thinking of establishing the same curriculum in their university or individuals willing to work as a physical therapist is that, PT as a profession is of a great demand and importance in serving the society by providing relieve from pain and disabilities and improving the overall quality of life.

AUTHOR'S CONTRIBUTION

Aksh Chahal and Mohammed Qasheesh undertook formulation of the article, Amr Shalaby, Mohammad Abu Shaphe and Marwa Hanny collected data, Junaid Ahmed Kirmani and Rashid Ali performed typing work while Pooja Chaudhuri and Nitesh Malhotra actively participated in editing the article.

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Mathematical Communication

Biosci. Biotech. Res. Comm. 12(2): 324-332 (2019)

BBRC
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Optimization of the variance of attribute by hybrid swarm intelligence and option price predication by cascading neural network

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ABSTRACT

The variation of attribute varies the prediction of option price in the stock market. The variation of attribute value creates uncertain behaviour in the stock market and increases the risk factors for buyers. For the minimization of risk and prediction accuracy used various neural network models and optimization algorithm. The optimization algorithm reduces the impact of variance and neural network increase the accuracy of prediction. In this paper proposed cascaded neural network-based classifier for the purification of data. For the optimization of attribute correlation used hybrid swarm intelligence algorithm. The hybrid swarm intelligence algorithm is a combination of plant grows optimization and ant colony optimization. The hybrid swarm intelligence algorithm reduces the variation of prices and proceeds the data for the prediction. For the validation of proposed algorithm used NSE stock banking data of recent years. The total settles price for the processing used 20871. For the evaluation of the performance used standard parameters such as MAE, MSE, RMSE and MI. The proposed algorithm implemented in MATLAB 14.0. the cascaded neural network classifier is the combination of SOM and RBF neural network model. The SOM neural network model basically proceeds the task of clustering and RBF neural network model used for prediction.

KEY WORDS: STOCK MARKET, OPTION PREDICTIONS, SWARM INTELLIGENCE, SOM, RBF, NSE

ARTICLE INFORMATION:

Corresponding Author: scholarpayalshrivastava@gmail.com
Received 27th March, 2019
Accepted after revision 25th May, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/15

INTRODUCTION

The forecasting of stock market implied the future of company assets and buyers of market. For the better forecasting and prediction of stock price used parametric and non-parametric model for option pricing. In option pricing, basically the role of variables is very high. The variation of variables changes the nature of prediction. For the better prediction, various authors and researcher used non-parametric model such as artificial neural network, deep learning, and optimization technique. Theattribute optimization process reduces the variance of variable value. The reduce variance implied the better prediction for the stock market. The impact of variance also impacts the performance of the prediction, (Andreou et al, 2008, Park et al, 2014; Verma et al, 2014; Hajizadeh et al, 2018, Liu et al, 2019).

In the process of prediction used various classifications and clustering technique such as neural network and support vector machine. In the process of prediction, the rate of option price prediction basically depends on the sampling of data. The process of sampling of data reduces the size of data and normalized the data. Thenormalized data gives better prediction Liang et al, 2009 and Jang et al, 2019). In this paper proposed the cascaded based classifier for the prediction of option pricing.The cascaded classifier is a combination of SOM and RBF neural network models. Self-organized map (SOM) is basically used for the process of clustering of stock data. Radialbias function (RBF) is used here for the process of classification. The combination of two models plays a role of prototype classifier for the prediction of option pricing. For the optimization of attribute(variables) used the hybrid swarm intelligence. The hybrid swarm intelligence is a combination of plant grows optimization algorithm and ant colony optimization. The plant grows optimization algorithm work in three phase morphogen, branch and termination (Verma et al, 2016). The ant colony optimization algorithm basically works in the nature of real ants. The behaviours of real ants are finding shortest path source to the food (Chou et al, 2015; Barumik et al, 2016). In our optimization algorithm the ant colony optimization plays role of distance reduction of attribute. The reduce distance of attributes creates a new set of data in the process of prediction (Olatomiwa et al, 2015; Kang et al, 2014; Mitra et al, 2012, Jang et al, 2019).

The proposed classification algorithm compares with deep learning-based option pricing model. For the experimental analysis used NSE dataset of 20871 thousand of data of different settle price. The contribution of this paper is summarized as follows : Wang et al, 2013; Burkovska et al, 2015; Al et al, 2015 Fridrich et al, 2017, Hirsa et al, 2019).

1. Optimized the value of variance of attribute (variable)
2. Based on cascaded classifier enhanced the performance of option pricing
3. A design hybrid optimization process based on plant grow optimization and ant colony optimization.
4. Conduct experimental simulation of an NSE dataset of different sample data unit and measure their performance.

MATERIAL AND METHODS:

Hybrid Swarm Optimization: In this section describe the process of hybrid swarm intelligence. The hybrid swarm intelligence is a combination of plant grows optimization algorithm and ant colony optimization. The plant grows optimization algorithm follow the nature of natural plant grows under sunlight. And ant colony optimization follows the real ant behaviors in environments (Heaton et al, 2017; Feuerriegel et al, 2015; Xiong et al, 2015; Yang et al, 2014).

The development of plants divided into three sections as describe below

1. Morphogen. In the case of morphogen check the status of plants for growing.
2. Branching. In the case of branching check the section condition of new leaf policy
3. Termination. Termination is the final process of plant theory. The termination process gives the optimal solution of given problems

The following parameter is used for the process of attribute, x_1, x_2,.....,x_n is the attribute of NSE data for option pricing. W is the Wight factor for the attribute, is the value of a morphogen, C_1 and C_2 is the contour value of attribute.

Step1. Define the value of attribute-set $S_1\{x_1, x_2,\ldots\ldots\ldots,x_n\}$ with population

Assign the value of the contour and weight of attribute C_1=0, C_2=0 and W=0

a. Morphogen selection of plant function

$$F(s) = \frac{(Ffd - Fpf)}{Fd * fp}, wi\epsilon S(x_1, x_2 \ldots\ldots x_n) \quad (1)$$

Here F_{fd} is initial attribute and F_{pf} is final attribute of the plant and w is set of attribute variable of sum sets

The attribute variables set the value of branch F = $\{f_{a1}\ldots\ldots\ldots\ldots_{an}\}$. Thesebranchvalues proceed for theestimation Competition condition of local leaf.

$$Fcom = \begin{cases} \frac{(T_i)^{\alpha}\left(LI_i^{S_j}\right)^{\beta}}{\sum_{g\in S_j}(\tau_g)^{\alpha}\left(LI_g^{S_j}\right)^{\beta}} if \quad i \notin S_j \\ 0 \qquad otherwise \end{cases} \quad (2)$$

Here T is the target value of attribute, and LI is the value of attribute difference.

Step 2. Branching condition

Input the selected attribute for the **Competition**

1. Calculate the value of relative attribute of C_1 and C_2 $Rf = \frac{LSI}{Wd}$ Here Lsi the difference of attribute set.
2. The PGO estimate the final attribute for selection.

$$FS = \begin{cases} \frac{\max\ (RF)\ -F(s)}{\max\limits_{h=1:}(WS)} & if\ s_i \in f_j \\ 0 \quad otherwise \end{cases} \tag{3}$$

3. Create the relative FS difference value of attribute

$$R_d = \sum_{fd=1}^{n} \sum_{pf=1}^{m} (x_i - fs) \tag{4}$$

4. if the value of Rd is zero the attribute termination condition is call

Step 3. Termination

Where Rd is the relative difference of $T(i)$; f_z is the fitness value; standard deviation S_z and local density D_z are defined in formula (5):

$$\begin{cases} R_d = \sqrt{\frac{\sum_{i=1}^{n}(z(i) - E(z))^2}{(n-1)}} \\ f_z = \sum_{i=1}^{n} \sum_{j=1}^{n} (R - r(i,j))\, u(R - r(i,j)) \end{cases} \tag{5}$$

Defining $D(z(k),z(h))$ as the absolute distance between the two-optimal attributes

$$d(z(k), z(h)) = \sqrt{(z(k) - z(h))(z(k) - z(h))} = \sqrt{(z(k) - z(h))^2}$$

k =1, 2,... ... ., N; h = 1, 2,... ... ., N and finally, the attribute is terminated.

5. The optimal features attributes set the value of ants F = {f_{a1}... ... . $_{an}$}. These ants value proceed for the estimation of variance, define an ant selection function as

$$Ants = \begin{cases} \frac{(\tau_i)^{\alpha} \left(LI_i^{S_j}\right)^{\beta}}{\sum_{g \notin S_j} (\tau_g)^{\alpha} \left(LI_g^{S_j}\right)^{\beta}} if\ i \notin S_j \\ 0 \quad otherwise \end{cases} \tag{6}$$

Here τ_i is phenomenal value of ants and LI is value of the least interface of ants.

6. The selected ants value proceeds for the process of classification.

Cascaded Classifier for Prediction: Cascading is a new area of neural network hybridization. In the process of cascading encapsulate two different neural network models based on requirement of the classification process. In option price prediction used two neural network models one is a SOM neural network and other is a RBF neural network model. In the process of cascading the SOM neural network model creates the clusters for the processing of RBF pattern. The RBF pattern is finally prediction value of option prices. The selection of settle price proceeds through the process of plant grows optimization. The RBF neural network model is a single hidden layer classifier and the process of classification is very fast and accurate. And other side used SOM network, it is self-organized map neural network and property of this network is unsupervised. Due to this property training process of the network is not required. In SOM network, optimal attribute which has been selected by selector passes to this network and create two different attribute vector one is winner attribute vector, and another is a successor attribute vector. The successor attributes vector passes through the RBF neural network in the process of training and pattern classification. The attributes vector passes through SOM acts as a clustering mechanism that projects N-dimensional attributes from the attribute matrix into an M-dimensional attribute space. The resulting vectors are fed into a SOM that categorizes them into one of the relearned optimal attributes. The transformed attribute vectors are fed into the SOM, which classifies them. We call the attribute space generated from the attribute selector function output as the primary attribute space and M-dimensional attribute space for SOM output as a secondary attribute space. The secondary attribute space passes through as vector input of RBF function.

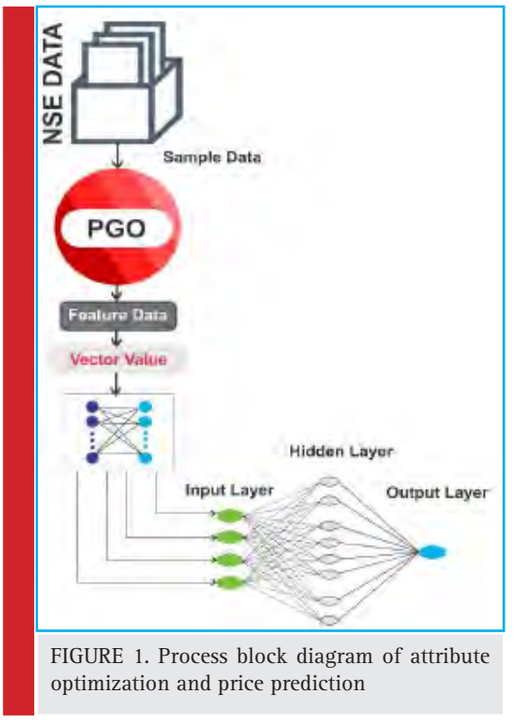

FIGURE 1. Process block diagram of attribute optimization and price prediction

The output of optimal data of the PGO map as $X = [x_1, x_2, \ldots, x_{N_l}, x_{N_l+1}, \ldots, x_N] \in R^{D \times N}$ that is corresponding to a cascaded, where each $x_i \in R^D$ represents the i[th] index of vector, D is the number of data samples and $N = (N_l + N_u)$ is the number of training samples. The first N_l samples $X_l = [X_1^l, X_2^l, \ldots . X_{N_l}^l]$ are labeled with the corresponding labels $Y_l = \{y_i\}_{i=1}^{N_l}$ the remaining N_u samples $X_u = [X_{N_l+1}^u, \ldots \ldots \ldots, X_N^u]$.

In the process of cascaded classifier, the value of matrix W map SOM output in RBF input vector and minimized the attribute variance value as

$$W_p = arg_w \min(\sum_{s=1}^{N} \sum_{t=1}^{k_s} \|W_{x_a} - W_{x_b}\|^2 rf) \quad (7)$$

Where X_b is the neighbor of X_a and k_s is the number of neighbors of X_s. rf is the relative feature difference values of sample X_a and X_b, A mapping of each sample $X_i^l (i = 1, 2, \ldots, N_l)$ can be as vector $a_i \in R^{N_l \times 1}$ under a sample of data $X_l = [X_1^l, X_2^l, \ldots . X_{N_l}^l] \in R^{N_l \times 1}$ that is composed as

$$\min_{a_i} \|a_i\|_1, s.t. \frac{1}{2} \|X_i^l - X_l a_i\|^2 < MAR \quad (8)$$

where MAR is the mean absolute error define the value of w_{dif}^i as the distance between the winner X_i^l and its successor data

$$w_{dif}^i = \sum_{k=1}^{k_{s2}} \|X_i^l - X_{ik}^l\|^2 \quad (9)$$

Where x_{ik}^l are the successor data of points x_i^l and k_{s2} is the number of selected winners?

The learning rate in should be time varying. This requirement can be satisfied by choosing an exponential decay for $n(t)$.

$$\eta(t) = \eta o exp\left(-\frac{t}{w1}\right), t = 0,1,2 \quad (10)$$

The update matrix value process after learning

$$updateWij = \sum_{k=1}^{k_{s2}} \|WX_i^l - WX_{ik}^l\|^2 - \sum_{j=1}^{k_{s1}} \|WX_i^l - WX_{ij}^l\|^2 \quad (11)$$

The updated winner's matrix data passes through the RBF interconnected input layers

$$Rfb = \sum_{i=1}^{N_l} updateWij, \\ = \sum_{i=1}^{N_l} \sum_{k=1}^{k_{s2}} \|WX_i^l - WX_{ik}^l\|^2 - \sum_{i=1}^{N_l} \sum_{j=1}^{k_{s1}} \|WX_i^l - WX_{ij}^l\|^2. \quad (12)$$

Calculating the deviation value of sample data points to measure the error rate

$$MSE = \frac{1}{n} \sqrt{\sum_{i=1}^{n} \sum_{j=1}^{m} (Rfbij - yij)^2} \quad (13)$$

RESULTS AND DISCUSSION

In this section discuss the process of result analysis of proposed algorithm and optimization of swarm intelli-

Table 1. Input Data taken from National Stock Exchange of India (NSE) Stock option of Andhra Bank

Symbol	Strike Price	Settle Price	Underlying Value
AXIXBANK	75	0.05	49.85
AXIXBANK	75	0.05	50.65
AXIXBANK	75	0.1	51.05
AXIXBANK	75	0.05	51.5
AXIXBANK	75	0.05	51.85
AXIXBANK	70	0.05	49.5
AXIXBANK	67.5	0.1	47.85
AXIXBANK	75	0.05	53.2
AXIXBANK	70	0.05	49.85
AXIXBANK	75	0.05	53.95
AXIXBANK	70	0.05	50.65
AXIXBANK	75	1.5	54.35
AXIXBANK	67.5	0.05	49.5
AXIXBANK	67.5	1.4	49.6

Table 2. Input Data taken from National Stock Exchange of India (NSE) Stock option of ICICI Bank

Symbol	Strike Price	Settle Price	Underlying Value
ICICIBANK	310	0.2	190.75
ICICIBANK	300	0.05	192
ICICIBANK	310	0.05	198.45
ICICIBANK	300	0.05	193.55
ICICIBANK	310	0.35	204.05
ICICIBANK	300	0.15	199.25
ICICIBANK	280	0.05	187
ICICIBANK	270	0.05	183
ICICIBANK	300	0.25	203.5
ICICIBANK	270	0.2	184.8
ICICIBANK	280	0.05	192
ICICIBANK	300	0.15	207.25
ICICIBANK	280	0.05	193.55
ICICIBANK	280	0.1	196.6
ICICIBANK	260	0.3	183
ICICIBANK	270	0.15	190.05
ICICIBANK	280	0.05	198.45
ICICIBANK	280	0.05	198.8

Table 3. Input Data taken from National Stock Exchange of India (NSE) Stock option of RBL Bank

Symbol	Strike Price	Settle Price	Underlying Value
RBLBANK	620	0.05	505.85
RBLBANK	600	0.75	490.3
RBLBANK	600	0.8	490.35
RBLBANK	620	2.3	508.15
RBLBANK	620	0.05	508.95
RBLBANK	620	1.75	509.5
RBLBANK	620	0.15	511.7
RBLBANK	600	0.9	497.2
RBLBANK	620	0.65	516.35
RBLBANK	620	3	516.55
RBLBANK	620	0.45	518.1
RBLBANK	600	0.45	501.65
RBLBANK	600	3.3	501.65
RBLBANK	600	0.05	505.35
RBLBANK	600	0.2	505.85

gence. The proposed algorithm implemented in MATLAB 14.0. For the process of analysis used NSE dataset. For the evaluation used standard parameters NMSE, RMSE, MAE and MI. The proposed result compares with Deep learning algorithms.

In Figure 2, indicates the variation of normalized mean square error between deep neural network methods and proposed cascaded algorithm for the Andhra Bank dataset. The result of variation distributed in different settle price such as 0.05, 0.15, 0.25, 0.30 and 0.45 the variation indicates the value of RMSE is optimized due to the process of optimization and better prediction of cascaded classifier. According to the settle price (0.05, 0.15, 0.25, 0.30 and 0.45) sequentially deep neural network method RMSE values are 1.988, 1.0627, 0.8776, 0.8313 and 0.7542 and similarly proposed cascaded algorithm RMSE values are 0.5388, 0.44627, 0.42776, 0.42313 and 0.41542.

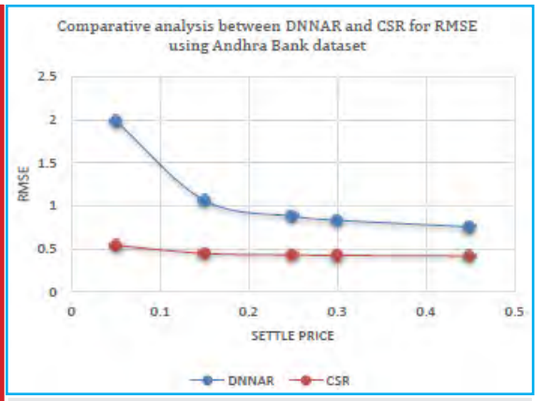

FIGURE 2. Comparison DNNAR and CSR for RMSE using Andhra Bank dataset

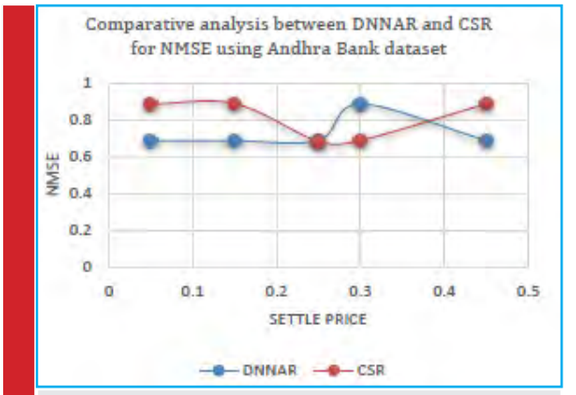

FIGURE 3. Comparison DNNAR and CSR for NMSE using Andhra Bank dataset

In Figure 3, indicates the variation of normalized mean square error between deep neural network methods and proposed cascaded algorithm for the Andhra Bank dataset. The result of variation distributed in different settle price such as 0.05, 0.15, 0.25, 0.30 and 0.45 the variation indicates the value of NMSE is optimized due to the process of optimization and better prediction of cascaded classifier. According to the settle price (0.05, 0.15, 0.25, 0.30 and 0.45) sequentially deep neural network method NMSE values are 0.68428, 0.68549, 0.68763, 0.88549 and 0.68756 and similarly proposed cascaded algorithm NMSE values are 0.88521, 0.88791, 0.68194, 0.68756 and 0.88634.

In Figure 4, indicates the variation of normalized mean square error between deep neural network methods and proposed cascaded algorithm for the Andhra Bank dataset. The result of variation distributed in different settle price such as 0.05, 0.15, 0.25, 0.30 and 0.45 the variation indicates the value of MI is optimized due to the process of optimization and better prediction of cascaded classifier. According to the settle price (0.05, 0.15, 0.25, 0.30 and 0.45) sequentially deep neural network method MI values are 1.988, 1.0627, 0.8776, 0.8313 and 0.7542 and similarly proposed cascaded algorithm MI values are 1.0388, 0.94627, 0.92776, 0.92313 and 0.91542.

In Figure 5, indicates the variation of normalized mean square error between deep neural network methods and proposed cascaded algorithm for the ICICI Bank dataset. The result of variation distributed in different

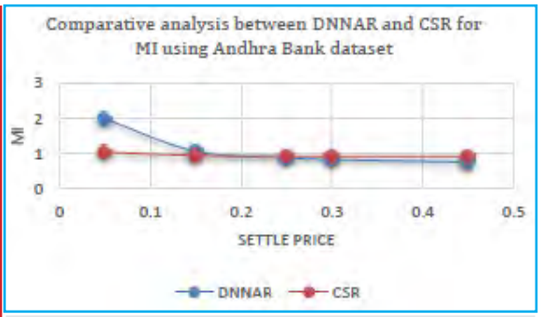

FIGURE 4. Comparison DNNAR and CSR for MI using Andhra Bank dataset

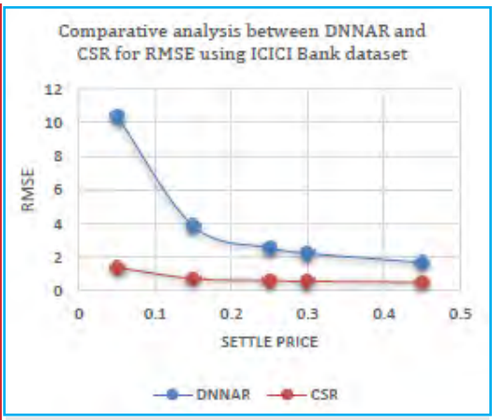

FIGURE 5. Comparison DNNAR and CSR for RMSE using ICICI Bank dataset

settle price such as 0.05, 0.15, 0.25, 0.30 and 0.45 the variation indicates the value of RMSE is optimized due to the process of optimization and better predictionof cascaded classifier. According to the settle price (0.05, 0.15, 0.25, 0.30 and 0.45) sequentially deep neural network method RMSE values are 10.358, 3.8527, 2.5516, 2.2263 and 1.6842 and similarly proposed cascaded algorithm RMSE values are 1.3758, 0.72527, 0.59516, 0.56263 and 0.50842.

In Figure 6, indicates the variation of normalized mean square error between deep neural network methods and proposed cascaded algorithm for the ICICI Bank dataset. The result of variation distributed in different settle price such as 0.05, 0.15, 0.25, 0.30 and 0.45 the variation indicates the value of NMSE is optimized due to the process of optimization and better prediction of cascaded classifier.

According to the settle price (0.05, 0.15, 0.25, 0.30 and 0.45) sequentially deep neural network method NMSE values are 0.58403, 0.5906, 0.61145, 0.61555 and 0.61272 and similarly proposed cascaded algorithm NMSE values are 0.81272, 0.75999, 0.81555, 0.7906 and 0.81438.

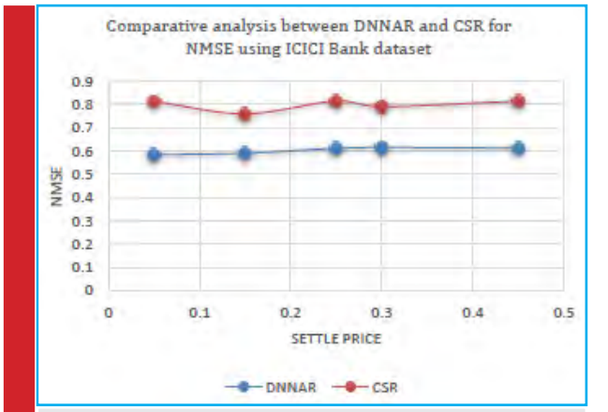

FIGURE 6. Comparison DNNAR and CSR for NMSE using ICICI Bank dataset

In Figure 7, indicates the variation of normalized mean square error between deep neural network methods and proposed cascaded algorithm for the RBL Bank dataset. The result of variation distributed in different settle price such as 0.05, 0.15, 0.25, 0.30 and 0.45 the variation indicates the value of NMSE is optimized due to the process of optimization and better predictionof cascaded classifier. According to the settle price (0.05, 0.15, 0.25, 0.30 and 0.45) sequentially deep neural network method NMSE values are 0.67728, 0.68376, 0.68195, 0.68393 and 0.68393 and similarly proposed cascaded algorithm NMSE values are 0.88311, 0.8824, 0.88025, 0.88325 and 0.87926.

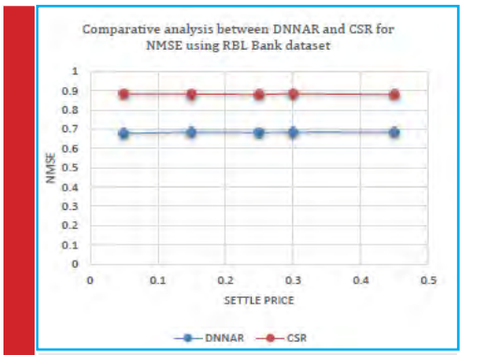

FIGURE 7. Comparison DNNAR and CSR for NMSE using RBL Bank dataset

In Figure 8, indicates the variation of normalized mean square error between deep neural network methods and proposed cascaded algorithm for Andhra Bank, ICICI Bank and RBL Bank dataset. The result of variation distributed in different settle price such as 0.05, 0.15, 0.25, 0.30 and 0.45 the variation indicates the value of

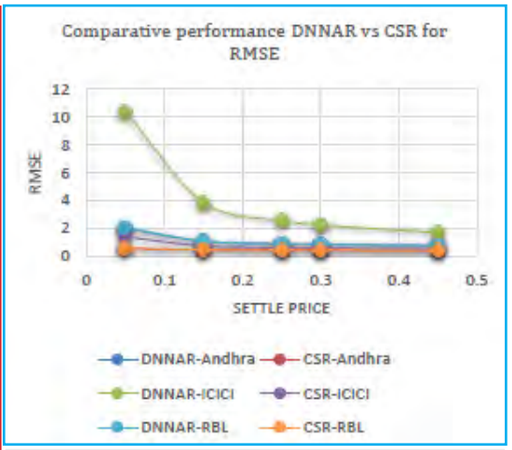

FIGURE 8. Comparison DNNAR vs CSR for RMSE

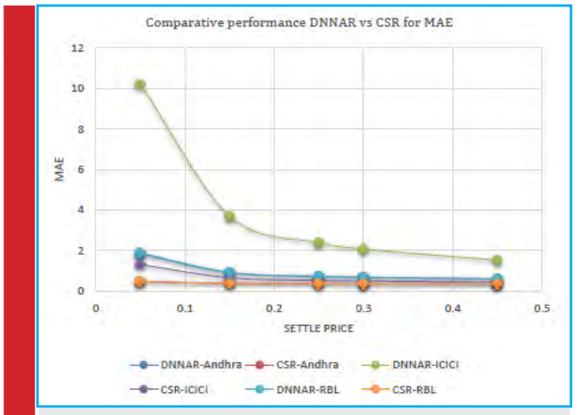

FIGURE 10. Comparison DNNAR vs CSR for MAE.

RMSE is optimized due to the process of optimization and better predictionof cascaded classifier.

In Figure 9, indicates the variation of normalized mean square error between deep neural network methods and proposed cascaded algorithm for Andhra Bank, ICICI Bank and RBL Bank dataset. The result of variation distributed in different settle price such as 0.05, 0.15, 0.25, 0.30 and 0.45 the variation indicates the value of NMSE is optimized due to the process of optimization and better prediction of cascaded classifier.

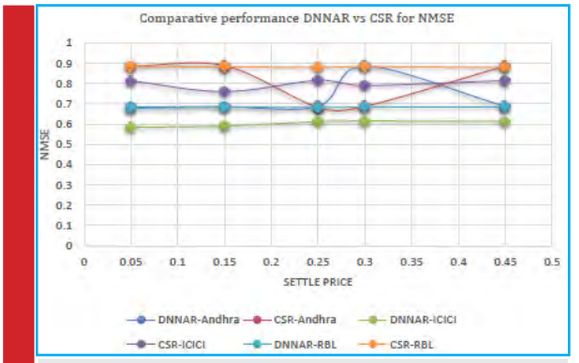

FIGURE 9. Comparison DNNAR vs CSR for NMSE

In Figure 10, indicates the variation of normalized mean square error between deep neural network methods and proposed cascaded algorithm for Andhra Bank, ICICI Bank and RBL Bank dataset. The result of variation distributed in different settle price such as 0.05, 0.15, 0.25, 0.30, 0.45 the variation indicates the value of MAE is optimized due to the process of optimization and better predictionof cascaded classifier.

In Figure 11, indicates the variation of normalized mean square error between deep neural network methods and proposed cascaded algorithm for Andhra Bank, ICICI Bank and RBL Bank dataset. The result of variation distributed in different settle price such as 0.05, 0.15, 0.25, 0.30 and 0.45 the variation indicates the value of MI is optimized due to the process of optimization and better prediction of cascaded classifier.

The cascaded classifier reduces the variation of price and enhances the process of prediction. For the validation and analysis of algorithm used NSE dataset in recent years. The total data instance is 20871. The total data are distributed into different settle prices for the measuring the variation of real value and predicated value. For the evaluation used 4 non-parametric parameters such as normalized mean square error, root mean square error, mean absolute error and mutual information. The value of mutual information indicates the independency of a variable during the process of cascaded classifier. In Figure 2, shows the variation value of NMSE (normalized mean square error), in case of CSR (cascaded classifier) the value of variation is decreased. In case of the deep

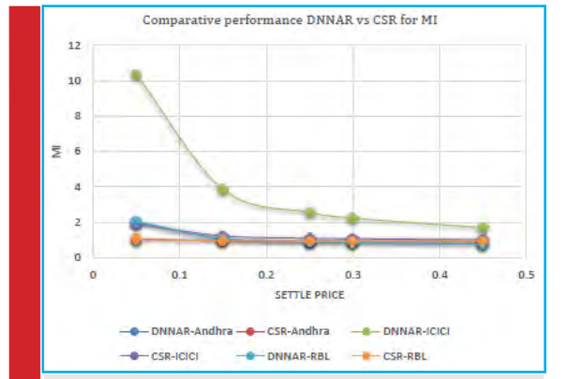

FIGURE 11. Comparison DNNAR vs CSR for MI

learning algorithm, the variation of error is increased in all distributed points for the selection of settle price.

The reduce value of NMSE increase the value of option price prediction. The enhance value of price also shows the best performance of the optimization of attribute selection and processing of data in the process of classification. In Figure 3, shows the value of RMSE (root mean square error). The variation value of RMSE is also decreasing in case of CSR. Due to the better selection and optimization of attribute according to the plant grow optimization algorithm, the deviation of attribute value is reduced. The reduced value of deviation indicates the minimum value of MSER. In Figure 4, describe the performance of mean absolute error, in case of CSR instead of DNNAR the value of MAE is down due to optimized patterns of price attribute of the RBF function. The processing of RBF function reduces the variation of attribute and enhance the predictionvalue of price and reduces the value of MAE. In Figure 5, describe the independency of price in the attribute selection process during the cascaded classifier. The target value of predictionis increase and value of error is decreasing, increase the value of MI shows that the better prediction ratio of classifier instead of DNNAR. In Figure 6 and 7, describe the process performance of individual algorithms satisfying the all parameters for the distribution of all settle prices. The interval ratio of settle price indicates that the variation in case of CSR is minimized instead of DNARR.

CONCLUSION

In this paper analyzed the performance of option price prediction using two algorithms. The used algorithms are a combination of optimization algorithm and cascaded neural network-based classification. The optimization algorithm reduces the variation of attribute selection in the process of classification. The optimization algorithm is called plant grow optimization. The plant grows optimization algorithm work in three phases. In a first phase basically dynamic define the population in the form of data loading for the process of optimization. In the second phase process used branch selection process for the new population, in this phase basically reduces the attribute variation relation of two distinct attributes. And finally measure the value of computation, for the final selection of the optimal value of the price of the input processing of cascaded classifier. The cascaded classifier is a combination of two neural network models. RBF and SOM neural network, here the SOM neural network model used for the process of clustering of input data by the optimization algorithms.

The rate of learning used 0.6 probability value and group data in good manners. The group of clusters passes through the RBF function, the RBF function generates the trained patterns and predict the value of option prices. For the validation and analysis of the optimization and cascaded classifier used NSE India dataset. The data interval is last 6 years. In all data instances used selected numbers of attributes. For the analysis of performance used 4 standard parameters NMSE, RMSE, MAE and MI. The values indicate better performance of cascaded classifier instead of deep neural network-based classifier. The deep neural network-based classifier suffers from the selection of feature attributes of price variation. The plant grow optimization algorithm reduces the variation of attributes and increase the value of option pricing.

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Technological Communication

Biosci. Biotech. Res. Comm. 12(2): 333-337 (2019)

BBRC Bioscience Biotechnology Research Communications

iGuard: mobile security guard system with infrared biosensor and google glass

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ABSTRACT

Nowadays, most security companies hire guards' team for different tasks to protect the company's assets. At least one guard should monitor the cameras or other systems. The goal of the proposed research in this paper is to add mobility to security guard by using Google Glass. The guard can do many tasks to control and keep the building safe from any damage. Accordingly, the guard doesn't have to stay in one place to monitor, and hence many security guards are required to cover all the building. The system would require supporting the building and the important rooms with the infrared sensor, light intensity sensor and camera, whilst supporting the guard with Google Glass. The sensory data will be dealt with through a sensor board that sends all the information to the main controller. The system has a look-up table that contains data, such as: temperature and light infrared readings; the sensors keep sending readings to the system. The system calculates the difference between the stored readings with the sent data. If the difference is greater than zero, then the alarm is sent to the guard and the image is displayed in the Google Glass to the guard

KEY WORDS: GOOGLE GLASS, INFRARED SENSORS, LIGHT INTENSITY SENSOR, MOBILE SECURITY

ARTICLE INFORMATION:

Corresponding Author: galhudhud@ksu.edu.sa
Received 15th April, 2019
Accepted after revision 19th June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
Crossref Clarivate Analytics
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/16

INTRODUCTION

Securing work, institutional, business, tourism or military facilities is highly demanded. Current security management systems rely on hiring teams of security guards supported by a set of surveillance cameras and monitoring devices. These systems verify the visited points by security guards in the building/facility and report the deviation of security check plans. Meanwhile, these systems require at least one guard should monitor the cameras displays in a control room. In addition, the currently available mobile security guards systems, for example: Pancomp, monitors the security guards' performance not the facilities.The main problem is the shortage of security guards and the high level of effort required from them. Controlling the whole building requires many guard rounds within the building. The second problem is that the guard who monitors the cameras or the systems has to stay all the time in the room. For this, the proposed solution is to add mobility to guards. One guard can control and keep the building safe from any damage without having to stay in one place to monitor. Therefore, we need to use some tools such as Google Glass and Motion sensors, sounds, and heat as a mobile security tower. The proposed system is composed of multi-sensor boards and a main controller using Arduino technology. Each sensor board retrieves different forms of data such as the light intensity, sound waves and temperature for example in the offices, labs and servers room also in order to monitoring room then sends all the information to the main controller which sends the data to a web server and its database. Hence, when any of these data changes by unauthorized access or especially out of working hours, the security guard will receive this data change from the second part of the network which displays the information retrieved to the security guard. One of the most functionalities that the proposed system offers is to let the guards keep their mobility and give them the ability to move and monitor at the same time. What we aim for in mobile security tower is incorporation the Arduino technology and Google glass to enhance security devices these days, (Safavi & Shukur 2014 He et al. 2015).

RELATED WORK

A. Internet of Things Status Monitoring with Augmented Reality on Google Glass

It is a system that implements the augmented reality capabilities of Google Glass and connects them together with the idea of internet-able items across an environment (Atzori, Iera & Morabito 2010), the Internet of Things (Jiang, Liu & Yang 2004) (Safavi, Shukur & Razali 2013). This system gathers information from an environment, and stores it to be accessed either by the web or by scanning QR codes on a custom app on the Google Glass. On the Glass interface, it brings up a real-time info-graphic for the information related to the scanned QR code. It is mirrored by the web interface with the addition of more in-depth analysis of the data, (Ghemawat, Gobioff & Leung 2003 and Safavi & Shukur 2014).

B. Immune chromatographic Diagnostic Test Analysis Using Google Glass

Google Glass-based fast symptomatic test (RDT) peruser stage equipped for subjective and quantitative estimations of different parallel stream invulnerable chromatographic measures and comparative biomedical diagnostics tests. Utilizing a custom-composed Glass application and with no outside equipment connections, at least one RDTs named with Quick Response (QR) code identifiers are at the same time imaged utilizing the inherent camera of the Google Glass that depends on a sans hands and voice-controlled interface and carefully transmitted to a server for computerized. The procured JPEG pictures are consequently prepared to find all the RDTs and, for each RDT, to deliver a quantitative analytic outcome, which is come back to the Google Glass (i.e., the user) Also, put away on a focal server alongside the RDT picture, QR code, and other related data (e.g., statistic information). A similar server additionally gives a dynamic spatiotemporal guide and ongoing insights for transferred RDT come about open through Internet browsers, (Feng et al. 2014).

C. Texting while driving using Google Glass

Texting while driving is risky but common. This investigation assessed how messaging utilizing a Head-Mounted Display, Google Glass, impacts driving performance. Experienced drivers played out a great auto following errand while utilizing three distinct interfaces to content: completely manual cooperation with a head-down cell phone, vocal collaboration with a cell phone, and vocal association with Google Glass. Completely manual collaboration created more awful driving execution than both of the collaboration strategies, prompting more path journeys and variable vehicle control, and higher workload. Contrasted with messaging vocally with a cell phone, messaging utilizing Google Glass delivered less path trips, all the more braking reactions, and lower workload. All types of messaging disabled driving execution contrasted with undistracted driving. These outcomes suggest that the utilization of Google Glass for messaging impedes driving; however its Head-Mounted Display setup and discourse acknowledgment innovation might be more secure than messaging utilizing a cell phone, (He et al. 2015).

D. Google Glass in pediatric surgery: An exploratory study

The utilization of innovation to help restorative training is very much portrayed. We report another such case in which wearable innovation could be utilized to help the educating of systems without a lot of costly gear (eg, video headsets, transmission wiring and screens). The creators presumed that Google Glass would be a decent path for students to effectively procure intra-operative film for self-audit though the more lumbering GoPro would be utilized to store up a video library of basic operations. A current public statement from the University of California, Irvine (UCI) has recommended that Google Glass might be incorporated into its therapeutic showing courses for precisely such reasons, (Muensterer et al. 2014).

E. XBee Wireless Sensor Networks for Temperature Monitoring

XBee is an embedded wireless sensor network (WSN) prototype system for temperature monitoring in a building. This network is used for management of air conditioning systems at SIIT. The ultimate goal is to help saving the energy cost and reducing energy consumption. The system provides a web user interface for any user to access the current and past temperature readings in different rooms. The network consists of a data gateway or coordinator which wirelessly polls each WSN temperature-monitoring node located in each classroom. Each WSN node consists of a microcontroller on Arduino board and an Xbee wireless communication module based on the IEEE 802.15.4/ Zigbee standards. The coordinator also has an Ethernet interface and runs a simple data web server. Hence, the coordinator allows data collection over Xbee and data access from web browsers, (Greenwald 2013).

MATERIAL AND METHODS

System Architecture: The proposed system design focuses on commonly known design pattern for security of the mobile connection providing the maximum availability of the services that the system provides: reliability, fast response, availability of secure connection, usability, and efficiency (Bialas 2011) (Modares, Moravejosharieh & Salleh 2013). After analyzing the system architecture, a layered pattern is most suitable to serve the system to meet up with its services, iGuard architecture is composed of the following layers:

- Presentation Layer

Presentation of the web pages, UI forms and end user interacting API's

- Business Layer

The logic behind the accessibility, security and authentication happens in this layer, middle ware and other various request interceptors to perform validations.

- Persistent layer

This is the presentation layer for the Data. This includes the DAO (Data Access Object) presentation, ORM (Object Relational Mappings) and other modes of presenting persistent data in the application level. In more meaningful words this demonstrates the persistent data in RAM which usually stays in Disks at the below layer.

- Database Layer

Simple Databases is expanding up to SANs (Storage Area Networks).

Transactional Model Components

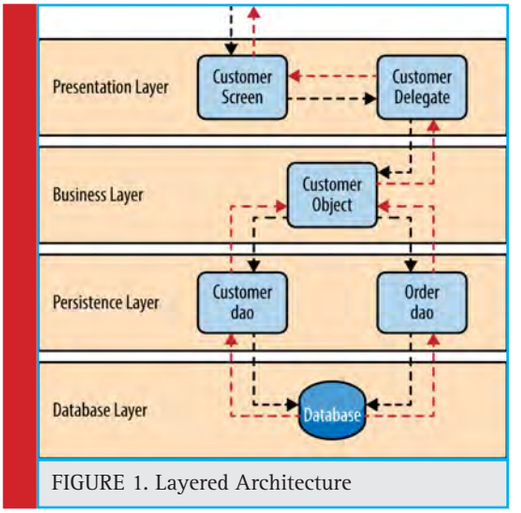

FIGURE 1. Layered Architecture

ALGORITHM

In this section, the algorithm according to which the alarm to the guard is issued will presented below

Do While
Sense the Office Temperature
If Motion Detector is triggered
If the Temperature is >24
Capture Image
Send Image to Google Glass
Else if the Temperature is <24
Check the AC and WIndows
Sense the Office Temperature
Endif
Send Notification to the Google Display
Display Image
End

FIGURE 2. Components transactions

Development Environment

The proposed system integrates multiple technologies. It includes motion detector board and a main controller using Arduino technology. The PIR sensor board detects the motion for example in the offices, labs and servers room then sends all the information to the main controller which sends the data to a web server and its database. Hence, if there are changes in the normal status by unauthorized access especially out of working hours, the security guard will receive alarm from the second part of the network, which displays the information retrieved to the security guard. One of the most functionalities that the proposed system offers is to let the guards keep their mobility and give them the ability to move and monitor at the same time. What we aim for in iGuard is to integrate Arduino technology and Google glass with mobile application to enhance security management applications as shown in the figure below.

Figure 3 shows how iGuard system works. The guard can select the location that he will monitor. The application sends the action to the Arduino which is connected to the application by Bluetooth and starts sense any motion. If any of the conditions below is detected, then the system sends to the camera the order to capture the scene. Finally, the application sends the captured image to the Google glass worn by the guard.

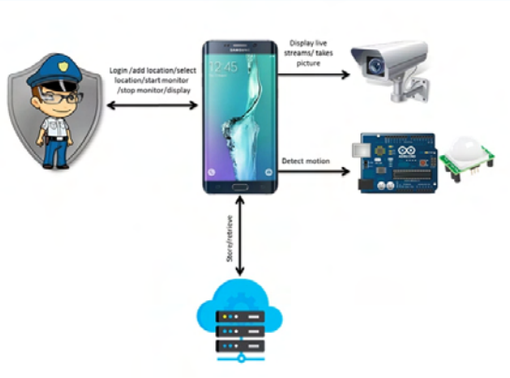

FIGURE 3. i-Guard system

In this paper, we proposed Mobile Security guard that improves the current state of security devices at most security companies. The system is composed of multi-sensor boards and a main controller. Each sensor board retrieves different forms of data such as the light intensity, sound waves and temperature for any location into the companies that will ensure the security at the building of any unauthorized access or especially out of working hours. One of the most functionalities provided in our system is to let the guards keep their mobility and give them the ability to move and monitor at the same time by applying that the efficiency of the job performance for the guard will be increased. What we aim for in mobile security tower is to reduce security guards at the company and increase their monitoring efficiency.

ACKNOWLEDGEMENTS

This research project was supported by a grant from the "Research Center of the Female Scientific and Medical Colleges Deanship of Scientific Research, King Saud University for funding this work through research group No (SMRC -1903)

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Technological Communication

Biosci. Biotech. Res. Comm. 12(2): 338-345 (2019)

BBRC Bioscience Biotechnology Research Communications

Development of an intelligent Arabic text translation model for deaf students using state of the art Information technology

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ABSTRACT

This paper presents an intelligent model to read, understand and translate Arabic text content into Arabic signs. The proposed model includes four main phases, preprocessing, modeling language, translation, and sign generation phases. In the preprocessing phase, the corpora and the stop words will be employed. The language model includes morphological, lexical and syntax, and semantic analysis. This is in addition to stem, root extraction with ontological support, and number indication will be involved. Consequently, we have different features that represent the analyzed Arabic text (words' meanings, words ordering, syntactic features, number features ...). Therefore, the generation phase takes place to generate the equivalent Arabic signs using the signer model. Accordingly, the deaf and hearing-impaired people are showing the generated stream of Arabic signs using video or 3D Avatar. The proposed solution uses programming corpus written in Arabic language, so the generated dictionary/lexicon has limited set of Arabic words with their meaning. After getting the content data from the course, the language model analyzes and understands the content and store it into deep structure or internal representation, consequently, the system will generate the Arabic stream signs based on the signer model.

KEY WORDS: ARABIC NLP, CORPUS, DEAF, HEARING-IMPAIRED, MACHINE TRANSLATION

ARTICLE INFORMATION:

Corresponding Author: Hassanin M. Al-Barhamtoshy
Received 9th May, 2019
Accepted after revision 25th June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
Crossref Clarivate Analytics
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/17

INTRODUCTION

Now a day, disability, deaf-blind, and hearing-impaired need a communication way to deal with teaching and communicating the community and society. The communication approach is to find the best way to speak, understand, visualize and deal what they are need. Sign language is the most effective way to communicate with those people. Sign language used for deaf and hearing-impaired people in order to facilitate the communication with community people (Abuzinadah, et al., 2017). A sign language recognition based on deep learning methodology from video sequences is proposed in (Konstantinidis, et al., 2018) and (Kong & Ranganath, 2008). However, face expression, two hands gestures and body language have been introduced to adopt the recognition and generation tasks with deaf and hearing-impaired (Lim, et al., 2016).

Other challenge point to work with the sign language is the dataset needed to work with sign recognition and sign generation. Some of this dataset uses only one hand (right hand), and others are signed with both hands, (Konstantinidis, et al., 2018) (Ronchetti, et al., 2016). Hand gestures provide important information to work with sign languages such as ASL, FSL, DSL, RSL and also Arabic Sign language (ArSL) (Patel & Ambekar, 2017) (Nikam & Ambekar, 2016) .

A more recent development in the academic, pedagogic and societal is a demand for legal recognition to emerge the hearing-impaired people into society (Harris, 2018). Therefore, sign languages are faced number of challenges related to socio-linguistic aspects for signing with the community (Quer & Steinbach, 2019).

A. Video Apprach

A bilingual corpus for Arabic sign language has been created in (ElMaazouzi, et al., 2016) concerned with developing a corpus for sign language that meets communication needs of the Arab deaf community. An alternative approach is presented to use video technology in Insign project to answer an online survey from 84 deaf over 22 diverse countries (Napier, et al., 2018). A deep learning method used to analyze and recognize video in sign language (Konstantinidis, et al., 2018) based on framework. In such work, video is examined and analyzed using images flow extracted features, and skeletal movement features (body, hand and face), in such case, each signed video corresponds separate word.

B. 3D Avatar Approach

An assistant education model with 3D avatar is presented in (Ulisses, et al., 2018) to incorporate *Virtual Sign* as a translator between sign language and oral language.In last decade, an example-based approach is implemented as a translator in American Sign language (ASL) with a deaf people (Morrissey & Way, 2005). In addition, leap motion can be converted and therefore, translated into text using assistant device such as tablet machine (Escudeiro, et al., 2017).

Simov (Simoy, et al., 2016) presented semantic-based approach to translate from Bulgarian and English in information technology domain. This approach is used for answering questions after employing of morphological analysis for Bulgarian nature. Some troubles and difficulties such as understanding the concept and the meaning of programming terminologies and related definitions need to additional elaboration. The idea behind that is, lexical units that have similar meanings should appear in similar context. Therefore, official repository for semantic information for course contents and terminologies definitions will be involved.

MACHINE TRANSLATION FRAMEWORK

A simple prototype framework for translate Arabic text content to Arabic sign language using video or 3D avatar will be described in this section. The framework of the proposed model accepts any Arabic text content and analyzes and understands such content and store such meaning into a deep structure or internal representation. The architecture of the proposed framework is illustrated in Fig 1. The proposed solution of ArSL signer based on rule and lexical-based approaches. It uses morphology phase and syntax phase in dependency trees, without any sophisticated methods (machine learning algorithms).

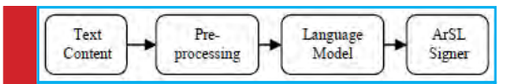

FIGURE 1. High Level of the Arabic Sign Langauge (ArSL) Translator Framework

C. Preprocessing Model

The proposed solution starts with retrieving Arabic text content, cleaning and preprocessing text data with analyzing, understanding with ways of language processing algorithms. Therefore, the “Arabic text content” must be aggressively filtered and corrected from obvious typos. So, syntactic analysis and semantic analysis will be explored. So, many NLP procedures will be involved, i.e.; word tokenization, stemming, POS tagging, word meaning, topic model or ontology model. Therefore, in this chapter various fundamentals in Arabic NLP coupled with other state of the art techniques will be covered. Consequently, the “Arabic text content” includes “Arabic stop words”, we need to remove these stop words before Arabic text analysis and understand. This in addition to make some corrections of the “Arabic text content”. After removing “stop words”, the language model takes

place to tokenize the "Arabic text content" stream before the POS tagger model.

D. Language model

The objective the language model is to analyze and understand the content text in order to extract the relevant information and create the internal features' representation of the analyzed text. This internal representation is very important to generate the equivalent of the signs of the Arabic sign language (ArSL). In this part, we are going to cover several procedures to process the Arabic text content along with examining content analysis. Therefore, ways of techniques to analyze and understand the Arabic content: text cleaning and text normalization, stop words removing, word tokenization, word stemming, and POS tagging. Consequently, the task of the language model includes several phases, tokenizer, morphological, syntactic and semantic analysis phases. Therefore, the language model uses a well-defined dictionary, lexicon and grammatical rules. The output results of the language model are used to achieve the internal deep structure (internal representation).

E. Phrase treatment and Arabic Grammar

The first step includes two entirely steps: sentence/phrase splitting and word derivations with affixes processing. Any Arabic text content will be segmented into separate sentences/ phrases (chunks). The Arabic phrases are classified into noun phrases (NP) or verb phrases (VP). We can extract the noun phrases from the Arabic text content, and consequently, identify noun, proper noun, and extra noun phrase from the text.

Phrase → < Noun Phrase > < Verb Phrase > | <
Verb Phrase > < Noun Phrase > | <
Noun Phrase > | < Verb Phrase > ..... (1)
Noun Phrase → < *Det* > < *Noun* > ..... (2)
Verb Phrase → < *Verb* > < *Noun Phrase* > | < *Verb* >
..... (3)
Det → < هذا > < هذه > ... ... < هؤلاء > ..... (4)
Noun → < حاسب > | < جهاز > | < برنامج > | < ملف >
| ... . < ملف > ..... (5)
Verb → < يحلل > | < تنفيذ > | < تتبع > | < سجل > | ... <
برمج > .....(6)

The Arabic grammar is flexible with phrase and sentence structure in word ordering. The simple Arabic phrase has three forms (read from right to left):

1. (فهم الطالب البرنامج) <Object: البرنامج> <Subject: الطالب> <Verb: فهم>
2. (الطالب فهم البرنامج) <Object: البرنامج> < Verb: فهم > < Subject الطالب>
3. (البرنامج فهمه الطالب) <Subject: الطالب> <Verb: فهمه> <Object البرنامج>

In English, the phrase might be (Student understood the program) => <Subject> <Verb> <Object>. Therefore, additional increase in complexity to understand and analyze the actual meaning for Arabic phrase structure happened.

F. Tokenizer Phase

After that, each sentence/phrase will be segmented into separate words (tokens). So, the morphological analyzer takes place to analyze and make derivations of the current word and remove affixes from it, and therefore, find root, stem, prefix, suffix, and infix (if it is existing). The affixes is processed to find out additional parameters or indictors to support the word type (verb, noun, character, etc.), gender, tense for verb, number "singular or plural" (Al-Barahamtoshy & Al-Barhamtoshy, 2017) (Al-Barhamtoshy, et al., 2014). Details of the preprocessing phase and the details of tokenizer module are shown in Fig 2 (Al-Barhamtoshy, et al., 2007). Arabic text content as inputting will be translated into stream of signs.

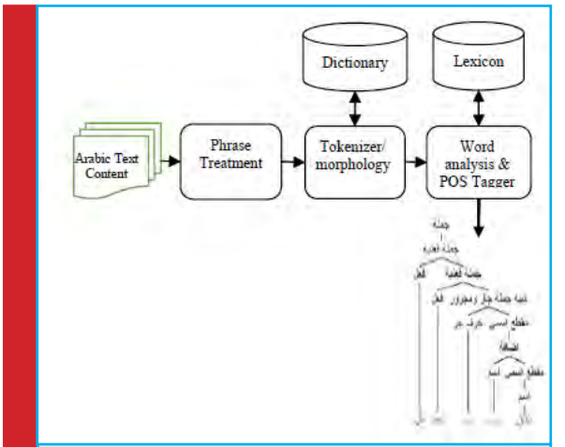

FIGURE 2. Language Model and Part of Speech Tagging (POS)

G. Ontology Phase

The ontology term is used to describe conceptualization of linguistic terms for representing entities in the domain. Other researchers are using ontology as resources of knowledge to measure the semantic similarity between analyzed tokens.

We are now moving from corpus data (unstructured data that includes programming contents) to deep structure or internal representation (semi structured data). If we know the course author name, we also know that author lives in country; author write books that are published on certain dates and written in a particular language, etc. There is a whole series of information to be drawn from this story scenario- this is the goal of the ontology. In addition, phonology helps us understand missing data. If the NLP analyst has realized the author and title, what has not been recognized? It seems that

the book is published in programming domain. So let's look for history - it's there. Moreover, it seems that the language is also involved - we can find it too.

This section presents additional formal linguistic information about word meaning (Arabic ontology). This formal representation describes the concepts of the Arabic programming terms within the course description. The formal representation includes terms concepts and their semantic relationships (see Fig. 3).

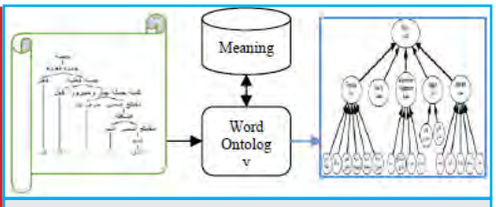

FIGURE 3. Word Ontology Parsing

The detailed design of the complete proposed solution is illustrated in Fig 4. We are now moving from corpus data (unstructured data that includes an introduction for Java programming contents) to deep structure or internal representation (semi structured data).

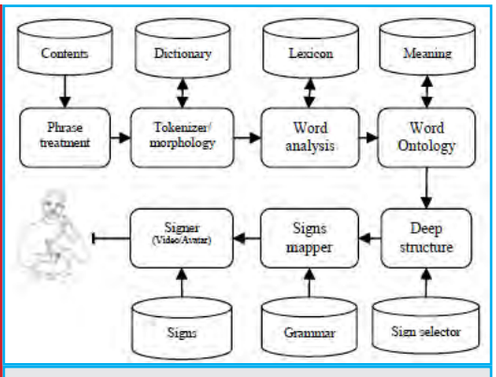

FIGURE 4. Arabic text 2 (ArSL) Sign Translator

The ontology provides semantic information about the analyzed words. Thus, the system performs "word sense disambiguation" (WSD) using the analyzed part of speech (POS) for deciding best notation. Therefore, an ontology-based in the information technology domain is created. It is used to enhance the semantic accuracy in the Arabic-signs translation. The syntactic analysis is done first (Al-Barahamtoshy & Al-Barhamtoshy, 2017) (Al-Barhamtoshy, et al., 2014) using Arabic grammatical rules (Al-Barhamtoshy, et al., 2007)].

H. Ontology Domain

The deep structure of the analyzed Arabic text is used to generate ontology programing domain. The proposed ArSL translator is mainly intended to provide translation process of the "primary programming content" to stream of Arabic signs at KAU university using both the two methodologies (video and Avatar). Therefore, SQL database can be used to generate the output stream using RDF, OWL and XML (visual ontology design). Fig 5 illustrates relation between the analyzed text content (internal/deep features' structure) and the visual ontology design module.

I. Sign Language Dictionary and the Signer

Many of bilingual dictionaries have been designed to support several sign languages over the world (Bouzid & Jmni, 2017). Each of these dictionaries tries to search and find the equivalent signs.

In this paper, we will use two methodologies of dictionary building. The first methodology is the video and written word utterance equivalent. The second is the sign written word with Avatar transcription. The ArSL translator try to find the video/avatar equivalent with the analyzed Arabic text. The only thing we need is large data. However, depending on how we plan to use our model, we need to be more or less satisfied about the quality of the dictionary and lexicon we use. When in doubt, the general rule is the more data we have, is the better.

Moreover, depending on the corpus size (text content), training can take several hours or even days, but fortunately we can store the analyzed data and extracted features on a storage disk. This way we do not have to do the analyzed tasks of model training every time we need to use it.

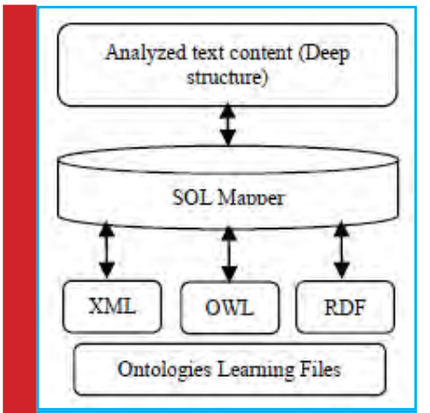

FIGURE 5. Anayzed text and Visual ontology Design

The solution takes the analyzed deep structure of the analyzed text and converts this structure into RDF using OWL and XML. Therefore, if ontology (repository) signer is ready with semantic concepts, the signer is ready to translate.

CORPORA CREATION

Many courses related to programming can be used to create dataset (dictionary and lexicon) of the proposed solution with the Arabic Sign Language (ArSL). The generated video terms with our corpus includes 149 samples. Table 1 and table 2 illustrate some examples that are selected from the created corpus. The following equations describes the tree domain of corpus regular expression that, includes our proposed corpora.

ArSL terms ⇒ <Computational> | <Control> | <Reserved> (7)
Computational ⇒ <Hardware/عتاد> | <Software/برمجيات> | … | <Race/تتبع> …… (8)
Control ⇒ <Package/حزمة> | <Class/فئة> | … | <While/طالما> …… (9)

TABLE I. ARABIC COMPUTATIONAL TERMS WITH ENGLISH EQUAVALENT DATA EXAMPLES

Terms		*Rating*	*Terms*		*Rating*
Arabic	*English*	*(1,2,3)*	*Arabic*	*English*	*(1,2,3)*
عتاد	Hardware	3	لغة برمجة	Language	3
برمجيات	Software	2	جافا	Java	3
تطبيق	Application	3	سي++	C++	3
برنامج	Program	3	وورد	Word	3
نظام تشغيل	Operating system	3	تصميم برنامج	Program Design	2
ويندوز	Windows	3	ترجمة برنامج	Program Compiling	2
يونكس	Lunix	2	نسخة احتياطية	Backup	3
أندرويد	Android	3	ملف	File	3
ذاكرة	Memory	3	سجل	Record	3
ذاكرة عشوائية	RAM	3	يحلل	Parse	3
حزمة	Package	3	خصائص	Properties	3
عام	Public	3	خاص	Private	1
قواعد	Syntax	3	اختبار	Test	3
تسمية	Label	3	رابط	Link	3
مفكرة	Notepad	3	تنفيذ	Execute	2
صحيح	True	3	مشغل	Operator	3
خطأ	false	3	تتبع	Trace	3

The equations in 7, 8, and 9 illustrate the different regular expression (in BNF grammars) to formulate the ArSL derivation grammars.

TABLE II. ARSL DEFINITIONS EXAMPLES

SN	*Original Content Topics*		*Rating (1,2,3)*	
	English	*Arabic*	*Video*	*Avatar*
1	Computer Compnents	مكونات الحاسوب	3	3
2	Hardware	المكونات المصنعة (العتاد)	3	3
3	Software	برمجيات أو تعليمات	2	2
4	Program Definition	تعريف البرنامج	2	2
5	Operating System	نظام التشغيل	3	3
6	Compiler	مترجم اللغة	3	3
7	Java Language	لغة جافا	3	3
8	Java Advantages	مميزات لغة جافا	2	2
9	IDE	بيئة التطوير المتكاملة	2	3
10	JDK	مكتبة تطوير جافا	3	3

The ArSL language is a derivative of spoken-written Arabic language, and therefore it is not a language by itself. This in addition to reordering of the signs or the position of signs. Such position or reordering of the signs' criteria will be taken into consideration, table 3 illustrates the word evaluation form, according to position or reordering of 10 Arabic signs.

TABLE III. DATASET SUBSET OF CONTROL STATEMENTS EVALUATION FORM

SN	*Arabic Word*	*English Word*	*Rating (1,2,3)*	
			Video	*Avatar*
1	حزمة	Package	*3*	*3*
2	فئة/صفيفة	Class	*3*	*3*
3	أمر الشرط إذ	If	*3*	*3*
4	أمر شرط إذ وإلا	If … else	*3*	*3*
5	أمر التكرار من	For	*3*	*3*
6	أمر حاول	Try … Catch	*3*	*2*
7	أمر حالة_ مفتاح	Switch … Case	*3*	*3*
8	توقف	Break	*3*	*3*
9	إفعل	Do	*3*	*3*
10	طالما/مادام	While	*3*	*3*

TESTING AND EVALUATION

To test and evaluate the proposed "Arabic Text to Arabic Signs" translation system, a human effort is used with respect to the equivalent translated signs. There are evaluation metrics that used to measure the quality between different machine translation solutions, but they are not used in sign translation. Therefore, an evaluation sheet has been prepared, includes the 10 technological translated definitions in the proposed dataset of the ArSL. Table 4 illustrates the proposed evaluation form.

Word Error Rate (WER) is used to evaluate machine translation and automatic speech recognitions (Al-Barhamtoshy, et al., 2014). The WER criterion based on the following:

$$WER = (I + D + S) / N \quad \ldots\ldots\ldots (10)$$

Where N represents total words in the dataset, I represents the number of words/signs that are inserted, D is the number of words/signs that are deleted, and S is the number of substituted words/signs in the translation process.

The WER is implemented based on the Levenshtein distance for words matching between original content (Java course) and the signed content (signed by ArSL).

Therefore, the accuracy is computed by:

$$\text{Accuracy} = (1\text{-WER}) \times 100 \quad \ldots\ldots\ldots\ldots\ldots (11)$$

The ArSL prototype system is implemented in Python language. The following figures (Fig 6 (a):(f)) and (Fig 7 (a): (f)) are the outputs of the two proposed models (video and Avatar).

FIGURE 6. Evaluated samples of output results for the Video approach

Next, we compare between the two models' video and avatar, using the predefined corpora. The deaf human expert evaluates each corpus' word by word using the created dataset. Table 4 describes the evaluation processes of the proposed dataset, considered word error rate (WER), sensitivity, and specificity.

TABLE IV. MEANING DATASET SUBSET OF ARSL EVALUATION FORM

SN	Arabic Meaning Definition Terms	Word Error Rate	
		Video	Avatar
1	مكونات أجهزة الحاسوب	0	0
2	مجموعة من المكونات المصنعة (العتاد)	0.2(1/5)	0.2(1/5)
3	مجموعة برامج أو تعليمات تشغل الكمبيوتر	0.3(2/6)	0.3(2/6)
4	مجموعة أوامر، تكتب وفق قواعد تُحدَّد بواسطة لغة برمجة، ثم تعمر هذه الأوامر بعدة مراحل وتنفذ على الحاسب	0.3 (6/18)	0.33 (6/18)
5	أحد مكونات البرمجيات الذي يقوم بتفسير التعليمات ومعالجة البيانات عن طريق الحاسب.	0.24 (3/12)	0.26 (3/12)
6	مترجم اللغة	0.5(1/2)	0.5(1/2)
7	لغة جاقا	0	0
8	مميزات لغة جاقا	0.3(1/3)	0.3(1/3)
9	بيئة التطوير المتكاملة	0.3(2/3)	0.3(2/3)
10	مكتبة تطوير جاقا	0	0

• Red color means that these words (characters) are not signed.

We analyzed the proposed corpora: Arabic text content corpus, video corpus and avatar corpus. We collected the errors, and then classified them into the two using approaches video and avatar. Table 4 illustrates the details of the evaluated work achieved by human experts. Therefore, the table demonstrates the WER, precision, and recall of the proposed translation using the two approaches. We found no significant difference between the two approaches video and avatar for the Arabic sign translation. Also, no statistically differences between the two approaches for precision and recall. The overall evaluation analysis of the output result demonstrates the effectiveness of our two approaches using the proposed translation solution of the Arabic text content to the Arabic sign language.

The accuracy performed through two experiments. Experiment # 1 using evaluator procedure with human

TABLE V. DATASET SUBSET OF ARSL EVALUATION FORM

Video Model			Avatar Model		
WER (I+D+S)/N	Sensitivity P/(P+N')	Specificity N'/(N'+P')	WER (I+D+S)/N	Sensitivity P/(P+N')	Specificity N'/(N'+P')
0	1.0	1.0	0	1.0	1.0
0.2	0.96	0.94	0.2	0.96	0.94
0.3	0.94	0.95	0.3	0.94	0.95
0.30	0.92	0.90	0.33	0.95	0.91
0.24	0.94	0.93	0.26	0.93	0.92
0.5	0.91	0.92	0.5	0.91	0.92
0	1.0	1.0	0	1.0	1.0
0.33	0.91	0.92	0.33	0.91	0.92
0.33	0.90	0.93	0.33	0.90	0.93
0	1.0	1.0	0	1.0	1.0

P = True positive value, N = True negative value.
P'= False positive value, N'= False negative value.

experts using WER to measure the errors in the generated Arabic sign results. We selected the original course text content compared to the generated (translated) signs output using ArSL. Fig. 5(a) illustrates the WER for the two approaches. Experiment #2 describes the precision and recall for the used corpora using the two approaches video and avatar.

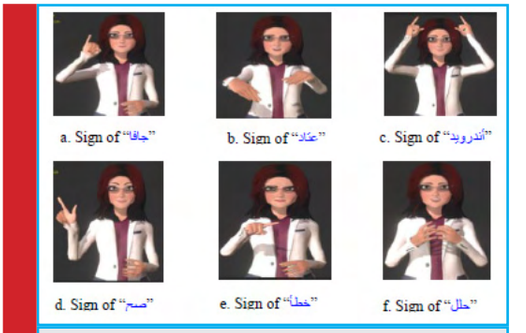

FIGURE 7. Evaluated samples of output results for the avatar approach

Fig 8 displays the comparison between the two approaches for the WER and the precision and recall for the evaluation form.

A. Experimental Testing

In real test, the ArSL corpus includes 150 of video signs stream in the domain of Java programming. It consists from 55 video for computational terms, 50 videos signs for reserved terms, and 45 terms for control and conditional statements. The ArSL signer test shows the number of correct and non-correct for deaf people at the committee student test (Table 6).

The total average value of WER for the sign understanding is derived with the proposed approach on the perception of the conducted human expert. The exper-

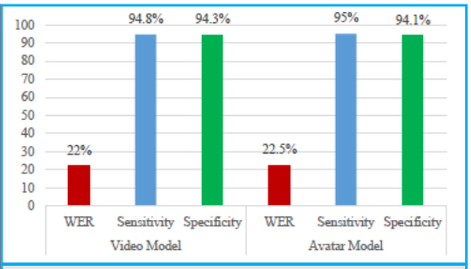

FIGURE 8. Video and Avatar evaluation for the WER, precision and recall

imental results show that the WER for signing of (1) computational-terms is 7.27%, (2) for Java reserved words (terms) is 4.00%, and (3) for control and conditional statement is 11.11%. The overall WER for the proposed work is 7.46 %.

TABLE VI. NUMBER OF CORRECT AND NON-CORRECT DURING SIGNER PRONOUNCIATION TEST

	Computational	*Reserved*	*Conditional*
Correct	51	48	40
Non-Correct	4	2	5
WER	7.27 %	4.00 %	11.11 %
Average WER	7.46 %		

TABLE VII. PERFORMANCE TESTING RESULTS

ArSL Terms	*Average time (Second)*	*Average File size (MB)*
Computational Term	3.0	3.150
Reserved Terms	1.2	1.750
Control and Conditional Terms	2.1	2.675

B. Performance Testing

In order to evaluate the speed of the proposed system, performance testing is done. Table 7 illustrates the performance testing results, during playing every term.

CONCLUSION

We have presented in this paper, a proposed machine translation model to translate from Arabic text content to Arabic sign language (ArSL). The real application domain includes an introduction to Java programming in the secondary school for deaf and hearing-impaired students. The solution build two corpus/corpora for Arabic sign language, one for avatar and second for real video in scope domain of programming.

This is the first corpora in Arabic sign language that covered the programming domain. Two prepared corpora approaches are implemented, one for video model and the second for Avatar model. The two models are used in translation for deaf and hearing-impaired children. For this purpose, 100 video and 100 avatars were tested and evaluated.

An expert human assessed manually, the two corpora of the proposed work. Then, this evaluator measured improvement according to the information technology and programming domain.

Future work will include well-known bilingual dictionaries/lexicons with mapping process to RDF and OWL for content translation. In addition, BLEU will be involved as metric to evaluate the meaning in the output result of the proposed system.

ACKNOWLEDGEMENT

The authors would like to pay special thanks to Ms. Khadija Kidwai for her valuable assistance during the research process.

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Technological
Communication

Biosci. Biotech. Res. Comm. 12(2): 346-355 (2019)

BBRC
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Tacit knowledge extracting in Holy Makkah municipality: An empirical study

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ABSTRACT

The present study dealt with the extraction of the implicit knowledge in the Secretariat of the Holy Makkah, which aimed to identify the experts of implicit knowledge in the Secretariat of the Holy Makkah, and the ways and methods used by the Secretariat of the Holy Makkah to draw knowledge from the knowledge experts working for them. The study relied on the use of the descriptive statistic approach to identify methods of extracting knowledge from knowledge experts located in the Secretariat of the Holy Makkah. The tool used is the interview, with the total population of the study (50) persons representing the knowledge experts working in the Secretariat of the Holy Makkah.The study sample consisted of 16 persons, 32% representing former secretaries and former leaders, as well as the second class of leaders in the Secretariat of the Holy Makkah. They were chosen by means of a intentional sample based on selective selection of the sample according to several criteria: Experience, qualification, scientific achievements, positions held. The study concluded that the Secretariat of the Holy Makkah is one of the first governmental sectors in the Kingdom of Saudi Arabia to introduce knowledge management, provide the necessary IT infrastructure for the implementation of knowledge management in the Holy Makkah Municipality, knowledge management, that there is a trend from the Secretariat in the transformation towards the application of information technology in all its works, the presence of awareness of the experts in the knowledge of the importance of information technology in facilitating the municipal work, support the secretariat of the Holy Makkah for electronic archiving projects that. The study showed that 56.25% of the sample have a scientific qualification in the field of engineering in various specialties, The study produced a set of recommendations, the most important of which are: To create a working environment in the Secretariat of the Holy City to transform the implicit knowledge available to institutional knowledge through the creation of efficient and effective policies and

ARTICLE INFORMATION:

Corresponding Author: mjdabusharha@gmail.com
Received 9th April, 2019
Accepted after revision 28th May, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/18

methodologies, to empower the work environment and encourage them to create new knowledge of knowledge gained and experience accumulated by retired or departing employees, which is one of the most important sources of implicit knowledge, which must be developed in the mechanisms of extraction, preservation and participation, the establishment of a database of implicit knowledge experts in the Secretariat of the Holy Makkah.

KEY WORDS: HOLY MAKKAH MUNICIPALITY - KNOWLEDGE MANAGEMENT - TACIT KNOWLEDGE - KNOWLEDGE EXTRACTION

INTRODUCTION

Access to and possession of knowledge is considered one of the most important challenges and difficulties faced by organizations that have adopted a trend towards implementing of knowledge management in their work, and there are many processes followed by organizations in obtaining knowledge that vary according to nature and environment. According to the tenth five-year plan of the Kingdom of Saudi Arabia, which was implemented in 2015-2019, the third item: "The transformation towards the knowledge-based economy and the knowledge society", and the Supreme Order No. 546 Minister of Economy and Planning "to come up with a comprehensive national and scientific strategy, to move to the knowledge society backed by time-limited operational and time programs" and implemented the "National Strategy for the transition to a knowledge society" (Ministry of Economy and Planning (2019) .

Recently, the number of organizations in different categories has increased, and competitiveness has become the strongest in order to maintain its survival in the labor market and gain more business. Knowledge is one of the main sources of competitive advantage of these organizations by transforming them into an added value by investing their existing knowledge Stored in the minds of individuals or what is known as human Makkah, as well as knowledge within those organizations stored in databases, research and reports. The process of knowledge extraction is one of the main activities of knowledge management, experienced and skilled workers who have acquired great knowledge in the work they have acquired during the course of their work and converted to explicit knowledge and are classified and coded to facilitate getting back to them and guide them within the organization and invest them as well, (Bawazie 2018).

Experts (the human element), or so-called intellectual Makkah, are the most important resources and knowledge assets within the organization. Knowledge organizations must give this element the greatest part of investment and development. What will be reviewed in this part of the paper falls under the concept of "implicit knowledge" Which means the set of experiences, skills and experiments lies in the minds of individuals, and are not written or encoded and difficult to move from person to person. One of the most important challenges facing organizations in implementing the concept of knowledge management is to work for the acquisition and gaining of implicit knowledge. Business organizations, economic, social, public and other organizations need implicit and explicit knowledge to gain added value in their work. The implicit knowledge is particularly important because the knowledge owned by an organization will necessarily be missing as soon as it is encoded and stored or simply distributed in directories and documents with products and services provided to beneficiaries or customers.It is necessary to define the implicit knowledge to be attracted, or to acquire and extract as well, and to establish controls that define the dimensions of the process of transforming implicit knowledge into explicit knowledge (Al-Salih, 2012).

There are a variety of methods and tools that organizations rely on in the process of deriving implicit knowledge, including exit interviews, best practices, post-action reviews, practice communities, social networking analysis, knowledge café, focus groups, organizational stories, etc., and there are multiple types of extraction are:

1. Extraction of knowledge from individuals (experts),
2. Extraction of knowledge from documents,
3. Extraction of knowledge from databases.

The Secretariat of the Holy Makkah (Makkah) was one of the first Saudi government sectors to adopt the concept of knowledge management and adopted the "Knowledge Management Project Implementation Initiative".In this study we will present the experience of the Secretariat of the Holy Makkah, The concept implementation of knowledge management and also experience in the process of deriving knowledge from its implicit knowledge experts and to document their achievements and experiences with a view to making use of them in sharing such knowledge with the rest of the Secretariat.The study includes the definition of the concept of extraction of knowledge and its characteristics, types and tools, and we will review the experience of the Secretariat of the Holy Makkah to apply the concept of knowledge management and experience in the process of extracting the knowledge from the experts of the implicit knowledge they have and documenting their achievements and experiences with a view to converting them into explicit knowledge and benefiting from them in sharing that knowledge with the rest of the Secretariat.

Next, we review the analytical aspect of the study, which is the analysis of the data collected through the interview tool with these experts and the most prominent findings. And then come up with a set of recommendations that contribute to the development of the performance of organizations and raise their productivity.

Objectives of the study:

1. The present study aims to identify:
2. Experts of implicit knowledge in Holy Makkah Municipality.
3. Ways and methods used by Holy Makkah Municipality to extract knowledge from its knowledge experts.

The importance of studying:
The importance of the study is as follows:

1. The need to activate and implement knowledge management in the Secretariat of the Holy Makkah, which includes the importance of investing in implicit knowledge and methods of extracting them.
2. Apply the study to the Secretariat of the Holy Makkah to reach results that contribute to the development of performance in the knowledge management unit.
3. The lack of Arabic studies that dealt with the extraction of implicit knowledge from individuals.

METHODOLOGY

The study relied on the use of the descriptive survey approach to identify methods of extracting knowledge from knowledge experts located in the Secretariat of the Holy Makkah. The method used is the interview, with the whole total of the study (50) representing the knowledge experts working in the Secretariat of the Holy Makkah. The study sample was composed of 16 people, 32% representing former secretaries and former leaders, as well as the second class of leaders in the Secretariat of the Holy City. And were selected in a intentional sample manner, which depends on the selective selection of the sample members based on several criteria, namely: Years of Experience, Qualification, Achievements and Positions held. The subject of extracting the implicit knowledge, according to the researcher's knowledge, is that it is one of the rare subjects that has not been studied by a small number of researchers, especially at the Arab level. This is one of the most important difficulties faced by the researcher to prepare this study, but there are some studies that dealt with the subject of implicit knowledge and its role in organizations and their relationship In making these decisions. The study (Al-Saleh, 2012), which aims to identify the role of implicit knowledge in the development of human resources in multinational companies under the concept of globalized management. The problem of the study was to try to answer the question: "does implicit knowledge have a role and an effect in the development of human resources under the concept of globalized management in the multinational company?

Pathirage et al (2007) aimed to highlight the importance of implicit knowledge in the construction industry and its impact on organizational performance., A theoretical study that showed the researchers' view of knowledge and its organized resources and the nature of implicit knowledge strategy, and described the characteristics of the construction industry in the UK and described the factors affecting people and the role of implicit knowledge. The study concluded that implicit knowledge plays a fundamental role in the changing business environment and contributes significantly to business continuity. The implicit knowledge is based on the skills, experience, and talent that people have and have to take into account. The study shows the important role of implicit knowledge in linking organizational performance and achieve competitive advantage.

Ribeiro, (2012) reported on the implicit knowledge management, where the purpose of the study was to study the theory of knowledge as well as to describe the implicit knowledge, problems and difficulties faced by organizations and companies in the process of polarization and composition and how to overcome them. The study also dealt with some of the practices that take place in some of the companies that have identified. In which the knowledge management has been implemented and the researcher used in this study the case study. It was about a Brazilian company working in the field of mining and iron industry, which employs about 1490 people and produces 50,000 tons of nickel annually. The researcher used in this study interviews as a tool to collect information and data, and the study reached several results, including:

That all types of implicit knowledge are important in the process of iron production or mining in general smoothly and safely, during the pre-operation stage. The results of the study also indicate the importance of training and education in developing the expertise, competencies and practices of the organizational workers and linking them to their existing knowledge and the factory's need for such knowledge, And the importance of previous experiences and their relationship to the nature of the current work and the extent of proficiency and skill in the performance of work and the measurement and identification of the existing experiences of workers and their ability to perform their work in the factory as a result of those experiences that were acquired in previous work.

Gavrilova & Andreeva (2012), emphasized the importance of deriving knowledge from the employees of the organizations (implicit - explicit) and also confirmed that part of the knowledge and experience is in the hands of the employees of the organization and belongs to them and not to the organization, Knowledge management tasks should therefore include a process of extraction from those who possess them. The study relied on a broad review of intellectual production on the subject of knowledge extraction, as well as twenty years of experience from one researcher in the application of different techniques and tools in the extraction of knowledge in many organizations. The study concluded that there is an urgent need for a specialist to obtain knowledge from Individuals (experts) ensure that these organizations benefit from existing knowledge and their participation and thus create new knowledge. The study suggested a new classification of the techniques and tools that must be relied upon in the process of knowledge extraction.

Extraction of implicit knowledge: In order to prepare this study, many studies and researches have been consulted on this concept. There are many terms used for the word extracting: Capture, Elicitation, Acquisition and Visualization. The following are the most important conclusions about the concept of knowledge extraction: The process of obtaining knowledge from its human resources (implicit knowledge of experts) and symbolic - explicit (knowledge in digital and physical media) and transfer and storage in the knowledge base or in knowledge management systems, the process by which the knowledge system development team to explore the knowledge that Used by FAO experts to accomplish the required tasks as a pilot and exploratory research process requiring interviews and protocol analysis to build knowledge management systems, (Yasin, 2006).

The process of retrieving and documenting explicit knowledge in its variousforms, or implicit in knowledge experts in organizational structures and organizations. The knowledge to be drawn outside the regulatory boundaries, including consultants, competitors, customers, suppliers and former employers of the organization, (Bisera and Rajeev 2014). A process through which knowledge is extracted from a huge number of data stored in digital repositories and databases using a set of statistical and mathematical styles and methods (Hayek, 2014).

Analysis and presentation of data: The accumulated experience of current employees, retirees or departures is one of the most important sources of knowledge that must be developed in order to extract, preserve and share mechanisms. In this regard, the current study in data analysis has been based on a number of procedures:

1. The total of the knowledge experts included in the Secretariat of the Holy Makkah of (50) persons (see Appendix 1) and the sample of the study was composed of 16 persons with 32% representing former secretaries and former leaders as well as the second row of leaders in the Makkah's secretariat. And they were chosen in a intentional sample manner, which depends on selective selection of the sample based on several criteria: Years of experience. Qualification. Achievements and Positions held.
2. Identify the appropriate study tool to extract the implicit knowledge of experts working in the Secretariat of the Holy Makkah, the interview.
3. Identify the axes of the interview that will be with the experts of the implicit knowledge, including:

- Curriculum vitae including (qualification - beginning of work in the Secretariat - positions held - period spent in the Secretariat of the Holy Makkah)
- What are the main achievements or projects that took place during your tenure in the Secretariat of the Holy City?
- What elements do you think were behind the success of these projects?
- Have you encountered difficulties in implementing these projects ... What are the most prominent?
- What projects have you implemented and have not been satisfied with?
- What are the causes of dissatisfaction?
- If you have the opportunity to implement the same projects again. Are you making changes to ensure success?
- What projects have you given or planned for and will not be conceived, approved or implemented?
- What are the reasons for non-implementation?
- How do you see the relationship between your administration, departments and other departments? Highlight the pros and cons or problems in this relationship?
- What are the main issues that concern you in your management?
- What important recommendations can you make to someone who may come after you in the same position?
- Are there any additions that you would like to make but not addressed?
- Experts of the implicit knowledge of the Secretariat of the Holy Makkah interviewed:

It is noted from Table (2) that the experts selected to derive the implicit knowledge that they are working in different areas of the work of the Secretariat, where we find that there are 15 administrations were selected from them, as Table 2 shows the diversity of their specialties, especially in the field of engineering, (9) of the experts interviewed have a scientific qualification in engineer-

Table 1. showing Experts of the implicit knowledge of the Secretariat of the Holy Makkah

M	Name	Management	qualification	Years of experience	position
1	Ibrahim Bin Sulaiman Abdullah	Information Technology	d. Assistant Information Technology	29	Assistant Information Technology
2	ENG. Gamal Ben Bakr Hariri	Former Undersecretary for Services (Retired)	BA - Civil Engineering	30	Undersecretary for Services
3	ENG. Ameen Naeb Al-Haram	General Directorate of Municipal Investments	Bachelor - Accounting	32	Assistant Secretary of Municipal Investments
4	Sharaf Al Abdali	Under Secretary for Studies and Projects (retired)	BA - Environmental Engineering	40	Undersecretary for Studies and Projects
5	Mtair Bin Menahi Al Qurashi	Personnel Management	Bachelor - General Administration	34	Assistant General Manager Personnel
6	ENG. Hisham Bin Abdulrahman Shali	Facilities Management and Environmental Management	Bachelor of Architecture	28	General Manager of Facilities and Environment
7	A. Samir Bin Mohammed Shafı	Personnel Management	BA - Administration and Economics	40	General Manager of Human Resources
8	ENG. Talaat Ben Salem Al Bar	General Manager	Land and Property Bachelor - Civil Engineering	32	General Manager Land and Property
9	Hani Hassan Faqiha	General Services Department	Bachelor - Mechanical Engineering	22	General Manager General Services
10	pm. Atef Ben Ali Mulla	Building and Engineering	Engineering Department Bachelor of Civil Engineering	21	Director of Buildings Affairs and Engineering Offices
11	Abdulaziz Al-Issaie	Office of the Secretary	Master - Civil Engineering	34	General Supervisor of the Office of His Excellency the Secretary
12	a. Saadi Bin Mohammed Al-Qarni	Office of the Secretary-General (Retired)	BA - General Administration	40	Advisor to the Secretary
13	ENG. Hassan Ben Ali Eid	Member of the Municipal Council (Retired)	Bachelor - Civil Engineering	40	Member of the Municipal Council
14	ENG. Gamal bin Abdullah Al - Hindi	Municipal Council	Bachelor - Computer Science and Engineering	37	Advisor to the Secretary of the Municipal Council
15	Dr. Saud Mohammed Al-Hitiri	General Manager of slaughterhouses	Bachelor of Veterinary Medicine Department of Interests		Management of slaughterhouses
16	ENG. Khalid Abdulhafid Fada	Development Authority Makkah	Bachelor - Architecture	35	Deputy Secretary of the development of Mecca

ing out of sixteen names representing 56.25% due to the nature of work in the field of the municipality as it needs the scientific qualification in most of the engineering disciplines needed by the Secretariat in the implementation of its tasks and objectives, While 43% of them hold qualifications in management , accounting and Veterinary Medicine. Table (2) shows that 100% of the selected persons have experience in the field of municipal work for at least 20 years. The selection of experts who are currently on the job is not limited to those who have been referred to but included the retirees who represent 37.5% in order to benefit from their experiences and extract their implicit knowledge and convert it into explicit knowledge.

Table 3 shows that most of the achievements made by tacit knowledge experts are the shift towards the

Table 2. showing Accomplishments of Knowledge Experts in the Secretariat of the Holy Makkah

id	Name of the expert	achievements
1	d. Ibrahim Sulaiman Abdullah	Transferring Transactions in the Secretariat to Electronic - Customer Service Systems represented by Sabeel, which provides 36 services - Geographic database SDI - Hajj Housing Services Project - Issuing shop licenses electronically - JRB project for financial purchases and monitoring of Inventory - Supervision of projects Electronic archiving in the Secretariat.
2	Mtair Bin Menahi Al Qurashi	Manual Job Description - Introducing Technology in the Work of Personnel Management - Employees 'Electronic Employees' Transfer Project
3	ENG. Hisham bin Abdulrahman Shali	Implementation of sports stadiums in Mecca - Increase green spaces in the streets and neighborhoods of Mecca - The allocation of the territory of the Secretariat in the neighborhoods to establish sports stadiums - Urban Park - Supervising the global competition to beautify Mecca.
4	Mr. Samir bin Mohammed Shafı	introduction of computer in the work of personnel management - recruitment of specialists in the management of personnel - archiving staff files in electronic - supervision of the installation of employees on the item of wages after the issuance of royal orders under the late King Abdullah bin Abdul Aziz - Special promotion items and competitions.
5	ENG. Talaat Ben Salem Al Bar	Supervising the improvement and beautification of the entrances to Madinah - Introducing GIS to land management in the Holy City Secretariat - Introducing the land expropriation system - Introducing the electronic archiving system in the land and property administration - Introducing the so-called land registry map (file containing all documents).
6	Hani Hassan Faqih	supervision of the return of electricity to the feelings in less than 24 hours after the fire incident Mona 1417 e - the addition of communication towers on the mountains in the feelings - privatization of equipment - the privatization of cameras - the modernization of the Secretariat's central program - the introduction of an automated system of the positions of the Secretariat.
7	ENG. Atef Bin Ali Mullah	Organizing the work of engineering offices - Developing the system of licenses (Licenses no hassle) - Establishment of an automated system for the registration of engineering offices.
8	Abdulaziz Al-Isaii	Management of the Kadi Streets Project - Increasing the streets and roads in Bahra Municipality - Supervising the cleanliness of the holy sites during the Hajj season - Establishing the advisory bodies in the office of His Excellency the Secretary
9	a. Saadi bin Mohammed Al-Qarni (retired)	Design of the organizational structure of the municipalities in the Kingdom of Saudi Arabia -Supervision of development Management in the Secretariat - Supervision of the work of Hajj.
10	pm. Hassan bin Ali Eid (retired)	Department of Municipal Investment in the Secretariat of the Holy Makkah - Supervision of the allocation of the central market (the ring) - Supervising the scheme of limited income - Development of the market Otaibia - Development of security and safety management and the introduction of uniforms uniform guards and the introduction of cars special security and safety.
11	ENG. Gamal bin Abdullah Al-Hindi	Development of the Information Technology Department in the Secretariat of the Holy Makkah - Development of municipal procedures such as shop licenses and linking the procedures of their extraction with the relevant authorities - Supervision of the new building of the Information Technology Department
12	Dr. Saud Mohammed Al-Hitiri	Launch of the Award of the Custodian of the Holy Mosques for Institutional Excellence - Establishment of control offices in the slaughterhouse - Imposition of violations of the meat shops violation - Establishment of two new slaughterhouses in Mecca - Dealing with the accumulation of sacrificial animals slaughtered during the Eid al-Adha.
13	Dr. Ashraf Al-Abdali (retired)	Improvement and development of land - Development of Al-Awali neighborhood - Development of Al-Azizia neighborhood and planning - Third ring line - Planning 60 thousand pieces of land - Establishment of sub-municipalities - Giving powers to municipalities to issue permits.
14	ENG. Ameen Naeb Al-Haram	Development of an automated system for the management of financial affairs - Establishment of a system to accelerate employee promotions - Increase the resources of the Secretariat - the collection of 100 million riyals during a short period. Investment of the new livestock market - Chairman of the Knowledge Committee of the Secretariat
15	ENG. Khalid bin Abdulhafid Fada	Supervising the implementation of 5 arterial roads in the Secretariat of the Holy Makkah Garden design - Supervision of the Fourth Ring Road - Supervision of consulting contracts - Supervision of the restoration of the historic Palace of Saqqaf
16	ENG. Jamal Hariri (retired)	Supervision of land management in the Secretariat of the Holy Makkah - Supervision of granting of limited income - Accelerate achievement in grant transactions and organization - Supervision of the expansion of the Malawi - Supervision of the allocation of the project of cleanliness - Afforestation of feelings(sites) - Offering maintenance and operation contracts for street planting.

application of information technology in the performance of their departments. Where most agreed and this is what was concluded through the interview that 60% of them had the largest role in the development of automated systems in the work of departments and the shift to automate their work.Dr. Ibrahim Sulaiman, Assistant Secretary of Information Technology, is the most prominent in accomplishing achievements in the work of the Secretariat because of the great orientation of the former secretaries and the current Secretary of the Secretariat to move towards the application of information technology and to provide the best means and methods that serve the clients benefiting from the work of the Secretariat. As evidenced by the interviewees. It also shows that there is a tendency from most departments towards electronic archiving, especially those that have archival sections and deal with important documents such as Instruments and financial management documents such as financial derivatives of Masha Which are valuable historical and reference in the case of need to make a decision. This is an important indicator of the application of the concept of knowledge management in organizations. The most important challenges facing organizations to implement knowledge management are the readiness of the IT infrastructure.

It is also shown from Table (3) that, according to the interview, there were experiments conducted in the Secretariat of the Holy Makkah and the first at the level of the departments in the governmental sector, especially the municipal work. The Secretariat is distinguished by the rest of the secretariats in Saudi Arabia for their presence in mecca, Of direct services to pilgrims in the Hajj season, as well as pilgrims for the whole year. The privatization experience in the government sector started early in the work of the Secretariat. For example, the Secretariat privatized heavy equipment and machines and delivered them to the private sector for operation and maintenance, as well as privatization of the cleaning sector, maintenance and irrigation of gardens, Safety cars, and photocopiers, They confirmed who were supervising these projects that the Secretariat of the Holy Makkah was the first in the Kingdom of Saudi Arabia in the implementation of these projects and benefited from the experience and then circulated to the rest of the secretariats and other government sectors.

As shown in Table (3), some of the experts of implicit knowledge interviewed during the period of their work in the Secretariat required them to deal with them more accurately and able to deal with crises, as happened in the fire incident in 1417 e and burning a large part of the camps in addition to the power outages and the burning of cables and control panels for the delivery of electricity was in the pilgrimage season that year and specifically on the seventh day of the month of Dhu Al-Hijjah This represents a major challenge to the parties involved in providing solutions, and these incidents are rare but

Table 3. Showing Level of relationship with other departments

id	Name	Relationship level		
		Excellent	Good	Poor
1	d. Ibrahim bin Suleiman Abdullah.	✓		
2	Mtair bin Menahi Al Qurashi	✓		
3	Hisham bin Abdul Rahman Shli		✓	
4	Mr. Samir Bin Mohammed Shafı	✓		
5	m. Talaat Ben Salem Al Bar		✓	
6	Hani Hassan Faqiha.	✓		
7	m. Atef bin Ali Mulla.		✓	
8	Abdul Aziz Al – Issaie	✓		
9	a. Saadi bin Mohammed al-Qarni.	✓		
10	pm. Hassan bin Ali Eid.		✓	
11	m. Jamal Abdullah Al Hindi		✓	
12	Dr. Saud Mohammed Al-Hitiri	✓		
13	Dr. A. shraf Al-Abdali	✓		
14	ENG. Ameen Naeb Al-Haram		✓	
15	M. Khalid bin Abdulhafid Fada.	✓		
16	m. Jamal Bakr Hariri	✓		

may occur again, It is important to document this incident To try to know the reasons and methods of prevention so as not to recur, and it was important to document the solutions reached in order to be used in the case of God forbid again.

As well as the experience of working in the slaughterhouses, especially in the Hajj season and the required preparation and processing of those abattoirs due to the large numbers of sacrifices that are slaughtered in this period and how to deal with and benefit from them to be distributed to the poor and needy of the poor Mecca and the world also instead of what was previously Leaving some to rot and become unfit for human consumption, and introducing nitrogen cooling technology as a solution to freeze the meat quickly, as well as benefit from the project to benefit from the meat of sacrifice. Through the interview with the experts of the implicit knowledge in the Secretariat of the Holy Makkah to extract their experiences and their desire not to lose the knowledge they hold and benefit from them in the current municipal work, where 30% of the 50 names proposed (see Appendix No. 1) have been referred to retirement After they reach the statutory age or have completed their period of employment in the Secretariat, and some of them prefer to leave the work in the Secretariat to practice in the private sector.

One of the most important experiences in the Secretariat of the Holy Makkah is the implementation of the job description manual and its electronic availability on the internal rules of the secretariat. It includes all the tasks required to be performed by the employee. This is one of the most important procedures practiced by organizations that have adopted the concept of knowledge management. This guide contributes to the dissemination of knowledge to be provided by the employee and he / she shall be given the means and channels of communication with the other departments and shall indicate the limits of his powers so as not to interfere with the functions of another function.

As shown in Table (3), the direction taken by the Secretariat of the Holy Makkah to develop municipal investments through the investment of land and the projects it owns. One of those who worked in this administration stated that the revenues that enter the budget of the Secretariat increased from 35 million riyals in the past 20 years to about 350 million riyals during the year and this is the largest number in the level of secretariats in Saudi Arabia because of its presence in Mecca and the high prices of real estate in them.

The study aims (from the table 3) to identify the level of relationship with other departments to show the extent, integration and participation in the dissemination of knowledge among the departments operating in the Secretariat of the Holy Makkah.Table 3 shows that through the interview with the experts of implicit knowledge in the Secretariat, A constant need for coordination and partnership between competent departments in order for the process to be integrated.During the interview, it was found that 62.5% of the departments have a high level of coordination with the other departments in the Secretariat. For example, the IT Department has direct interaction with all departments and administrations in the Secretariat due to the shift in the application of IT in all the works of the Secretariat, whether it is an electronic archiving process or the introduction of automated systems in the work of these departments. The interview also revealed that the IT Department is responsible for managing the secretariat electronic portal, which includes providing all services and news of departments, as well as facilitate electronic transactions such as issuing licenses Construction, customer services, etc.

The level of relationship between personnel departments and all other departments in the Secretariat is excellent because it is the administrative reference for all employees of the Secretariat of the recruitment and administrative transactions and promotions and leave and save the files of staff and other services provided by the Department, while Land and Property Management has relationships with IT departments, document management and urban planning management as they need to coordinate with them on facilitating access to information on the nature of their work.

Difficulties and Challenges:The study found that, according to the axes of the interview, there are a number of difficulties and challenges faced by implicit knowledge experts in their implementation of some projects in the Secretariat: The following are the most prominent difficulties:

1. Resistance of some leaders in the Secretariat of the Holy Makkah and staff to change and shift to the use of information technology in the provision of municipal services.
2. Lack of awareness among intermediate administrations of the role of technology in accomplishing work.
3. The inability of some employees to describe the appropriate procedures in their work to delay the issuance of the job description manual.
4. The weakness of electronic archiving projects which have not been completed as required, the difficulty of retrieving information, the lack of comprehensiveness of bibliographic data of the document, and the loss of some important documents during the archiving process.
5. The loss of a lot of implicit knowledge because of the departure of experts to the Secretariat.

6. Lack of financial allocations for the implementation of some electronic archiving projects.
7. Weak provision of qualified national cadres, especially in the field of programming and information networks.
8. Lack of awareness of some of the experts and implicit knowledge in the importance of sharing knowledge and transfer to others.
9. Not to benefit from the projects and consultancy studies that worked in the Secretariat.
10. Lack of young leaders qualified to complete the process of the secretariat in municipal work.

CONCLUSIONS AND RECOMMENDATIONS:

First- Results: The study reached a number of results, as follows:

1. The Secretariat of the Holy Makkah is one of the first government sectors in Saudi Arabia that initiated the implementation of knowledge management.
2. the importance of implicit knowledge in the development and advance of work in all areas.
3. Availability of the IT infrastructure required to implement knowledge management in the Holy Makkah Municipality.
4. The study found that there is understanding and awareness of employees in the Secretariat of the Holy Makkah towards the concept of knowledge management.
5. Support the senior management in the Secretariat of the Holy Makkah to implement the concept of knowledge management, and this is reflected in the Official correspondence that are made to all those working in cooperation and facilitate the task of the Department concerned with the application of knowledge management.
6. The Secretariat is moving towards the application of information technology in all its works.
7. The existence of awareness of knowledge experts about the importance of information technology in the facilitation of municipal work.
8. Support the Secretariat of the Holy Makkah for electronic archiving projects that contribute to the preservation of important documents from damage and loss and also help in decision-making.
9. The study found that the IT Department has played a major role in developing the performance of many departments in introducing the systems they need and supervising electronic archiving projects.
10. The study found the need for the Secretariat of the Holy Makkah to extract and document knowledge from the experts of implicit knowledge before losing it due to the loss of the person.
11. The study found that the Secretariat of the Holy Makkah in its Personnel Department has a job description manual for all posts in the Secretariat and is available electronically.
12. The study showed that 56.25% of the sample of the study have a scientific qualification in the field of engineering in various specialties.
13. Absence of encouraging incentives for sharing knowledge among individuals.

SECOND: RECOMMENDATIONS:

Based on the results of the study, it is possible to come up with a set of recommendations and proposals that can be used by organizations and bodies to invest their resources and knowledge assets, through the following:

1. Creating the working environment in the Secretariat of the Holy City to transform the implicit knowledge available to institutional knowledge assets through the creation of efficient and effective policies and methodologies.
2. The working environment will empower staff and motivate them to create new knowledge of the acquired knowledge.
3. Utilizing the accumulated knowledge and experience of retired or departing staff, which is one of the most important sources of implicit knowledge, which must work to develop mechanisms of extraction, conservation and participation.
4. The establishment of a database of implicit knowledge experts in the Secretariat of the Holy Makkah represent most of the specialties needed by the Secretariat and be a reference when needed to take a decision or solve a problem.
5. Attracting qualified and trained professionals to implement knowledge management requirements.
6. To work on spreading and strengthening the knowledge culture among the employees of the secretariat, and embed them within the institutional culture.
7. The Secretariat of the Holy Makkah has to benefit from the sources of explicit knowledge available in it, such as: the Documentation Center and the Library of knowledge that was created recently and the portal of knowledge of the Secretariat.
8. Creating special programs to activate the Knowledge Café, which was established at the Secretariat's Knowledge Management Headquarters, with the aim of extracting, transferring and sharing the knowledge of the experts to the rest of the employees.

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Technological Communication

Biosci. Biotech. Res. Comm. 12(2): 356-361 (2019)

BBRC Bioscience Biotechnology Research Communications

3D Echolocating system for the visually impaired based on bat SONAR approach

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ABSTRACT

Video games are becoming widely used today for entertainment and socializing. However, with all the technology there are few games for the blind or the visually impaired people. In the literature, research attempts for wearable and/or assistive systems designed to support visually impaired people are limited in terms of accuracy and support only limited tasks; such as: learning activities, sending SMS, ... etc.. They use predefined tasks and train visually impaired individual to use the system. For navigation and detecting obstacles whilst moving in uncertain environment, visually impaired people would need fruitful research regarding training visually impaired people to understand and interpret the 3D sounds; commonly known as BATS based software. BATS software supports independent mobility for visual impairments individuals benefit as it provides means to navigate, detect objects, and react to uncertain issues in surrounding using sound navigation and ranging (SONAR). In this paper, we propose a game that allows visually impaired/blind individual to be trained to interpret sounds through the game by play against a sighted opponent (teacher/ relevant or mother for the child). The main idea of the game is to let the sighted player raise sounds; programmed in the game players attack each other's area. The sighted player mode will have both visuals and audio. According to the Bat system, the blind/visually impaired will have 3D audio, Ultrasonic sensors are used for detecting the obstacles whilst the Servo motors are used to give a precise position and the google glasses to make object type recognition. The sighted opponent will enforce objects occurrences inside the game and the blind/ visually impaired user would be trained use the system in order to be able determine types of the objects, distance to objects compensate the visuals.

KEY WORDS: ECHOLOCATION, EDUCATION, BLIND, VISUALLY IMPAIRMENT, BAT HEARING SYSTEMS, SPATIAL SOUND, AUDIO DESCRIPTIONS, PERCEPTIBLE FEEDBACK, GOOGLE MAPS, GOOGLE GLASSES, ULTRASOUND SENSORS

ARTICLE INFORMATION:

Corresponding Author: galhudhud@ksu.edu.sa
Received 10th April, 2019
Accepted after revision 7th June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
Crossref Clarivate Analytics
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/19

INTRODUCTION

Video games industry has grown quickly since it emerged in 1970s. People play video games for entertainment and socializing. In 2016, the industry generated more than $30.4 billion in revenue from selling games. In 2018, frequent gamers play multiplayer and online games spend an average of 7 hours a week. The average gamer age is 34 years (2019). The attractive design of the user interface and the idea of the game keep people playing. Today, most of the games interface and audio are 3D that gives the game more reality. Also, the accessories that used to play the game with like virtual reality, controller, and headphone. However, with all this technology there are few games for the blind or the visually impaired people (Kolarik et al. 2014). According to the *world health organization*, the visually impaired population worldwide exceeds 1.3 billion and 36 million of this population are totally blind (2019). Accessibility refers to the design of products, devices, services, or environments for people who experience disabilities (Henry, Abou-Zahra & Brewer 2014). Game accessibility allows people with visual disabilities the chance to have access to multimedia games equivalent to sighted people. It is challenging since the main feedback channel in games is usually visual while it is difficult or even impossible for visually impaired players to receive feedback from games (Giannakopoulos et al. 2018).

In fact, game accessibility a more challenging and complex problem than computer accessibility and web accessibility (Archambault et al. 2007). To make games accessible to blind players, visual feedback needs to be replace with another form of feedback that is perceivable by a blind player, such as auditory or haptic. Option for visual replacement with audio include: screen readers, Audio cues: using real world sounds, or Sonication. In some cases it is difficult to replace visual with audio such as in music games, in such cases haptic feedback is preferred over audio. (Archambault et al. 2007) (Yuan, Folmer & Harris 2011):

Echolocation is an acoustical process for both object location and identification by means of sending sound pulses and receiving the reflected echoes (Kim 2015). Bats are known to be able of echolocation (Sumiya et al. 2019). They would give a short whistle and estimate the distance from the shoreline by the returning echo. If the echo came back from both sides at the same time they'd know that they were in the middle of the channel. They could recognize different shorelines by the different echoes - a rocky cliff, for example, would give a clear distinctive echo, whereas a sandy beach would give a more prolonged echo. They could even pick up an echo from logs. Echolocation is mostly used to discuss the responses of bats and dolphins, which are known for their echolocating abilities. It was first used by Griffin in 1944 (Griffin 1944) to describe the exceptional ability of bats to navigate in the darkness, experiments have shown that this ability was based on the principles of echolocation (Kolarik et al. 2014) (Koning 2014). Humans are not considered among the echolocating species. However, some blind human can develop echolocation skills and show remarkable spatial abilities and become an expert echolocator (Yu et al. 2018).

Human echolocation is the ability to locate objects in the environment through interpreting acoustic echoes (Thaler & Goodale 2016). A human trained in echolocation can obtain information about the environment around him such as objects, he can also accurately identify distance and size. Most of us have encountered a blind person walking alone and able to avoid obstacles while navigating (Sohl-Dickstein et al. 2015) (Milne 2014) (Thaler & Goodale 2016). There are two different types of echolocation: passive and active (Flanagin et al. 2017). Passive echolocation is interpreting the echoes of the natural sounds produced around you. On the other hand, in active echolocation, you will actively produce sounds and then receive and interpret the reflected echoes to extract localization information (Koning 2014). Studies have revealed that blind and visually impaired people are more sensitive to acoustic reverberations echoes than sighted people (Thaler 2015). They use the natural surrounding echoes or the reflected sound waves to sense details about their environment and build a mental image of it. Therefore, they have the potential to use echolocation system like Bats to detect where objects are. To actively echolocate, blind people have to learn how to visualize their surroundings by making clicking sounds with their tongue and using their echoes to gauge information about their environment and move about. Blind people who become experts in click-based human echolocation are able to determine an object's distance, size, texture and density. Echolocation can be seen as an effective mobility, location and orientation aid for blind and visually impaired people, with which they can improve their independencies and quality of life (Milne 2014) (Thaler & Goodale 2016).

Each Audio technology made it possible to present audio with a 3D effect. 3D audio effects are sounds that play through a stereo output, surround sound speakers, speaker arrays, and through smart phones and handheld gaming systems, headphones. 3D audio is very important in gaming and is more important in designing games for the blind (Russo, Sacks & Vandal 2012). It gives people the ability to hear voices from different positions and allow them to have a realistic feeling of the environment of a particular app or a game. Adding a 3D audio to the game will provide users with a more real experience allowing them to know what their surroundings

are, what is close to them and what is far from them. With the help of 3D audio, game developers can create different games for blind or visually impaired players by using it to help the player imagine the games environment and providing a 3D audio to their surroundings or enemies and their movement to help the blind/visually impaired players to locate them. There are different ways to stimulate the 3D sound, but the most practical way is to use a game engine with the ability to take a sound and create spatial perspective by placing it in the scene of the action such as Unity (Technologies no date) and Unreal (Epic Games no date) game development kit.

In the last few years, the world became aware of the need for technologies and software that is designed specifically for the blind and visually impaired people. The growth of these technologies and software is slow in game industry. A number of games currently employ mechanisms to assist players who are blind. Here, we will survey some e-games that are developed for the blind. A Blind Legend (Dowino no date) is an audio-based game that offers a 3D audio experience to the player. The user play as a blind knight whose wife has been kidnapped and must rescue her with the help of his daughter who give the blind knight the directions to where to go next. The daughters sound is 3D, while wearing your head phones the user can hear where she is and follow her voice. At the beginning the player is given instructions on how to play using a human audio that introduce him the basic controls to play the game, for example, to move you need to drag your finger on the screen to the direction you want to go next, forward, backward, left or right, and to move faster you need to drag your finger farther and hold it on the screen. Lifting your finger from the screen will stop the knight from moving. Papa Sangre II (Webster no date) is an audio game for sighted and blind people for only iOS users. Papa Sangre II doesn't have a graphical user interface which makes players use their fiction to imagine. In this game, player navigate a dark world using sound only, guided by a narrator voiced by Sean Bean who is Lord of the Rings and Game of Thrones star. Even without graphics, the game is one of the biggest selling points for games. The first thing that player told in Papa Sangre II is that "you are dead" and they must find the way to the land of living. There are set of instructions that let gamer navigate the game like to move forward tap the two lower corners of your iPhone or iPad. Entombed (Driftwood Games no date) is a game for blind and visually impaired designed by driftwood audio entertainment company. This game has been in development since 2008. In Entombed, you battle to escape a deep and brutal dungeon and having been thrown into the infamous pit then you have to find a passage that returns you to the surface. Along the way, you will face ogres, menacing goblins and living statues. Depending on the sound, you keeping away from hurt and try again to reach the safety surface. Blindfold Racer(Kid Friendly Software, Inc no date) is a driving game where you drive with ears instead of eyes. It is a free game for blind and sighted people. Game depends on the audio instead of graphical user interface but if player sighted can see road after level over. It fit kids and adults.

The idea of the game is the player avoids hitting animals while driving. The player drives his/her car by listing to what on the road. It is a multi-level game and require headphones to have fully enjoy them. SixthSense (Hyun Soo KIM no date) is a game for both blind and sighted people. It is free application on app store. The SixthSense is an action-horror game. The game idea is to fight the zombies with various of weapons. The player can control the game by sliding and tapping the screen. The player required to wear headphone to play the game because it is provided with 3D surround sound to make the player feels like he is really attacked by the zombies. Also, it has voice over function for blind player. In an attempt to enhance racing games accessibility and allowing players who are blind to play the same racing games as do sighted players, RAD was developed as a racing auditory display (Smith & Nayar 2018). It is an audio-based user interface. It works with a standard pair of headphones and comprises two novel sonication techniques: the sound slider for understanding a cars speed and trajectory on a racetrack and the turn indicator system for alerting players of upcoming turns.

In this paper, we present an e-game application that allows the blind/visually impaired player to play with sighted opponent. The main idea of the game is to let the players attack each other's area. The game will have two different modes. The first one is the blind mode; it will have 3D audio for the blind/visually impaired player to compensate the visuals. The second mode is the sighted player mode where it will have both visuals and audio. To create the 3D model for sighted people in the game, Unity 3D is chosen so that it can attract sighted people to the game and let them share fun with blind people.

MATERIAL & METHODS

Our game interfaces are 3D interfaces. First interface's aim is to define if the user is blind or sighted and it is an audio interface. Second interface contains registration form. Third interface is the main menu which contains two buttons: Play (to start new game) and Tutorial (to play the tutorial). Fourth interface is level of difficulty to let the player choose which level she/he wants to play. Fifth interface is choosing opponent to let the player choose which available opponents to play against. Last interface is result interface, to inform both players about

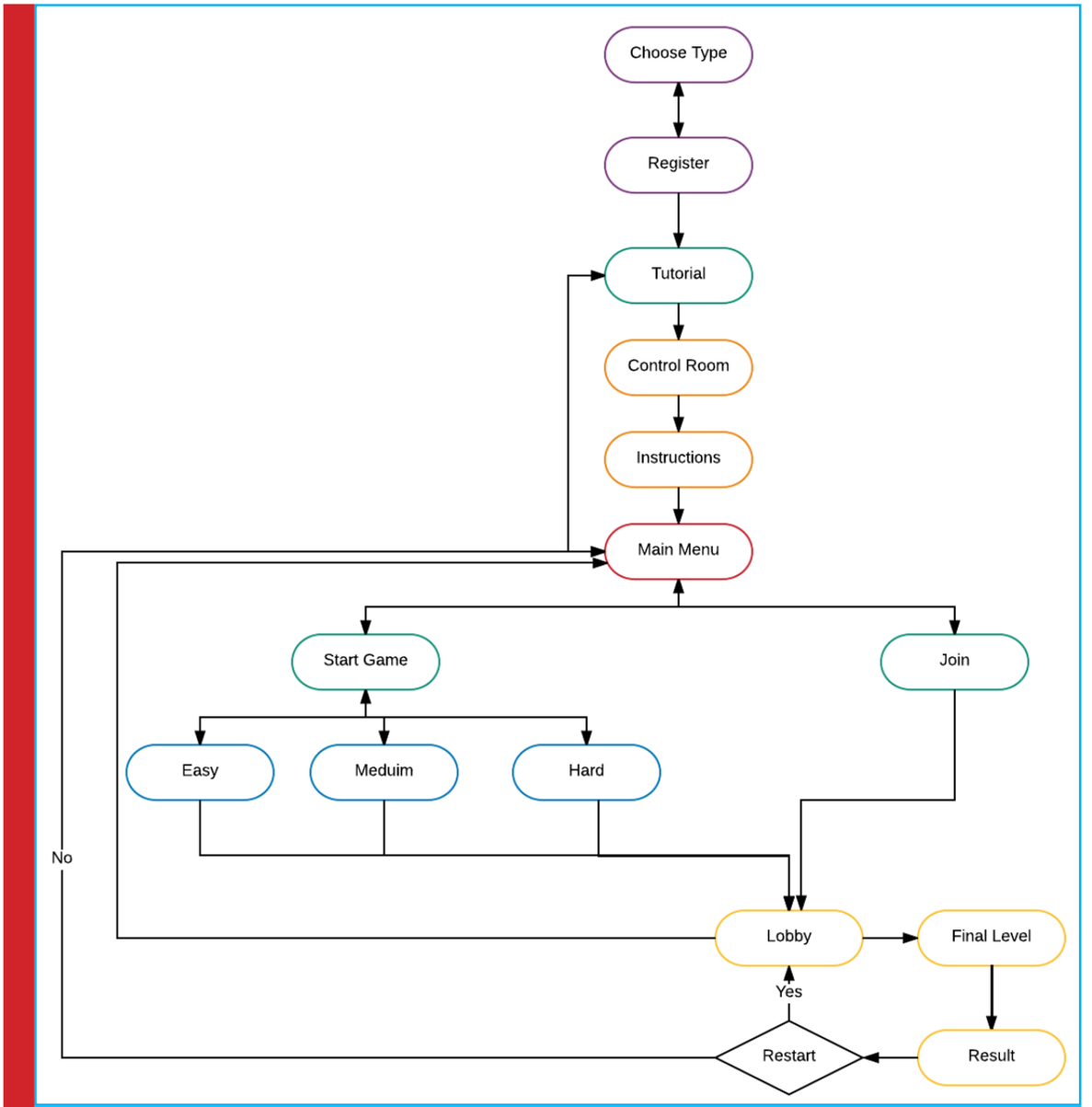

FIGURE 1. System Interfaces hierarchy

game's result and to let the players play again if they want to. Also, there are three different levels on the game.

The proposed game as an assistive system is based on using echo processing techniques for interpreting the echo from the surrounding objects, people in order to be able to decide direction, changing position, and or performing an action.For this purpose, Ultrasonic sensors are used for detecting the obstacles whilst the Servo motors are modeled and used inside the game used to give a precise position and the google glasses to make object type recognition. Accordingly, the system assists the user's avatar to sense the environment through the ultrasound and servo motors and hence the sensory data will be used for input to a simple fuzzy controller in order to calculate and make real time accurate decisions based on the information in order to enable the user to navigate safely in uncertain environment.

The algorithm is used for indoors and outdoors for the purpose of wider coverage over also day time as at night time

The game provides a range of distance between the user foot radius circle. The system would allow the user to

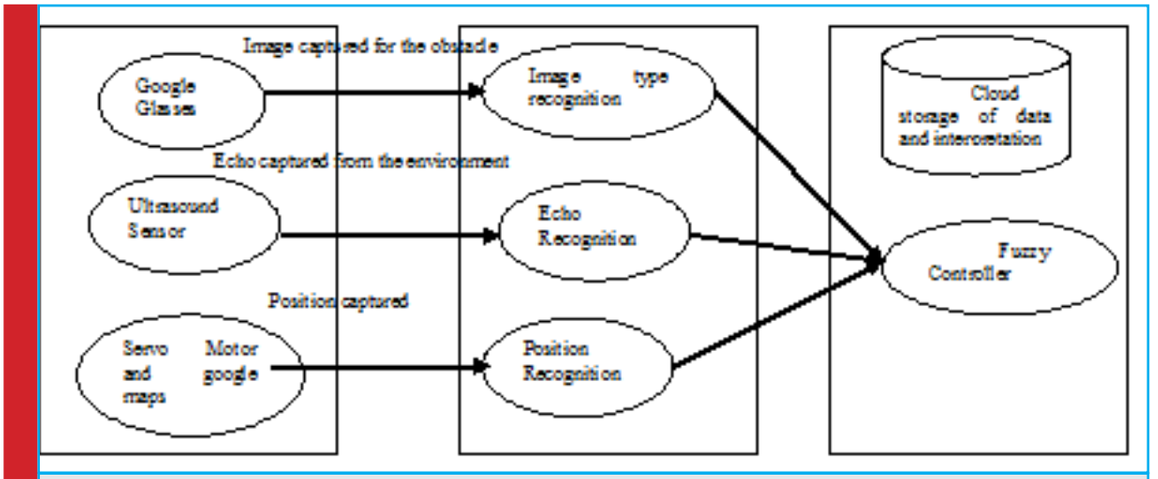

FIGURE 2. Process Flow

detect dynamic and static objects of different sizes(ranging from 8 cubic cms to 2 cubic meters). In addition, the object to be detected by the system from 20 cm to 6 m.

The algorithm would first detect one of the following user's modes:

- Orientation mode (routes instructions, tracing the user's location by tracing the bath, improving learnability of user's brain cognition of the environment and mapping the instructions to the experiences objects)
- Position Locator Mode (this mode enables tracking algorithm to map the user's path to original position in order to precisely determine the current position of user using GPS technology.
- Travel Mode: in this mode the system detects the user's steps and switched to the travel mode. The ultrasound and servomotor starts to gather information about the surrounding environment and interprets the information according to the position, size of the obstacles around the user body from the ground to the head; detecting the surrounding the obstacles; calculating the distance between the user and the obstacle; Prepare a rout or path plan and translates the path into a set of instructions.

RESULTS AND DISCUSSION

The experiment was designed to get 10 participants involved. Ages of the participants ranged from 5 years to 20 years. The participants were asked to attend an orientation session to learn about the game. There were more than one scenario to run the game with the teacher opponent (teacher avatar inside the game) navigates and creates obstacles and other avatars in the game. Hence, the visually impaired player would start with unknown environment to him and then will start with receiving few instructions then he will start build and recognize his instructions under the mentor of the teacher or relatives opponents. Real time response and recognition of the system was under evaluation within parts of the

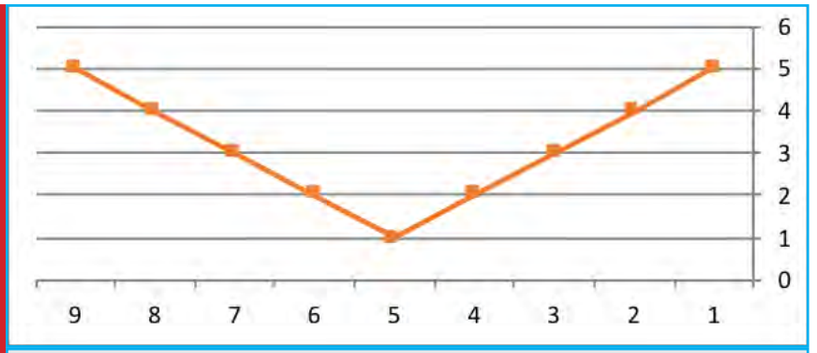

FIGURE 3. Sound Reflection in db over .9 seconds

second. Figure 3 below presents the system allowed the visually impaired avatar to recognize the object within 0.9 second.

CONCLUSION

The research in this paper focuses on the use of Bats hearing system together with the google glasses in order to make an assistive technology to aid the visually impaired people moving in uncertain environment. The proposed game allows the blind/visually impaired player to play with sighted opponent in order to recognize objects created by the sighted opponent. The system uses ultrasound sensors,

In future work, a new level could be added with more enemies for more excitement. Furthermore, we aim to provide an online voice chat between the players so they can communicate with each others during the game if they want. In addition, we will create a friend list to allow the player to add his friends and family.

ACKNOWLEDGEMENTS:

This research project was supported by a grant from the "Research Center of the Female Scientific and Medical Colleges Deanship of Scientific Research, King Saud University for funding this work through research group No (SMRC -1903)

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Medical
Communication

Biosci. Biotech. Res. Comm. 12(2): 362-376 (2019)

BBRC
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Tuberculosis: A comprehensive study on its evolution, variants, causes and treatment related challenges

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ABSTRACT

Tuberculosis, one of the most appalling disorders of the ancient times and still the major fatality affecting bacterial infection, is the condition principally concerned with the pulmonary complexities as well as sometimes damaging extra-pulmonary regions in the body also, demands proper medication and monitoring to be rectified effectively. In countries like India, the incremental prevalence of the resistant TB makes it obligatory to act rigorously in order to control the TB cases and adhere to the TB management programmes. Resistant tuberculosis represents the irrepressible state where the available TB drugs become insufficient for the treatment, worsening the lives of the sufferers. The situation is emerging in such a way that there would the time when the encountered totally-drug resistant cases become effectively high, emphasizing the necessity to develop drugs with improvised antagonistic capabilities to eradicate the existence of Mycobacterium. This review represents a thorough study of the dreadful existence of the pathogen providing all the required information for a better understanding of the disease and its resistance.

KEY WORDS: TUBERCULOSIS, MYCOBACTERIUM TUBERCULOSIS, RESISTANT TUBERCULOSIS, ACTIVE TUBERCULOSIS, LATENT TUBERCULOSIS

INTRODUCTION

The paleopathological evidence suggests the ubiquity of a scourging lung disease about thousand years back, invoked the terror of death amongst the people since then. The dynamicity of the disease, Tuberculosis, prevails in the names which were used in the history to indicate the presence of disease, namely, phthisis, consumption and the great white plague among many others (Frith, 2014). Tuberculosis is a contagious disease in which the lungs get severely damaged by the invasion of Mycobacterium tuberculosis, the causative agent of pul-

ARTICLE INFORMATION:

Corresponding Author: priyaranjan.biet07@gmail.com
Received 22th March, 2019
Accepted after revision 15th May, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/20

monary disease, results in the lethality to the host (Adigun and Bhimji, 2018). TB is a prominent cause of mortality enlisted in the top ten fatal diseases worldwide, reported in 2018 WHO Global Tuberculosis report. The emergence of the disease has caused 1.3 million deaths and 10 million new TB cases in 2017, required 10.4 US Dollar funding in the year 2018 for TB interventions. The severity of the incidence of the disease is prone to the South-East Asia Region (44%), followed by Africa (25%), whereas the Region of the Americas and Europe contributed least with 2.8% and 2.7% incidence respectively (WHO Key facts, 2018;WHO Global Tuberculosis Report, 2018).

The subjected risk factors include bacillary load, proximity to an infectious case, immunosuppressive conditions, malnutrition, alcohol and many others, create a favourable environment to increase the chances of contagion (Narasimhan, *et al*., 2013). The symptoms encountered at the onset of active TB disease are prolonged cough, fever and weight loss, eventually worsens with time (Heemskerk, *et al*., 2015; Shanmuganathan and Shanmuganathan, 2015). Mycobacterium, a member of the actinomycete group, classified into fast and slow-growers, collectively estimates for 150 identified species, possess the pathological ability to cause critical pulmonary, disseminated and cutaneous diseases to the host (Todar, 2008-2012; Talbot and Raffa, 2015; King, *et al*., 2017). The mycobacterial invasion of the host organism not necessarily culminates in the active infection due to the robust host immune system, thus results in latent tuberculosis. The latent tuberculosis infection (LTBI) persists in one-third of the global population, but consequently, 5-10% develops active tuberculosis within the foremost five years of initial infection, a process termed as TB reactivation (Flynn and Chan, 2001; WHO Latent tuberculosis infection (LTBI) – FAQS, 2019).

The first milestone research happened to combat TB was BacilleCalmette-Gue´rin (BCG) vaccine which took 13 years (1908-1921), discovered by Calmette and Gue´rin, followed by the development of antimycobacterial drugs starting from the discovery of streptomycin in 1943 (Luca and Mihaescu, 2013; Podany and Swindells, 2016). Besides an efficient treatment regimen implemented all over the world, the unexpected multidrug-resistant tuberculosis (MDR-TB) outrage in 1980 due to lacking docility towards the continued regimens along with the repeated use of already existed drugs. The concern has become tremendous due to the rise of extensively drug-resistant tuberculosis (XDR-TB), and currently totally drug-resistant tuberculosis (TDR-TB) (Smith, *et al*., 2013).

In response to the prevailing failed treatment and persisting resistant TB, in the early years of the 1990s, WHO has suggested a short course, Directly Observed Treatment (DOTS), to ensure the improvement in the adherence towards the treatment (Rabahi, *et al*., 2017). Globally every year the rate of TB is deteriorating with 2%, saving approximately 54 million lives, between 2000-2017, with the help of appropriate diagnosis and treatment provided (WHO Key facts, 2018). The extensive duration and complex treatment, its side effects, HIV coinfection, are amongst the many profound challenges in the TB treatment which are discussed later in the article, subjecting an individual prone to develop TB (Shehzad, *et al*., 2013; Boogaard, *et al*., 2008).

HISTORY OF TUBERCULOSIS

Tuberculosis discovery cannot be attributed to a single individual as it took the involvement of a huge number of people to recognise the etiology and pathogeny of such a dreadful illness. Pioneers for this were Aristotle (384-322 BCE), Hippocrates (410- 400 BCE), Cassius Felix (447 CE) and Aretaeus of Cappadocia who autonomously identified the disease, specifying it with different names such as Scrofula, Phthisis and Pott's disease (Frith, 2014). Phthisis, as described by Hippocrates, was the weakness of the lungs, a lethal and contagious disorder affecting youthful inhabitants, causing cough, fever and characteristic lung injuries. Scrofula signified the malady of throat's lymph nodes, also termed as King's evil and assumed to be healed by royal touching in England and France (Barberis, *et al*., 2017) while Pott's disease was the one where spines were damaged. Consumption, Robber of the youth, The great white plaque and Graveyard cough are some of the other names used, expressing the discouragement and miseries people were undergoing, Frith, 2014; Frith, 2014). The primary archaeological shreds of evidence for the prevalence of consumption have been observed by the investigations conducted on the Egyptian mummies, implying disease incidence during 3000–2400 BC (Al-Humadi, *et al*., 2017), due to the presence of spinal and rib tubercular wounds, as well as the distorted bones found at several Neolithic localities in the Middle East, Italy and Denmark (4000 years ago) (Daniel, 2006; Smith, 2003). The initially available written documents on TB were reported in India and China, dating back to 3300 and 2300 years ago, respectively (Daniel, 2006 Barberis, *et al*., 2017).

The 19th centenary witnessed magnificent contribution in the field of tuberculosis studies commencing with the invention of the stethoscope by Laennec in 1816, facilitating a proper analysis of subjects by hearing their chests recognising any sort of abnormalities present. He persuaded that the factor accountable for the numerous forms of the disease is the same Tubercle, possessing the capabilities to produce pulmonary as well as the extrapulmonary infections and rendered detailed information

on the miliary and caseous(cheese-like) forms directing the pus or cavity formation within the lungs or in the different organs. He himself died grieving from the same disease. In 1834, a German physician Johann Lukas Schönlein described the disease with tubercle as Tuberculosis but did not correlate it with phthisis or scrofula. Jean Antoine Villemin, a French military surgeon in 1865 identified that the soldiers from the countryside or who worked more on the fields were healthier and had fewer possibilities of phthisis contrasting to those at closed spaces for long times. He illustrated that this ailment is contagious by confirming its transfer to the rabbits from human or cattle (Sakula, 1983 Frith, 2014).

Pasteur's germ theory of infectious disease (1862) appeared as the basis to conclude the presence of a microbiological entity liable for disease outbreaks, consequently search for cause of TB began, preceding to which Robert Koch in 1882 identified the Tubercle bacillus, the causative agent for the consumption and proposed the etiological perspectives of the tuberculosis infection (Frith, 2014; Murray, 2004). This led to the classification of the disease as Koch's disease or Koch's bacillus (Al-Humadi, *et al*., 2017; Sakula, 1983). Afterwards, the importance of immunisation for the prevention of TB was highlighted with the experiments conducted by Clemens Freiherr von Pirquet who scouted an outline of Latent Tuberculosis and commenced the development of the BCG vaccine, (Daniel, 2006). Search for curative medications for the treatment started with the development of Streptomycin and Para-aminosalicylic acids proving advantageous for subduing the adverse consequences of the disorder (Zhang, 2005 Frith, 2014).

MYCOBACTERIUM

Mycobacterium tuberculosis (MTB), a dreaded agent of TB infection, belongs to the family Mycobacteriaceae, in phylum Actinobacteria.

Table 1. Chronological events of tuberculosis discovery (Frith, 2014; Frith, 2014; Al-Humadi, *et al*., 2017; Sakula, 1983; Murray, 2004; Youmans, *et al*., 1947; Chakraborty and Rhee, 2015)

Year	Event
410 BCE	Hippocrates discovered Phthisis
2400 BC	Evidence of TB in Egyptian Mummies
174 CE	Claudius Galen of Pergamum suspected TB as contagious
1363	French surgeon Guy de Chauliac proposed the removal of Scroufulos organ as the cure
1650	Sylvius described Tubercle
1689	Richard Morton explained the pathogeny of consumptive and additional forms of the infection
1702	Jean-Jacques Manget gave the term "miliary tuberculosis"
1720	Benjamin Marten, in his publication "A new theory of Consumption" speculated contagious nature of TB
1779	Sir Percivall Pott identified as Pott's disease
1793	Matthew Bailledescribed the caseous ("cheese-like") characteristics of phthitic ulcers
1803	Gaspard-Laurent Bayle of Vernet defined the pulmonary and other kinds of tubercle infection
1816	Stethoscope invented by Laennec
1834	Tuberculosis term coined by Johann Lukas Schönlein
1843	Philipp Friedrich Hermann Klenckeinoculated rabbits with TB
1844	Friedrich Gustav Jakob Henle postulated phthisis as infectious
1854	Sanatorium cure started
1862	Pasteur's germ theory of infectious disease
1865	Jean Antoine Villemin inoculated guinea pigs and rabbits with tubercle from cows and humans
1882	Koch discovered Tubercle Bacillus
1907	Clemens Freiherr von Pirquet introduced Latent TB
1908	Albert Calmette and Camille Gue developed BCG vaccine
1921	BCG vaccine first used on humans
1943	Streptomycin discovered
1946	PAS(para-aminosalicylic acid) discovered by Gerhard Domagk
1950	Golden era of Anti-TB Drug

FIGURE 1. Taxonomical classification of Mycobacterium tuberculosis (Todar, 2008-2012)

MTB is a rod-shaped, non-motile obligate aerobic bacterium, which is neither Gram-positive nor Gram-negative and thus classified as acid-fast bacteria (Todar, 2008-2012). There are nearly 150 known species under Mycobacterium genus which are further classified as fast-growing (visible growth within 7 days) and slow-growing species (more than 7 days for visible growth) (King, 2017; Stahl and Urbance, 1990). The slow growers are comparatively more host pathogenic which comprises human and animal infectious pathogens namely, Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium paratuberculosis and members of the Mycobacterium avium complex. Nevertheless, both groups involve pathogens that can cause pulmonary, disseminated and cutaneous diseases (Wards and Collins, 2000; King, 2017).

The Mycobacterium tuberculosis complex (MTBC) covers all the closely related humans and animal infecting species with an interrelated genome. They vary on the basis of epidemiology, pathogenicity, host specificity and ability to resist drug. MTBC involves pathogens M. tuberculosis, M. bovis, M. bovis BCG, M. africanum, M. microti, and M. canetti amongst which M. tuberculosis and M. africanum targets human as host whereas M. bovis infects wild and domestic animals and may infect humans too (Richter, et al., 2003; Bayraktar, et al., 2011). Mycobacterium leprae, another species of Mycobacterium genus, has the preference for the skin and nerves and causes Leprosy (Hansen's disease) (Bhat and Prakash, 2012). Mycobacterium avium complex (MAC) is different from MTBC, which comprises multiple nontuberculosis mycobacterial species (NTM) and is ubiquitous in nature. MAC infects HIV-immunocompromised human, children with cystic fibrosis, people with existing pulmonary disease and as well as lung disease and bronchiectasis in patients without underlying lung disease. Apart from pulmonary infections, MAC also causes skin and soft tissue infections, musculoskeletal infections, and lym-

Table 2. LTBI vs ATBI vs NTMI (Akram and Attia, 2018; Schluger, 2008; Kendall, *et al.*, 2011; Ryu, *et al.*, 2016; Centers for Disease Control and Prevention, 2013)

Features	Latent Tuberculosis Infection (Ltbi)	Active Tuberculosis Infection	Non-Tuberculous Infection (Ntmi)
Causative organism	Mycobacterium tuberculosis complex including: Mycobacterium tuberculosis	Mycobacterium tuberculosis complex including: Mycobacterium tuberculosis	Mycobacterium avium complex including: M. avium, Mycobacterium intracellular, Mycobacterium paraintracellulare
Type of infection	Inactive or dormant MTB sequester inside granuloma	Activated MTB causes chronic pulmonary and extra pulmonary infection	NTM causes pulmonary infections, Hypersensitivity pneumonitis (HP), musculoskeletal infections, and lymphadenitis
Transmission	Not contagious	Contagious to other individual	Not contagious
Test for indication	TB skin test or TB blood test reaction	TB skin test or TB blood test reaction	Positive sputum culture, Bronchoscopy, transbronchial or lung biopsy
Sputum or Smear test	Negative	positive	Positive
Radiography reports	Normal	May be abnormal	Radiological patterns visible
Symptoms	asymptomatic	cough, fever, and/or weight loss	common clinical symptoms with pulmonary TB like chronic cough, dyspnea, low-grade fever, malaise and weight loss
Risk factors	Direct contact with infected person	Immunosuppressive drug consumption, malnutrition, HIV co-infection	HIV immunocompromised individual, Children with cystic fibrosis

phadenitis as well (Field, et al., 2004; Shin, et al., 2010; Akram and Attia, 2018).

PATHOGENESIS

The primary exposure of MTB follows dormancy or latency in 90% of the human host, determined as latent tuberculosis infection (LTBI) while the remaining 10% human develops the clinical disease (Ilievska-Poposka, *et al*., 2018). The Latent TB is an asymptomatic clinical condition in which the person lacks the active disease by remaining in non-replicating or dormant bacilli state in response to the host immune system (Schraufnagel, 2016). The dormant tubercle bacilli persist in the human host for years, develops LTBI and consequently emerge as an active TB infection and thus becomes contagious to non-infected individuals (Centers for Disease Control and Prevention, 2013). The pathogeny of TB infection initiates when an infected person releases aerosol in the environment in the form of tiny droplets, carries loads of MTB in it to spread, enters the pulmonary region of the non-infected person when inhaled. The immune response is generated by the existing resident alveolar macrophages, by developing bacterial-macrophage interaction to phagocytose MTB. Pathogenic MTB can also enter and thrives in Alveolar epithelial type II pneumocytes, which is present in plenty of number than macrophage (Smith, 2003; Kaplan, *et al*., 2003). When the MTB ingested by immune cells including macrophages and dendritic cells, they get killed often but other times succeed in escaping the bactericidal effects of the alveolar macrophages. It is due to the characteristic property of the MTB to prevent phagolysosomal fusion and thus hinder phagosome acidification which leads to the formation of inactivated macrophages, where the MTB multiplies and continuously damages it. The infected macrophage triggers the activation of other immune response including oxygen and nitrogen reactive species, blood monocytes and other inflammatory cells, which slows down the replication rate but cannot eradicate the bacteria efficiently. The granular structure develops finally, in which the activated T-cells and macrophages accumulate to restrict the infection within the structure, which is the consequence of host's efficient immune response, to limit the growth and spread of MTB to surrounding cells, denoted as latency (Warner, 2015; Sasindran and Torrelles, 2011). It requires both innate and adaptive immunity of a host organism to form a granuloma, keep the MTB growth intact inside the granuloma and its rupture (Ehlers and Schaible, 2012).

Intracellularly, the granular structure becomes necrotic and hypoxic due to which MTB undergoes dormancy and stays asymptomatic. Although the granuloma can effectively restrict the infection with the host's strong

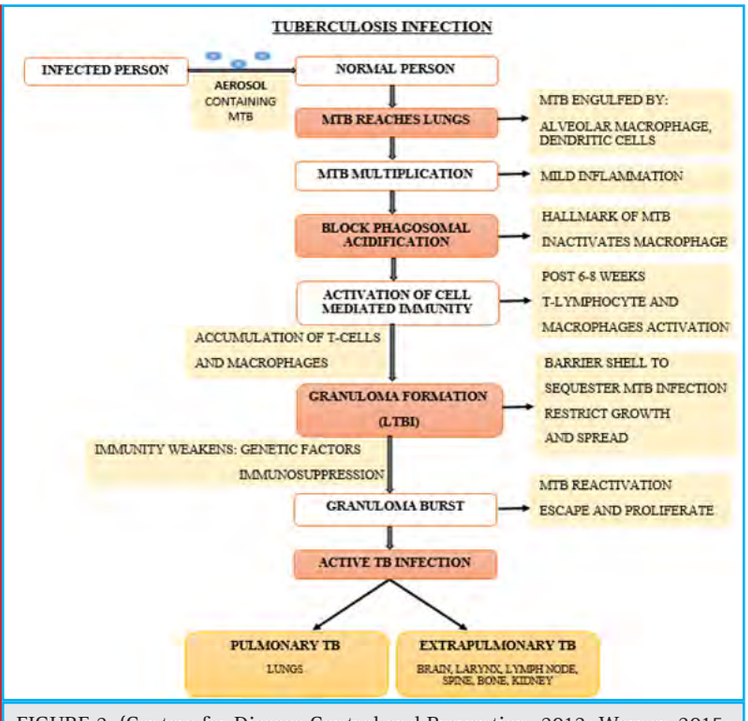

FIGURE 2. (Centers for Disease Control and Prevention, 2013; Warner, 2015; Sasindran and Torrelles, 2011)

immunity but if the host's immunity fails due to various factors including consumption of immunosuppressant drugs, HIV coinfection, malnutrition, may eventually lead to the reactivation and uncontrolled proliferation of the bacteria, followed by bursting of granuloma. At this stage, the escaped bacteria from granuloma start infecting the surrounding pulmonary cavity and consequently emerges as an active TB infection from LTBI. The active TB infection may also infect other tissues and emerges as extra pulmonary TB or military TB (Smith, 2003; Sasindran and Torrelles, 2011 and Warner 2015).

TB COMORBIDITIES AND ASSOCIATED RISK FACTORS

Tuberculosis, being a chronic contagious disease, gets clinically active due to the breakdown in immunity surveillance of host organism, reflecting a positive association with both communicable diseases (CDs) or non-communicable diseases (NCDs) (Bates, *et al*., 2015). Studies revealed NCD's can be easily developed by the TB patients (including Diabetes mellitus, cancer, chronic lung disease, alcohol-abuse disorder) (Peltzer, 2018), demonstrating a strong association with high TB mortality rates (Zerbini, *et al*., 2017). These medical conditions which attribute towards the risk factors for TB and the resulting poor outcomes in the treatment of TB disease are the TB associated comorbidities (Peltzer, 2018; TB comorbidities and risk factors, WHO, 2019). The emerging cases of comorbidities in the general population contribute to the TB burden and result in the enormous challenge faced by health care services (Peltzer, 2018).

Consequently, preventing the failure of recognizing the comorbidities can help to encourage joint management approaches and to control the TB epidemic (Bates, *et al*., 2015). Risk factor induces the occurrence of clinically active TB disease due to the fact, not every individual with existing bacterial infection develops TB disease (Shanmuganathan and Shanmuganathan, 2015). The risk factors associated with TB constitutes distal factors and proximate factors. The distal factors include socioeconomic status while proximate determinants are the host and environmental factors (Taha, *et al*., 2011). HIV coinfection, most critical immunity compromising factor, amongst other considerable risk factors including alcohol abuse, poverty, overcrowding, malnutrition and many others needed to be addressed to control the TB disease progression (Narasimhan, *et al*., 2013).

RESISTANT TB

Drug resistance Tuberculosis, a condition when the causative microbiological agent for TB develops endurance due to the spontaneous modifications happening within the genetic constitution of the bacteria itself, resulting in the failure of the patient's immune system to respond to the drugs prescribed for treatment. Resistant TB is a man-made phenomenon; non-adherence to the drug therapy regimen, single drug or improper combination of prescribed drugs, delayed diagnosis, inadequate quality of drugs, first-hand contact with a drug-resistant TB sufferer, incompetent drug formulations, are some of the reasons for its development making it worse to treat (Davies, 2001). It can develop in two distinct manners, Primary and Secondary Resistance. Primary resistance, when the patient is originally affected with the resistant bacterial strain, capable of forwarding this drug resistance to others as well, having no known prior treatment history while Secondary resistance is acquired during the course of treatment due to negligence in proper treatment (Centers for Disease Control and Prevention, 2013). Mutations in the target genes accountable for drug activation are the significant basis for a reduction in drug efficacy, leading to interference with numerous crucial biochemical processes (Seifert *et al.*, 2015).

Mechanism of drug resistance involves Acquired antibiotic resistance and Intrinsic antibiotic resistance; Acquired antibiotic resistance, when the once susceptive strains of Mycobacterium underwent chromosomal alterations, enhancing the likelihoods of survival against the potent drugs by causing cellular mutagenesis, chiefly attributed to the higher release of reactive oxygen species (Smith *et al*., 2013) Intrinsic antibiotic resistance involves numerous intracellular mechanisms which help the mycobacterium to strive against the antibiotics, neutralising their effect and enhancing its survival potentialities. This boosts the competence of the Mycobacteria to tackle the drug activity as well as affecting the generation of the new drugs against this bacteriological agent. Intrinsic resistance, primarily concerned with diminishing receptiveness of the targets, can be categorised into passive resistance and specialized resistance mechanisms. Passive mechanisms include cell wall impermeability, the envelope comprising of an outer cell membrane, mycolic acid, arabinogalactan, peptidoglycan, arabinomannan and plasma membrane layers around the mycobacterial cell rendering it further protection from the antibiotic action. The non-availability of porin channels lessens the permeability of cell wall for hydrophilic antibiotics, MTB's capability of existing in Latent or dormant states also favours the survival possibilities against drugs as the metabolic activity of the bacteria is remarkably low during this phase, resulting in lower production of ordinarily targeted molecules (Nasiri *et al*., 2017).

Specialized mechanism involves Modification of drug targets, either via conformational variations in the target to depreciate the binding affinity of antibiotics or by directly inactivating the drug with some chemical modi-

fication, Enzymatic degeneration of drugs, usually done by degrading the antibiotics with hydrolases, Molecular mimicry of drug targets, Enzymatic modification of antibiotics, where the mycobacterial modifying enzymes restrict the drug from binding to its target site by opportunistically altering the antibiotic (Smith *et al*., 2013) and the presence of efflux pumps supports the expulsion of the drug molecule outside the cell, contributing survival advantage and a low-level of drug tolerance (Szumowski *et al*., 2013).

Resistant Tuberculosis can be further differentiated into MDR (Multidrug-resistant tuberculosis), XDR (Extensively drug-resistant tuberculosis) and TDR (Totally drug-resistant tuberculosis) depending upon the perceptivity of the drugs towards treatment. The emergence of MDR and XDR has been observed to expand globally amongst the new TB subjects as well as the already treated ones. MDR-TB, resistance to the principal first-line drugs for tuberculosis, isoniazid (INH) and rifampicin (RIF), diminishing the potency of these anti-tuberculous drugs (Dash,2013; Eker *et al*., 2008). According to WHO 2018 statistics, among the entire encountered cases of TB, 3.5% new cases and 18% earlier treated TB patients had MDR-TB, the major shareholding countries include India, China and Russia (WHO Global Tuberculosis Report, 2018).

Rifampicin resistance, termed as RR-TB is observed to be more prevalent, it functions by repressing the transcription of the MTB to obstruct its functioning but mutation in the rpoB (β-subunit of RNA polymerase) gene limits rifampicin interaction and makes the cells resistant (Seung *et al*., 2015; Hameed *et al*., 2018) while isoniazid functions by inhibiting mycolic acid biosynthesis, which is hindered by the alterations in the katG (catalase-peroxidase), inhA (enoyl-acyl carrier protein reductase) or ahpC (alkyl hydroperoxide reductase) genes (Chhabra *et al*., 2012; Gillespie, 2002; Telenti, 1998). INH is a pro-drug activated by the katG gene whereas the inhA gene facilitates fatty acid elongation in mycolic acid biosynthesis, any modification in these can lead to resistance. The probability of resistance development is more in case of INH as compared to other anti-TB drugs (Shehzad, *et al*., 2013). This resistance towards existing drugs elevates the complexities in the treatment, prolonging the remedial period and the risks of contamination. XDR-TB is the resistance to either isoniazid or rifampicin accompanied with fluoroquinolones (ofloxacin, levofloxacin or moxifloxacin) and second line injectables such as kanamycin, capreomycin or amikacin. Social factors such as smoking, alcohol abuse, unemployment are few additional risk determinants for XDR-TB, Studies reveal that possibilities of MDR transforming to XDR-TB are comparatively higher in females than males (Kurz *et al*., 2016).

Its treatment relies on more noxious, expensive drugs with reduced potency (Calligaro *et al*., 2014). TDR-TB, the extreme situation when no response for any of the first line or second-line drugs is there, all the accessible drugs fail to cure the disease. Bedaquiline and delamanid, the recently developed drugs are also incapable to produce some sort of effect on TDR patients, hence declared untreatable by the Centre of disease control and prevention (Matteelli *et al*., 2014; Prasad *et al*., 2007). Numerous morphological variations are also identified in the TDR strains such as MTB with thicker walls and a highly branched structure (Velayati *et al*., 2013). Henceforth, minimising the probabilities for its occurrence is the only permissible alternative to tackle this condition, by getting the MDR and XDR-TB properly treated with the correct dose of drugs for appropriate time duration.

DIAGNOSIS

Diagnosing Tuberculosis comprises numerous screening and affirmative analyses to detect the presence of the contagious microbial factor within the subject's body. The choice of the diagnostic procedure is dependent on the site of infection either Pulmonary, Latent or Extra-pulmonary. Fundamental screening methods for analysis involves Chest X-Ray (CXR), the most followed microbiological identification test, intimating the presence of unusual deformities within the lungs or other body organs due to the growing tuberculin. It has been used for primary evaluation of TB for a long time, usually accompanied by the culturing of the sputum smear for confirmation. CXR, the diagnostic radiological test for severity prediction, provides the stage of infection depending upon the cavitation in the lungs (Ryu, 2015). In case of Primary TB, X-ray displays abnormalities in the mid and lower lung portions, for Reactivated TB, nodules are found in the upper lung region and for Extra-pulmonary TB, X-ray shows nodules in the affected body organs. If immunosuppression is there, CXR may appear normal despite the presence of active bacteria (Cudahy and Shenoi, 2016; Murthy *et al*., 2018).

Mantoux skin test, also named Tuberculin skin testing, standard for Latent TB infection identification (Brodie and Schluger, 2005), is primarily a screening approach in the speculated individuals indicating the infection where a purified protein derivative (PPD) is introduced intradermally and skin induration at the site of reaction within subsequent hours indicates exposure to the bacteria. PPD is an antigenic amalgamation of mycobacteria leading to a hypersensitive skin response (Pai, 2005). It is cheap and comparatively easier to perform, although it might yield misleading results in the person previously immunised with the BCG vaccine,

Table 3. Comparison between Drug susceptible and Drug resistant TB (WHO Global Tuberculosis Report, 2018; Seung *et al.*, 2015; Louw *et al.*, 2009; Chang and Yew, 2013; Collantes *et al.*, 2016; Yang *et al.*, 2017)

Parameter	Drug Susceptible Tb	Mdr-Tb	Xdr-Tb
Definition	Mycobacterial infection sensitive to available drugs	TB resistant to the two main first-line drugs	Resistance to few first-line drugs along with some second-line injectables
Resistant drugs	Sensitive to the available drugs; no resistance	Isoniazid, Rifampicin	Isoniazid, Rifampicin, Fluoroquinolones, kanamycin, capreomycin, amikacin
Mutated Genes	-	katG, InhA, ahpC, kasA, ndh, rpoB	katG, InhA, ahpC, kasA, ndh,rpoB, gyrA, gyrB, rrs, tlyA
Symptoms	Chronic cough, Blood in sputum, weight loss	Same as drug susceptible TB	Same as drug susceptible TB
Cases notified (WHO 2018)	6.7 million	1,60,684	10,800
Burdened countries	Angola,Brazil, Cambodia, China, Central African Republic, Ethiopia, India, Indonesia, Kenya, Liberia, Mexico, Mozambique, Myanmar, Namibia, Nigeria, Papua New Guinea, Philippines, Russian, Federation, South Africa, Thailand, VietNam, Zambia, Zimbabwe	Angola, Bangladesh, China, Democratic People's Republic of Korea, DR Congo, Ethiopia, Indonesia, Kazakhstan, Kenya, Mozambique, Myanmar, Nigeria, Pakistan, Philippines, Russian Federation, Somalia, South Africa, Thailand Ukraine, Uzbekistan	The Russian Federation, India, Ukraine South Africa and Belarus
Preventive measures	Vaccination, strong immunity, early diagnosis, ventilated surroundings	Adherence to the provided treatment regimen	No negligence in MDR-TB treatment
Diagnostic technique	Chest X-ray, Microscopy, Tuberculin skin testing	Line Probe Assay, Drug Sensitivity Testing, Microscopic Observation Drug Susceptibility	Drug Sensitivity Testing, Microscopic Observation Drug Susceptibility
Side effects of Drugs	Nausea, vomiting, weight loss, hepatotoxicity, fatigue, abdominal pain, ataxia, anorexia, immunological reactions	High toxicity, hepatitis, joint pain, raised uric acid levels, renal insuffiency, allergic reactions, blurred vision, hypothyroidism, arthritis	Compartively more toxic than MDR drugs, longer treatment duration

also in cases of inappropriate dosing of the PPD (The National Institute for Health and Care Excellence, 2016).

Microscopy, the culturing of the mycobacteria collected from the sputum of the sufferer, evaluating the comprehensive TB burden, including viable as well the non-viable lifeless cells (Cudahy and Shenoi, 2016). The cells grown in the laboratory conditions are stained using the Ziel- Neelson stain, staining the acid-fast species into bright red coloured cells. Since the mycobacteria is an acid-fast organism, it can be distinguished easily using this method (Parsons *et al.*, 2011). It produces results with high specificity but minimum sensitivity. LED Microscopy is the latest advancement substituting the already existing microscopic technique (Cudahy and Shenoi, 2016; Nema, 2012).

Confirmatory tests for Tuberculosis covers several molecular approaches such as, Interferon Release Assays for quantification of the fraction of inflammatory cytokines particularly interferon-gamma (IFN-gamma) which are produced when mycobacterial cells are incubated into the patient's blood only if the bacteria is already present in that system (Pai, 2005; The National Institute for Health and Care Excellence, 2016). Currently, available tests involve T-SPOT.TB and the Quantiferon-TB assay, where isolated blood mononuclear cells and the complete blood are utilised respectively, helps in the detection of Latent as well as Active TB infection (Cudahy and Shenoi, 2016; Brodie and Schluger, 2005). Adenosine deaminase assays (ADAs) detect adenosine deaminase activity in serum and plasma samples,

highly used in the detection of Extra-pulmonary TB (The National Institute for Health and Care Excellence, 2016).

Nuclear Amplification and Gene-Based Tests are the most advanced testing methods used for the confirmation of tuberculosis, DNA based molecular methods are employed to investigate the presence of Mycobacterium by identifying its genetic material which is amplified with the application of PCR (Ryu, 2015; Parsons *et al*., 2011). Polymerase Chain Reaction finds its use in yet another molecular detection method termed as Line Probe Assay, facilitating accelerated identification of Mycobacterium Tuberculosis along with characterisation of rifampicin and isoniazid drug resistance in patients by the analysis of mutated gene sequences which make the bacteria resistant, with the aid of hybridization techniques (Lawn, 2015; Desikan *et al*., 2017).

Drug Sensitivity Testing involves use of solid media cultures along with antibiotics to confirm the MTB growth and sensitivity towards the drugs (Cudahy and Shenoi, 2016). Colorimetric Redox Indicator (CRI), Nitrate Reductase Assay (NRA) and Microscopic Observation Drug Susceptibility (MODS) are some of the non- commercial drug susceptibility testing approaches providing phenotypic characterization of the mycobacterium by testing mainly the rifampicin and isoniazid resistance in the cells. MODS analyses the early growth of bacteria in the culture with use of inverted microscope, aiding for faster detection of MDR-TB especially in high burden countries (Migliori *et al.,* 2008). CRI and NRA are based on the colorimetric identification of the infectious agent present in the sputum directly or in the developed culture while NRA specifically focuses on the utilisation of the nitrate reducing capability of mycobacterium, further detected colorimetrically (Ahmad, 2009). These both methods are used in the low- resource countries for faster and economical outcomes, still not endorsed by WHO due to insufficient evidences available (Heemskerk, *et al*., 2015; Desikan *et al*., 2017).

TREATMENT AND ITS CHALLENGES

The discovery of drugs and vaccine in history have been the evidence in the struggle against tuberculosis prevalence and the complexities still persist. Before the onset of TB drugs, remedies in the past relied on herbal solutions, dietary support and climatic prescripts along with some measures implemented during the 17th-19th century which included ameliorate lifestyle and suggesting sanatorium to the infected person, resulted in reduced incidences (Al-Humadi, *et al*., 2017; Iseman, 2002; Zhang, 2005).

Starting from 1908, the work for developing BCG with the help of a virulent bovine strain implemented and finally reached to the human for trial in 1921 (Luca and Mihaescu, 2013). It was only after World War II, the first-ever vaccine for tuberculosis was especially encouraged by UNICEF, World Health Organization (WHO), and by Scandinavian Red Cross Societies, which then extensively adopted in the US, Great Britain, and Germany (Al-Humadi, *et al*., 2017; Luca and Mihaescu, 2013). The current 2017 data indicates high coverage by the BCG vaccine, provided to 158 countries out of which 120 reported 90% coverage (WHO Global Report, 2018).

The development of anti-TB drugs around the 1930s embarked the age of modern TB chemotherapy, derived from two anti-microbial sources of chemical and antibiotic origin (Zhang, 2005). The magnificent discovery of penicillin in 1928 and sulphonamide-prontosil rubrum in 1935 was the drastic move in the antimicrobial therapy (Iseman, 2002; Tan and Tatsumura, 2015; Jesman, 2011; Lesch, 2008). Later in 1938, sulfa drug was used to treat TB infection in an experimental guinea pig which showed a significant result but didn't perform well in humans. The drug was then further formulated to produce thiosemicarbazones, which was comparatively effective than sulfa drugs but not more than streptomycin (Zhang, 2005).

Modern chemotherapy took a steep turn in the range of anti-TB drugs with the discovery of streptomycin in 1943 by Waksman (Gonzales, 1994). Being an active anti-mycobacterial agent, it possessed the hindrance property towards the tubercle and its infection in both the in vitro and in vivo conditions respectively (Hinshaw, 1947). Soon after the discovery of streptomycin, Lehmann reported bacteriostatic property of para-aminosalicylic acid (PAS) on tubercle bacillus in 1943 with the first clinical trial on 1944 (Lehmann, 1964; Kanabus, 2018). Streptomycin and PAS, when provided individually, were responsible for drug resistance due to which combined dose prescribed for effective TB therapy but had certain side-effects. The year of coincidence 1951, released a potential anti-TB drug, isoniazid, which was coincidentally discovered by two different pharmaceutical specialists around the same time in 1912. Isoniazid demonstrated enormous supremacy among existing anti-TB drugs, with immense potency, mycobacterial specificity, tolerance and reasonability. Despite its superiority, the resistant TB infection still prevailed due to its individual practice, which subsequently induced the necessity of multiple drug treatment. The "triple therapy" recommended as the standard regimen for about 15 years, which included the combination of oral isoniazid altogether PAS for nearly two-years with intramuscular streptomycin for six months, was a great success (Chakraborty and Rhee, 2015; Iseman, 2002; Murray *et al*., 2015).

The decade after the 1950s remarkably experienced augmented number of TB drugs and symbolized the

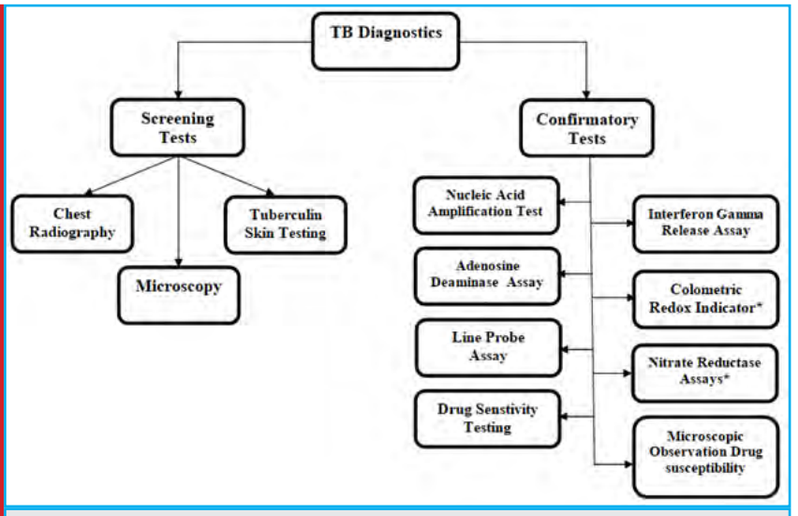

FIGURE 3. Tuberculosis Diagnostic Techniques (Cudahy and Shenoi, 2016; Lawn, 2015)
*represents Techniques not recommended by WHO

Golden era in the history of Tb drug discoveries. The nicotinamide derived analogue, Pyrazinamide (PZA) (Zhang *et al*., 2013), synthesized in 1936 but recognized in 1952, followed by the development of Rifamycins (1957), ethambutol (1961) and clinical trial of rifampin in 1966 (Zhang, 2005; Murray et al., 2015).

CURRENT TB REGIMEN

DOTS, Directly Observed Treatment Short course, an efficient and economic internationally approved supervision method strongly recommended by the WHO to combat global tuberculosis (Tuberculosis, What is DOTS, WHO). The WHO's Global Tuberculosis programme (GTB) promoted DOTS in 1993, which represented an amalgamation of a technical and managerial constituent, employed to prevent TB transmission by effectively restoring infectious cases (What is DOTS? : A guide to understanding the WHO-recommended TB control strategy known as DOTS. Geneva: WHO, 1999). With the launch of Stop TB strategy in 2006, the performance of DOTS accelerated (Treatment of Tuberculosis: Guidelines,WHO, 2010). Currently, the WHO has executed End TB Strategy in 2014, targeted to curb the

Table 4. TB cases and its regimens (Treatment of Tuberculosis: Guidelines, WHO, 2010; Guidelines for treatment of drug-susceptible tuberculosis and patient care, 2017 update, WHO, 2017)

Types Of Tb Cases	Tb Treatment Regimen			
	Intensive Phase		Continuation Phase	
	Months	Drugs	Months	Drugs
New case New patients	2	HRZE	4	HR
New patients with high level of Isoniazid resistance	2	HRZE	4	HRE
Previously Treated case (no documented resistance)	2	HRZE	4	HR
HIV-positive TB patients	6 months standard regimen (2HRZE+4HR) followed by ART within 8 weeks of treatment or 2 weeks for profound immune-compromised patients.			

HRZE: Isoniazid, Rifampicin, Pyrazinamide and Ethambutol.
ART: Antiretroviral treatment

global TB pandemic by 2035 (Implementing the end TB strategy: the essentials, WHO, 2015). The ongoing WHO approved regimens categorized on the basis of types of TB cases which is depicted in the table:

CHALLENGES

MTB CLINICAL MANIFESTATION

It demonstrates atypical properties possessed by the pathogen which involves high lipid content, unique physicochemical cell wall composition, intrinsic and acquired drug resistant properties, virulence, dormancy at a complex pulmonary cavity to avoid drug interaction and also the ability to cause extra pulmonary infection in the brain, kidney and bones (Al-Humadi, *et al.*, 2017; Nasiri *et al.*, 2017; Rabahi *et al.*, 2017).

TREATMENT DRAWBACKS

Despite being a globally adopted regimen, the lag in the efficacy of DOTS prevails due to the non-adherence by the patients. The possible reasons for the non-adherence involve the non-economic, long-term chemotherapy (18-24 months) as seen in the case of MDR-TB. The standard treatment regimen involves multiple drugs to eradicate the pathogen effectively and their lengthy courses cause the pile-up of side-effects and related toxicity in the patient's body. The first line drugs cause Hepatotoxicity, rashes, peripheral neuritis, sideroblastic anaemia whereas second-line drugs cause more toxicity to the patients including ototoxicity, vertigo, ataxia, and nystagmus, hearing loss, gastrointestinal side effects (nausea, vomiting), psychiatric disorder, depression psychosis, arthralgia, arthritis and gout. Due to the following reasons, the patient gets more detached from the therapy leading to either mortality or more drug resistant cases such as XDR-TB and even worse in the condition of Totally Drug Resistant (TDR), also there are increased chances of TB re-infection among the TB patients (Yang *et al.*, 2017; Rabahi *et al.*, 2017; Padgilwar *et al.*, 2016; Tousif *et al.*, 2015).

HIV CO-INFECTION AND ROLE OF DIABETES

HIV is an immunosuppressive virus, which drastically hampers the immunity of the person, causes the co-infection with TB more scourging and fosters profoundly resistant MTB strains which makes the TB management exigent and tough. The HIV coinfection with active TB, resistant TB or latent TB cases develops more fatal conditions due to which the provided treatment needs to be more effective, less time-consuming and achieving minimum probable drug-drug interaction between antitubercular and antiretroviral drugs (D'Ambrosio, 2015; Padgilwar, 2016; Wells, 2007). Diabetes also raises the probability of active TB development due to depleted immunity of an individual, which may account for emerging active TB cases (Shehzad, *et al.*, 2013). There are certain factors which overpower the TB prevalence including poverty, exposure to the mass population, age, sex, immune response in an individual, nutritional level (Al-Humadi, *et al.*, 2017).

CONCLUSION

Tuberculosis is an ever-growing disease, amongst ten lethal diseases, with the tendency to develop drug-resistant TB which intensifies the public health crisis. The 3 burden countries with resistant TB cases accounts for nearly half of world's resistant cases, which involves India, China, and the Russian Federations. Mycobacterium Tuberculosis strain causes TB infection to the human beings, primarily inside the lungs for the majority of the cases whereas Extra pulmonary cases also accounts to the disease. MTB possesses inherent property and can acquire mechanisms through which they can thrive inside the host organism. There are various variables which accounts for the risk factors to the development of clinically active TB, by triggering immunosuppressive condition to the patient. Despite being the prominent cause of mortality, TB is preventable as well as curable, involving 6 months standard TB treatment regimen so as to control and manage TB. WHO introduced various strategies so as to eradicate the disease completely but due to various challenges it is still difficult to overcome the disease. The detailed updated information about the mycobacteria and its mode of action helps to understand the resistance mechanism as well as to develop drugs which could overcome them effectively. It is very important to account the various challenges confronted which hinders the effective TB treatment. The idea of creating this review is to provide the thorough information about the disease so that the maximum information can be available at a particular platform.

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Agricultural Communication

Biosci. Biotech. Res. Comm. 12(2): 377-383 (2019)

BBRC Bioscience Biotechnology Research Communications

Effect of inoculation of salt tolerant *Rhizobium* on nodulation and leghaemoglobin content of soybean

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ABSTRACT

A field experiment was conducted with a view to see the effect of inoculation of salt tolerant *Rhizobium* on nodulation and leghaemoglobin content of soybean at Post Graduate Institute, Mahatma Phule Krishi Vidyapeeth, Rahuri (M.S) during the year 2015-2017. Legumes and the process of nodule initiation are both more sensitive to salinity than are rhizobia Both N_2 fixation activity and nodule respiration were inhibited sharply on exposure of plants to saline condition. The decrease in N2 fixation has been ascribed to direct effect on nitrogenase activity or an indirect effect to decrease in leghaemoglobin content, respiratory rate, malate concentration, nodules and altered their ultrastructure. Treatment T_7 (Liquid consortium + 100 % N) was singnificantly superior in number of effective nodules over rest of treatments however it was at par with treatment T_6 (Liquid consortium + 75 % N). It was also found that treatment T_7 (Liquid consortium + 100 % N) significantly superior in number of non- effective nodules over rest of the treatments however it was at par with treatment T_6 (Liquid consortium + 75 % N). The treatment absolute control recorded the least number of effective and highest number of non-effective nodules during flowering and harvesting stages respectively. The treatment T_7 (Liquid consortium + 100 % N) was significantly superior in Leghaemoglobin content of nodule over rest of all the treatments and it was at par with treatment T_6 (Liquid consortium + 75 % N). The treatment absolute control recorded the least in Leghaemoglobin content of nodule at 45 and 60 days. So there need to isolate strains and inoculation of salt tolerant *Rhizobium* which will enhance the nodule formation in legume crops.

KEY WORDS: SOYBEAN, LEGHAEMOGLOBIN CONTENT, SALT TOLERANT RHIZOBIUM, NODULATION

ARTICLE INFORMATION:

Corresponding Author: Krishna_pagare@rediffmail.com
Received 25th March, 2019
Accepted after revision 17th June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/21

INTRODUCTION

The decrease in N2 fixation activity has been ascribed to a direct effect on nitrogenase (Burns *et al.* 1985) or an indirect effect through decreases in leghaemoglobin content, respiration rate and malate concentration in nodules (Delgado *et al.* 1993, 1994). Legumes and the process of nodule initiation are both more sensitive to osmotic stress than are rhizobia (Russell, 1976; Tu, 1981; Velagaleti *et al.*, 1990). Salt tolerant rhizobia might have the potential to improve yield of legumes under salinity stress (EI-Mokadem *et al.*, 1991). Numerous studies have shown that soil salinity decreased rhizobial colonization and nodulation and dramatically reduced N2 fixation and nitrogenase activity of nodulated legumes (Elsheikh and Wood, 1995; Zahran, 1999). The osmotic environment within the rhizosphere may affect root colonization, infection thread development, nodule development, and the formation of effective N2-fixing nodules (Miller and Wood, 1996). Rhizobia induce the formation of nodules on the roots of legume plants, in which atmospheric nitrogen is fixed and supplied to the host plant, thereby enhancing growth under nitrogen-limiting conditions. The symbiotic interaction between rhizobia and their cognate leguminous plants is important for agricultural productivity. However, physiological stresses, such as salinity, negatively affect these symbiotic interactions by limiting nitrogen fixation (Zahran, 1999). An efficient Rhizobium-legume symbiosis under salt stress required also the selection of salt-tolerant rhizobia (Zahran, 1999). Nodulation and nodule dry weight was promoted markedly by inoculation with *Rhizobium triolii* in Berseem crop and depressed significantly with consistent increase in salinity (Hussain *et al.*, 2002). There is now increasing evidence that the use of beneficial microbes could enhance the resistance of plants to adverse environmental stresses, e.g., drought, salts, nutrient deficiency, and heavy metal contaminations (Glick *et al.*, 2007). An increase of tolerance to salinity of rhizobial bacteria might constitute another approach to improve plant productivity under symbiosis (Kenenil *et al.*, 2010).

Inoculation with RhM11 improved plant and nodule growth compared with those inoculated with RhM14 and CIAT 899 under saline condition in some common bean (Faghire *et al.* 2011). An alternate strategy to improve crop plants for salt tolerance is to introduce salt-tolerant plant growth promoting rhizobacteria (PGPR) that enhance crop growth in saline soil. It is suggested that root-colonizing bacteria that produce phytohormones may stimulate plant growth and help in nutrient recycling in the rhizosphere microcosm and thus the microbes can alleviate the effects of salinity in the environment. The evaluation of saline - tolerant bacterial strains to stimulate saline tolerance and promote growth of crop plants leading to better productivity in saline soil (Vivekanandan *et al.* 2015).

Therefore, inoculation of the salt tolerant *Rhizobium* under conditions of slat stress may help in nodule formation promote biological nitrogen fixation as well as leghaemoglobin content leguminous crops.

MATERIAL AND METHODS

Total 40 root nodules samples along with rhizosphere soil samples with intact root nodules of soybean plants were collected from saline tract of five districts of Western Maharashtra viz., Kolhapur, Sangali, Satara, Solapur and Ahmednagar to isolate salt tolerant *Rhizobium*. Total of 33 salt tolerant *Rhizobium* isolates were obtained from the root nodules of soybean grown in saline soils of Western Maharashtra. Isolation of Rhizobium from root nodule was done by the method of Samosegaran and Hoben (1985). The reference salt tolerant Rhizobium strain was used for comparison. To confirm the salt tolerance of *Rhizobium* isolates, they were tested against different concentrations of NaCl salt. For this, YEMA medium supplemented with 0.075, 0.15, 0.3, 0.6, 1.2, 1.8, 2.1, 2.4, 3.0, 3.6, 4.2, 4.8, 5.4, 6.0, 7.2, 8.4, 9.6 and 10.8 per cent NaCl. Out of 33 salt tolerant isolates, 20 isolates were categorized under extremely salt tolerance, (more than 5.4 % salt tolerance limit) only three efficient salt tolerant Rhizobium (STR-4. STR-14, STR-18) were selected to develop the liquid consortium. Classification of salt tolerant Rhizobium was done on the basis of salt tolerance limit (Cardoso *et al.*, 2014).

Using liquid consortium of salt tolerant *Rhizobium* in comparison with reference strain liquid formulation obtained from liquid biofertilizers production unit, Department of Plant Pathology and Agricultural Microbiology, M.P.K.V., Rahuri to study their performance on nodulation and leghaemoglobin content of soybean as detailed below. The land selection for experimental purpose was ploughed once and two harrowing were given. The farm yard manure (FYM) @ 10 ton ha^{-1} was uniformly spread all over the land before preparatory tillage operation. The soil was brought to fine tilth condition. The experiment was carried out in *kharif* -2016 in Randomized Block Design with three replications and eight treatments as given in (Table No. 1). The gross and net plot size were 1.80 x 3.0 m^2 and 1.20 x 2.60 m^2 respectively. The initial chemical properties of soil in terms of salinity, pH (1:2.5) and EC (dSm^{-1}) was 8.24 and 2.74 respectively.

The seeds of soybean (JS-335) were treated with consortium of salt tolerant Rhizobium @ 25 ml/kg of seeds at the time of sowing seed were dried in shade for 30

Table 1. Treatment details

Treatment No.	Treatment details
T_1	Absolute control
T_2	Reference strain + 50 % N
T_3	Reference strain + 75 % N
T_4	Reference strain + 100 % N
T_5	Liquid consortium + 50 % N
T_6	Liquid consortium +75 % N
T_7	Liquid consortium+100 % N
T_8	Only 100 % N

T- Treatment
N- Nitrogen dose

minutes and were sown in lines with spacing 30 cm x10 cm and 1.5 cm deep) in each plot. Salt tolerant *Rhizobium* consortium was applied to the seeds and through drenching @ 3.0 lit/ha @ 10^8 cfu/ml after 45 days of sowing as per the application of N. 50: 75:00 N:P_2O_5:K_2O (kg ha^{-1}) were used to supply N, P_2O_5 and K_2O also initial chemical and biological properties of soil were studied.

The observations on nodulation were recorded at the flowering and harvesting stage. Before uprooting plants, light irrigation was given to plot so that it became easy for uprooting. The root system was dipped in water for removal of soil adhered to roots and then washed with water. The nodulation count for effective and non-effective nodules was taken for randomly selected five plants and then average figure was taken. The observations on lehaemoglobin content were recorded at 45 days and 60 days after sowing.

Colorimetric estimation of Leghaemoglobin as pyridine haemochromogen

Leghaemoglobin content in the nodules of soybean was determined by using pyridine reagent as described by Hartree (1957). The standard working solution af haemin (1 mg haemin per ml solution) was prepared. The standard solution was pipette out corresponding to (0, 0.1, 0.2, 0.3....1.0 mg) concentrations. The colour was developed and the absorbance was measured at 500 nm as above. The calibration curve of optical density (O.D.) against mg of haemin was plotted.

The statistical analysis of data was carried out by employing Randomized Block Design (RBD). The critical differences were calculated at P = 0.05 level of significance for the *in-vivo* experiment. Wherever F test were significant and interpretation of the results was carried out in accordance by Panse and Sukhatme (1967).

RESULTS AND DISCUSSION

Number of effective and non-effective nodule: It is revealed from the data that, the mean number effective nodules of soybean decreased with increase in the age of crop up to harvesting stage and the mean number non-effective nodules of soybean increased with increase in the age of crop up to harvesting stage. Effect of application of salt tolerant Rhizobium consortium on effective and non-effective nodules was significant at all intervals.

At flowering: From the data presented in (Table 2 and Fig. 2) it was found that treatment T_7 (Liquid consortium + 100 % N) was singnificantly superior in number of effective nodules (25.25) over rest of treatments how-

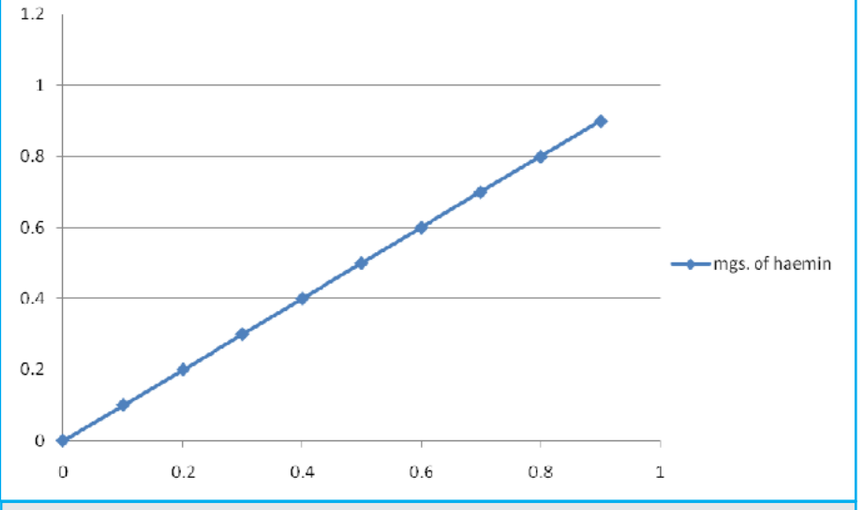

FIGURE 1. Calibration of a standard curve for leghaemoglobin (haemin) determination.

Table 2. Effect of liquid consortium of salt tolerant Rhizobium on number of effective and non effective nodules per plant of soybean at flowering and harvesting stages

Treatment		Number of effective nodules/plant		Number of non-effective nodules/plant	
		At flowering	At harvesting	At flowering	At Harvesting
T_1	Absolute control	15.88	5.79	16.60	21.80
T_2	Reference strain + 50 % N	17.74	8.20	15.12	19.17
T_3	Reference strain + 75 % N	21.64	11.33	11.55	15.47
T_4	Reference strain + 100 % N	23.32	12.24	10.76	14.21
T_5	Liquid consortium + 50 % N	18.49	9.12	13.87	18.33
T_6	Liquid consortium + 75 % N	24.70	14.93	8.75	12.20
T_7	Liquid consortium + 100% N	25.25	15.24	7.52	11.55
T_8	Only 100 % N	19.25	10.53	12.61	16.70
	S.E. +	0.18	0.10	0.41	0.21
	CD at 5 %	0.55	0.32	1.25	0.65

ever it was at par with treatment T_6 (Liquid consortium + 75 % N) (24.70). It was also found that treatment T_7 (Liquid consortium + 100 % N) significantly superior in number of non- effective nodules (7.52) over rest of the treatments however it was at par with treatment T_6 (Liquid consortium + 75 % N) (8.75). The treatment absolute control recorded the least number of effective and highest number of non-effective nodules (15.88) and (16.60) respectively.

At harvesting: The treatment (Table 2 and Fig. 2) T_7 (Liquid consortium +100 % N) was significantly superior in number of effective nodules (15.24) over rest of treatments however it was at par treatment T_6 (Liquid consortium + 75 % N) (14.93). Also it was found that treatment T_7 (Liquid consortium + 100 % N) significantly superior in number of non- effective nodules (7.52) over rest of treatments however it was at par treatment T_6 (Liquid consortium + 75 % N) (12.20). The treatment absolute control recorded the least number of effective and highest number non-effective nodule (5.79) and (21.80) respectively.

Effect of liquid consortium of salt tolerant Rhizobium on Lehaemoglobin content of nodule

The data (Table 3 and Fig. 3) showed that, the mean Leghaemoglobin content of soybean nodule increased with increase in the age of crop. Effect of application of salt tolerant Rhizobium consortium on Leghaemoglobin content was significant at all intervals.

The treatment T_7 (Liquid consortium + 100 % N) which was significantly superior in Leghaemoglobin content of nodule (0.290 mg g^{-1} fresh of nodule weight) over rest of all the treatments and it was at par with treatment T_6 (Liquid consortium + 75 % N) (0.276 mg g^{-1} fresh of nodule weight). The treatment absolute control recorded the least in Leghaemoglobin content of nodule (0.184 mg g^{-1} fresh of nodule weight).

The treatment T_7 (Liquid consortium + 100 % N) was significantly superior in Leghaemoglobin content of nodule (0.321 mg g^{-1} fresh of nodule weight) over rest of all the treatments and it was at par with treatment T_6 (Liquid consortium + 75 % N) (0.305 mg g^{-1} fresh of nodule weight). The treatment absolute control recorded the least in Leghaemoglobin content of nodule (0.202 mg g^{-1} fresh of nodule weight).

Similarly, Hussain *et al.* (2002) reported that nodule formation inferred that mean number of nodules decreases significantly with an increase in salinity level of soil and increased significantly by seed inoculation with *Rhizobium*. Inoculation of seed increased the nodule formation significantly at control, 12 dS m^{-1} and 16 dS m^{-1} salinity levels, but at 8 dS m^{-1} increase in number of nodules per pot is non-significant statistically. Similarly, Adewusi *et al.*, (2008) reported that rhizobial inoculation increased nodule biomass thus encouraged sustainable environmental friendly agriculture by responding perfectly in biological nitrogen fixation. Similarly Faghire *et al.* (2011) showed that in controls, inoculation with RhM11 improved plant and nodule growth compared with those inoculated with RhM14 and CIAT 899. NaCl treatment generally had a negative affect on plant and nodule growth. Under The nodular phosphoenolpyruvate carboxylase (PEPC) and malate dehydrogenase (MDH) exhibited higher activities and were less affected by salinity in plants inoculated with the reference strain CIAT899 than those inoculated with local strains and concluded that plants inoculated with CIAT899 and RhM11 showed more salinity stress tolerance than those inoculated with RhM14.

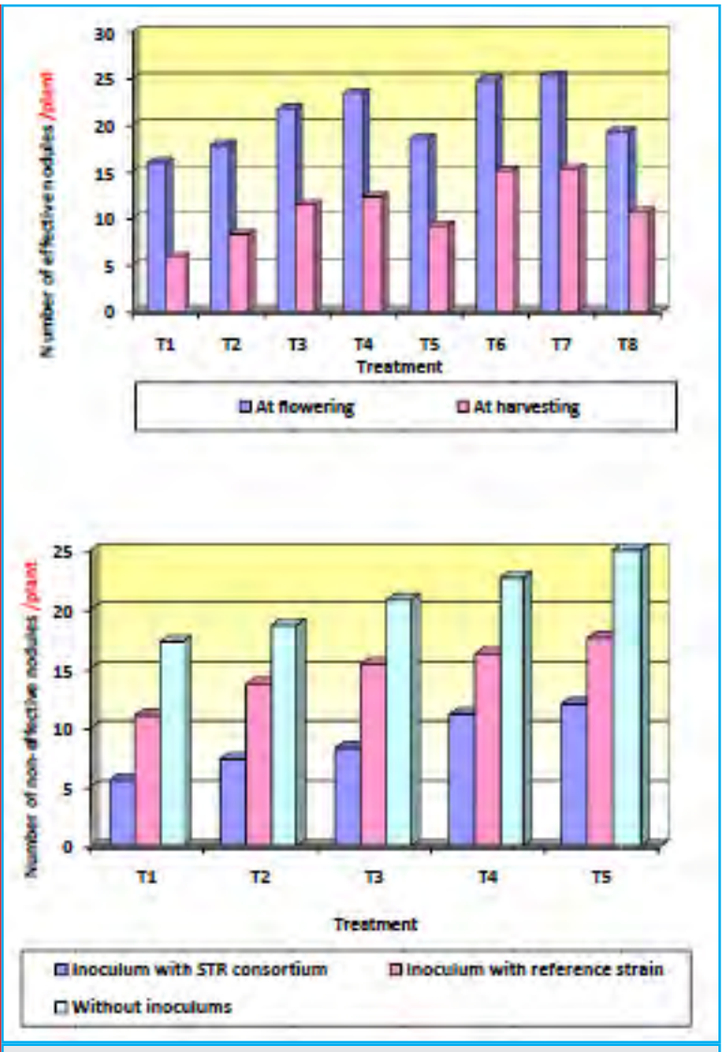

FIGURE 2. Effect of liquid consortium of salt tolerant Rhizobium on number of effective and non-effective nodulesper plant of Soybean at flowering and harvesting stages

The results are in line with, Rejili *et al.* (2012) reported that on the selection and characterization of salt-tolerant Rhizobia strains, able to fix nitrogen symbiotically under salt conditions, might constitute a strategy for improving legume symbiosis in adverse conditions and might constitute a better economic and sustainable alternative to chemical fertilization. Similarly, Sharma *et al.* (2013) reported that on the salinity tolerance of naturally occurring rhizobia, isolated from the root nodules of three leguminous plants, viz., sesbania (*Sesbania sesban*), lablab (*Lablab purpureus*) and pigeonpea (*Cajanus cajan*), growing at research farm in Dubai (United Arab Emirates). The Rhizobial isolates were also found to be effective in nodulating 21-day old seedlings grown in potting soil and irrigated with saline water up to 12 dSm^{-1} after inoculation. The tolerance to high levels of salinity and the survival and persistence in severe and harsh desert conditions made these Rhizobia highly valuable inoculum to improve productivity of the leguminous plants cultivated under extreme environments.

Present finding correlates, Vishal *et al.* (2013) reported that the inoculation with *Rhizobium* culture had invariably and significantly promoted nodulation and leghaemoglobin content at both durations particularly at lower EC levels and minimized the deleterious effect of salinity at10 to 14 dSm-1.Salinity is considered a limiting factor

Table 3. Effect of liquid consortium of salt tolerant Rhizobium on Leghaemoglobin content of soybean at 45 and 60 days

Treatment		Leghaemoglobin content (mg g^{-1} fresh weight of nodule)	
		At 45 days	At 60 days
T_1	Absolute control	0.184	0.202
T_2	Reference strain + 50 % N	0.204	0.224
T_3	Reference strain + 75 % N	0.251	0.276
T_4	Reference strain + 100 % N	0.269	0.293
T_5	Liquid consortium + 50 % N	0.219	0.243
T_6	Liquid consortium + 75 % N	0.276	0.305
T_7	Liquid consortium + 100% N	0.290	0.321
T_8	Only 100 % N	0.235	0.257
	S.E. +	0.001	0.001
	CD at 5 %	0.004	0.003

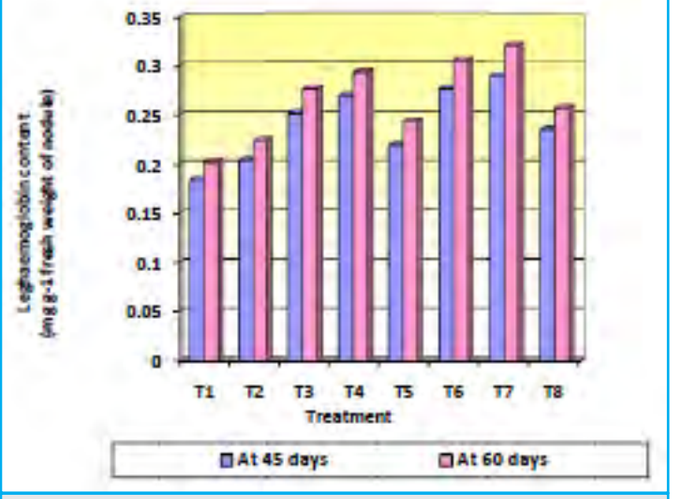

FIGURE 3. Effect of liquid consortium of salt tolerant Rhizobium on fresh weight of shoot and root of soybean at flowering and harvesting stages

in nodulation and leghaemoglobin content in legume-Rhizobium associations, which can adversely affect the yield of legume crops. Rhizobia can tolerate high concentrations unlike legume plants. Therefore, in saline soils, the multiplication of these Rhizobium strains will not be affected in the rhizosphere of the plant host. So, there is currently need isolation and development salt tolerant Rhizobium strains would enhance the plant growth through nodulation and leghaemoglobin content of plants under saline conditions which indirectly increase the nitrogen fixing ability of legume crop.

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Nutritional Communication

Biosci. Biotech. Res. Comm. 12(2): 384-389 (2019)

BBRC Bioscience Biotechnology Research Communications

Antioxidant capacity, oxidative stability and sensory evaluation of peanut oils produced using different processing techniques

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ABSTRACT

Four differently processed peanut oils viz. refined, filtered, cold pressed and organic were selected for the study. The total antioxidant capacity was estimated by DPPH method, Ferric Reducing Antioxidant Power and *in vitro* digestibility of antioxidants was determined. The oxidative stability of the samples was tested by biodiesel Rancimat analysis. Organoleptic evaluation was done using a 5 point hedonic scale. When analyzed by DPPH method, and FRAP method, filtered peanut oil showed highest antioxidant capacity followed by refined oil. The percent digestibility of antioxidants was highest in case of organic peanut oil (92.3%). The oxidative stability results showed that cold pressed oil was most stable at all temperatures followed by filtered oil. Although, the oil samples showed difference in the sensory scores, the potato chips deep fried in these peanut oils did not show statistically significant difference ($P<0.05$). Filtered peanut oil had highest antioxidant capacity but the digestibility of antioxidants was higher in case of organic peanut oil. The results need to be validated by *in vivo* studies. Cold pressed oil was most stable at all temperatures so it is more suitable for deep frying.

KEY WORDS: ANTIOXIDANT CAPACITY, COLD PRESSED OIL, FILTERED OIL, ORGANIC OIL, REFINED OIL

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Corresponding Author: vaijayanthikanabur@gmail.com
Received 23th March, 2019
Accepted after revision 25th May, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/22

INTRODUCTION

Peanut or groundnut (*Arachis hypogaea)* is eulogized as king of oil seeds in India as it is an important source of edible oil. It contributes 25 per cent to the total share of vegetable oil production in India (National Food Security Mission). Oils and fats are an important source of energy providing 900 K Cal/100g. Peanut oil has very good lipid profile. It has saturated, monounsaturated and polyunsaturated (Saturated Fatty Acid: Mono Unsaturated Fatty Acid: Poly Unsaturated Fatty Acid = 20: 54: 26) fatty acids in healthy proportions. They are an excellent source of fat soluble vitamins such as Vitamin A, D, E and K. Nutrients such as vitamin E, vitamin C, Vitamin A, selenium have antioxidant abilities. Oils are a good source of natural antioxidants, (Longvah *et. al*. 2017 Durmaz and **Gökmen** 2019).

Antioxidants are the chemical substances which neutralize free radicals. They can prevent damage to the body cells or repair damage that has already been done by free radicals. Free radicals are generated by the body during the course of normal metabolism. Some conditions such as diabetes, obesity, exercise, high fat and high sugar diet, stress, infections, air pollution, UV rays, smoke etc increase free radical generation. The balance between antioxidants and oxidants decides the health and of a person (Lien Ai Pham-Hay *et. al*. 2008). The combined ability of all antioxidants in a given food to neutralize the free radicals is referred to as total antioxidant capacity of a food. A number of factors such as soil type, chemistry, plant nutrients, climatic conditions, pest pressure, post harvest treatments influence the total antioxidant capacity of a food (Brandt *et al.,* 2002). Oils do not occur free in nature. They occur in seeds from which they are isolated, refined, and processed for specific use. Processing of oil brings about changes in the composition, properties of oil (Akhtar *et al* 2014 and Durmaz and Gökmen 2019).

There are different techniques employed to extract oil and based on this, different types of oils such as filtered oil, refined oil, cold pressed oil, organic oil are available in the market. Filtered peanut oil is made by pressing peanuts through Expeller or extracted using solvents such as hexane. In case of refined peanut oil, after filtration, the constituents such as free fatty acids, unsaponifiable matter, gums, waxes, mucilaginous matter, a variety of colouring matter, metallic contaminants, undesirable odoriferous constituents etc are removed by bleaching, neutralization, deodorization using chemicals. Most highly refined peanut oils remove the peanut allergens and have been shown to be safe for "the vast majority of peanut-allergic individuals," (Marvin et al 1998). Cold pressed peanut oils are produced by pressing the peanuts using heavy granite millstones or modern stainless steel presses. Although pressing and grinding produces heat through friction, the temperature must not rise above 120°F (49°C) for any oil to be considered cold pressed. The extraction of oil at lower temperature is believed to retain their flavor, aroma, and nutritional value, (Wang 2016).

Organic peanut oils are the oils produced using peanuts which are grown using organic substances during production, storage or processing. No synthetic chemicals are used in extraction and processing and extraction takes place at a lower temperature. Organic oils in general are 25% more expensive and consumers have a strong perception that they are tastier, nutritious and healthy compared to the conventional foods. But there are very few scientific studies on oils to prove or disprove this claim.

In this study an attempt was made to know if these differences in extraction methods and processing of oil influence the antioxidant capacity, oxidative stability and organoleptic properties of peanut oil. This study was carried out with the following objectives: To estimate total antioxidant capacity of the selected peanut oils, to determine the differential oxidative stability of selected peanut oils and to carry out organoleptic evaluation of some selected recipes using selected peanut oils. Limitations of the study: All brands of peanut oils available in the market have not been tested. Sensory evaluation was done on a small scale.

MATERIALS AND METHODS

The methodology of the study is presented under the following headings. The research was carried out in 4 phases: Selection and procurement peanut oils samples, Estimation of antioxidant capacity of the selected samples, determination of oxidative stability of the peanut oil samples and sensory evaluation of deep fried food using 4 peanut oil samples.

I) Selection of and Procurement of Peanut Oil Samples: Four differently processed peanut oil samples viz. Refined, filtered, cold pressed, and organic peanut oil samples were selected for the study. One popular brand (safola) of refined and filtered ground nut oil, cold pressed oil (24 Mantra cold pressed) were obtained from a hypermarket. Organic sample was procured from organic outlet certified by the horticultural department. All the chemicals used for analysis were of the analytical grade. Triple distilled water was used for the study.

II) Estimation Of Antioxidant Capacity Of The Selected Samples: About twenty different methods of antioxidant assay are currently being used. But no single method is sufficient to quantify the total antioxidant capacity. Scientists have opined that 2-3 methods rather than a single method can give better indication of total anti-

oxidant capacity of a food. In the present study the following methods have been used for estimation of total antioxidant capacity of the samples.

a) Free radical scavenging activity using 2, 2-Diphenyl-1-picrylhydrazyl (DPPH method): The ability of antioxidants to reduce DPPH was determined according to the procedure described by Moreno *et al* (1998) with some modifications. 10 ml of oil sample was dissolved in 20 ml of n-hexane. The solutions were prepared in n-hexane because the methonolic solution of DPPH causes turbidity in the reaction mixture and hence interferes with the results. The solution was kept in magnetic stirrer for one hour and filtered through Whatman # 1. The filtrate was used for analysis. To the above sample extract, 40 µM of DPPH solution was added. The reaction mixture was incubated with varying concentrations of sample in different dilutions. The reaction mixture was incubated for 20 minutes at room temperature in dark and the absorbance of the resulting solution was read at 517 nm against a blank using a spectrophotometer (Double beam UV VIS Spectrophotometer (AU2701 Systronics). Trolox was used as a standard. All the analyses were carried out in triplicate. Free radical scavenging ability of the sample was calculated according to the following equation.

DPPH radical scavenging activity (%) =

{(Abs Control-Abs Sample)/Abs of Control} X 100

b) Ferric Reducing Antioxidant Power (FRAP): Ferric-reducing antioxidant power was measured following the procedure originally described by Benzie and Strain (1996), in which Fe^{3+} is reduced to Fe^{2+} ion reduction, at low pH, causes the formation of a coloured ferrous-TPTZ complex (2,4,6 tripyridyl-s-triazine) resulting in an increase in absorbance at 593 nm.

Sample extraction: Sample was extracted according to procedure used by Sreeramulu and Raghunath (2011). 5 ml of the oil sample was mixed with 20 ml of 70% methanol containing 0.1% HCl by shaking vigorously for four hours at room temperature. The solution was centrifuged at 10,000 g for 15 min at 10°C. The supernatant was collected and filtered through Whatman # 1 filter paper and the filtrate was stored at –20 °C. Samples in the range of 30 to 70 µl were added to 4500 µl of FRAP reagent. FRAP reagent consists of the following. 1) 0.3 M acetate buffer, pH 3.6,2)10 mM TPTZ in 40mM hydrochloric acid and 3)20 mM ferric chloride in 40 m M hydrochloric acid.All the above were mixed in the ratio of 10:1:1(v/v/v) to obtain FRAP reagent. The reagent was preheated to 38°C and the initial absorbance was measured using acetate buffer blank. The reaction mixture was shaken vigorously for 15 sec and incubated at 27°C for 90 min. The absorbance was measured at 593 nm at the end of 90 min. Control experiments without the sample or TPTZ were carried out to exclude the effect of the added test compounds. Higher absorbance indicates higher ferric reducing power. The results are expressed as Trolox equivalent reducing power.

c) *In vitro* digestibility of antioxidants

In vitro digestibility of the samples was determined according to a method described by Luten *et al*. (1996).

III) Determination Of Oxidative Stability Of The Peanut Oil Samples: The oxidative stability of the four samples was tested by biodiesel rancimat analysis. This was out sourced from CSIR- Central Salt & Marine Chemicals Research Institute, Bhavnagar, Gujarat.

IV) Sensory Evaluation Of Deep Fried Food Using 4 Peanut Oil Samples: The preparation and sensory evaluation was done in Food and Nutrition Laboratory of the College a) Development of the score card: A score card was prepared keeping in mind the quality characteristics of the test drink. A 5- point hedonic rating scale was used for rating attributes such as colour, taste, odour and overall acceptability. Highest score (5) was assigned to the most preferred characteristic and 1 to the most undesired characteristic. Mean score for each attribute was calculated.b) Preparation of one deep fried product: Potato finger chips of uniform size and thickness were deep fried separately in each of the peanut oil samples and were presented for sensory evaluation.c) Sensory evaluation of the product: Acceptability of the product was tested by a selected panel of judges. A panel of 40 judges from the staff and post graduate students of Department of Food and Nutrition were selected for the evaluation of the product.Statistical analysis of data was done using suitable methods

RESULTS AND DISCUSSION

The results are presented under the following headings. Antioxidant Capacity of the samples: The DPPH free radical scavenging activities of peanut oil samples at different sample concentrations are shown in Table 1.

The free radical scavenging ability expressed as inhibition percentage was maximum in case of standard (trolox). Of the four samples tested, filtered peanut oil showed highest inhibition followed by refined oil. Cold pressed oil and organic peanut oil showed the low antioxidant activity. There was statistically significant difference between standard and peanut oil samples ($P<0.05$).

Antioxidant activity by Ferric Reducing Antioxidant Power (FRAP): The mean antioxidant activity value as assessed by ferric reducing power is given in Fig 1. One way ANOVA shows that there was a significant difference ($P<0.05$) between the absorbance values of the four oils tested. It was highest in case of filtered oil, followed by refined oil, organic oil and cold pressed oil.

Table 1. Antioxidant capacity of different peanut oils by DPPH Method

Sl. No	Concentration of sample (µM/g)	Standard (%)	Refined Peanut oil (%)	Filtered Peanut oil (%)	Cold Pressed peanut Oil (%)	Organic Peanut oil (%)
1	25	82	21	24	19	20
2	50	84	23	41	20	21
3	75	90	43	63	31	37
4	100	93	51	61	47	50

Gökhan Durmaz, VuralGökmen (2019) found that the process of refining of oils in case of hazelnut oil brought about a decrease in bioactive compounds. Lutein and zeaxanthin were lost completely whereas phenolic compounds and tocopherols were partly lost during bleaching step of the refining. This resulted in significant reduction of antioxidant capacity.

In vitro Digestibility/Bioavailability of peanut oil samples: The antioxidant capacity in terms of trolox equivalents before and after *in vitro* digestion in case of peanut oil samples is shown in Fig. 2.

There was statistically significant difference between the samples with respect to digestibility of antioxidants. It is interesting to note that the percent digestibility of antioxidants was highest in case of organic peanut oil (92.3%), closely followed by filtered oil (85.7%) and was quite low in case of refined oil (63.24%) and cold pressed oil (66.30%). There are hardly any studies on digestibility of antioxidants and so the result could not be compared with other studies.

Oxidative stability of oils: Freshness and oxidative stability of fats are often determined on the basis of the induction period, meaning the period in which peroxide creation is untraceable or very small, until the point of its sudden increase in the volume of the analysed sample. The results of oxidative stability as assessed by biodiesel rancimat method are given in table 2.

The induction period --the time taken to the onset of oxidation – is the indicator of oxidative stability in this method. One way ANOVA shows that there is significant difference between induction time of peanut oils at different temperatures. At room temperature the oxidative stability of cold pressed oil was highest followed by filtered oil and refined oil. Cold pressed oil was found to be most stable at all temperatures followed by filtered oil. Organic oil showed least stability at higher temperature. Arranz *et. al*. (2008) studied the relation between DPPH free radical scavenging capacity and oxidative stability (Rancimat method) in different hazelnut, peanut, pistachio, walnut and almond oils. They found that highest

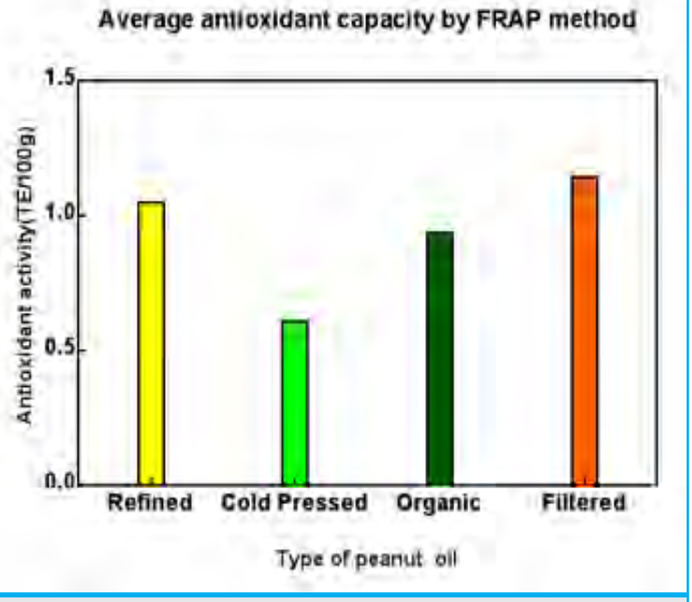

FIGURE 1. Antioxidant activity of the peanut oil samples using FRAP method

Table 2. Oxidative stability in terms of induction time for peanut oil samples at different temperatures.

Type of peanut oil	Induction time at 30° C (hours)	Induction time at 110° C (hours)	Induction time at 120° C(hours)
Refined Oil	3,649	7.08	3.3
Filtered Oil	5,160	8.47	3.8
Cold pressed Oil	7,741	9.5	4.11
Organic Oil	3,178	6.37	2.88

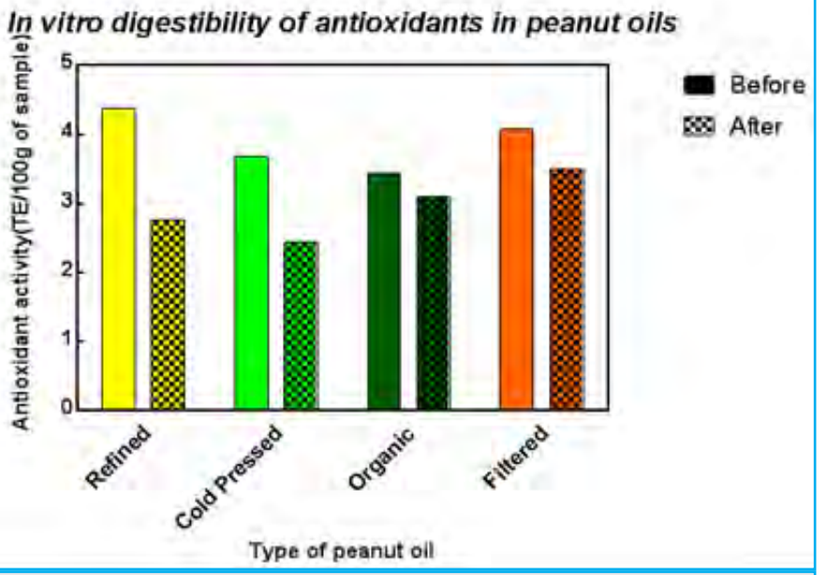

FIGURE 2. *In vitro* digestion of organic and conventional spinach

free radical scavenging capacity was in case of pistachio and least was in case of peanut oil. They also found a significant correlation between DPPH and Rancimat methods assays. In case of hazelnut oil the neutralization process increased oxidative stability whereas deodorization slightly decreased oxidative stability as there was a partial removal of tocopherols during deodorization, (Durmaz and Gökmen 2019).

Organoleptic Properties of peanut oils: Peanut oil is one of the cooking oils with a high smoke point; 450 °F. Therefore it is preferred oil for deep frying. List (2016) opines that the flavour, crispness and mouth feel of foods deep fried in peanut oil are excellent because of high levels of oleic acid and absence of linolenic acid and optimum amount of linoleic acid. Table 3. gives the mean sensory scores for the potato chips deep fried in different peanut oil samples as tested by the panellists.

Although, the cold pressed peanut oil and organic peanut oil had a deep yellow colour with pleasant nutty aroma and sweet taste and refined oil had light yellow with the neutral taste, the sensory scores of the potato chips deep fried in these oils did not show statistically significant difference ($P<0.05$). People with peanut allergy should be cautious in consuming cold

Table 3. Mean sensory scores for potato chips deep fried in peanut oil samples

Type of peanut oil	Colour	Taste	odour	Overall acceptability
Refined oil	4.8	3.8	4.0	4.1
Filtered oil	4.7	3.7	3.9	4.2
Cold pressed oil	4.68	4.3	4.5	4.3
Organic oil	4.8	4.5	4.5	4.3

pressed and organic peanut oils as the processing may not remove the allergens. Studies have shown that the process of refining alters the organoleptic properties. Peanut oil which is, pale yellow in colour with distinctive nutty taste and odour obtained from the processing of its kernel becomes odourless after refining (Sanders, 2002). DuPlessis et al. (1981) compared the performance of peanut oil and cottonseed oil and concluded that potato chips deep fried in peanut oil had significantly higher flavour scores. The study has not compared the effect of refining of peanut oil on organoleptic properties of foods deep fried in peanut oil.

CONCLUSION

Processing of oil influences the properties of the peanut oil. Cold pressed oil was found to be most stable at all temperatures followed by filtered oil. Organic oil showed least stability at higher temperature. Although there was a significant difference in the sensory attributes of peanut oil samples, there was no significant difference in the deep fried food among the four samples when used as a medium for deep frying.

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Biotechnological Communication

Biosci. Biotech. Res. Comm. 12(2): 390-396 (2019)

BBRC Bioscience Biotechnology Research Communications

Isolation and biochemical characterization of heavy metal resistant bacteria from dye industry effluent in Faridabad, Haryana, India

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ABSTRACT

Dye industry effluent containing toxic compounds, heavy metals and pigments are polluting agricultural soil and water by incorporating toxic, mutagenic and carcinogenic heavy metals from the dye. This study aims to investigate the heavy metal resistant pattern of bacterial diversity present in effluents of dye industry containing various heavy metal in Faridabad, Haryana (India). After carrying out the primary screening, five bacterial isolates KL1-KL5 were selected on the basis of resistant showed against initial concentration (80 µg) of different heavy metal salts. Strain KL1 was highly resistant to all the test heavy metals Nickel (380 µg/ml), Lithium (420 µg/ml), Copper (360 µg/ml), Ferric (400 µg/ml) and Zinc (300 µg/ml) when cultured on nutrient agar medium. Optimized growth conditions for KL1 were at 35°C, pH 7 and 4% NaCl concentration. Strain KL1 was identified as *E.coli* on the basis of morphological, physiological and biochemical characteristics from IMTECH, Chandigarh. This study revealed that the isolated bacterial strain KL can be efficiently used in the removal of heavy metals in contaminated industrial effluents.

KEY WORDS: ANTIBIOTIC, CARCINOGENIC, HEAVY METALS, MUTAGENIC, EFFLUENT

ARTICLE INFORMATION:

Corresponding Author: amitakuk@gmail.com
Received 27th March, 2019
Accepted after revision 1st June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
Crossref Clarivate Analytics
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/23

INTRODUCTION

Constantly increasing population and industrialization activities are polluting air, water and soil by depositing heavy metals (Marzan *et al*., 2017; Shifaw, 2018). Various dyes are being used in the textile industry having toxic properties. Azo dye is one of the main pollutants which contribute up to 70% of the textile and paper dyes. Even after using a number of traditional effluent treatment methods these carcinogenic dyes are non-degradable and discharged into the water resources (Carliell *et al*., 1995; Karatas and Durusun, 2007; Islam *et al*., 2017). The textile dye effluent consists of heavy metals such as Cadmium, Lead and Zinc either in free ionic metals or complex metals, said to mostly originate from the dyeing process which are very toxic and carcinogenic (Basha and Rajaganesh, 2014). Natural water resources have been polluted by toxic heavy metal containing effluent. These toxic compounds are accumulated in living organisms including microorganisms, plants, animals and human, resulting into serious threat to the health of living organisms, in the present scenario the dye industry effluent is a major source of heavy metal toxicity, (Sarker *et al*., 2015, Ayangbenro and Babalola, 2017 and Shifaw, 2018).

Textile dye effluents cause serious environmental problems by absorbing light in receiving water bodies like streams, rivers and lakes etc and ultimately interfering with aquatic biological processes.The water containing textile effluent used for irrigation contains heavy metals like Cd, Pb and Zn, which accumulate in various parts of plans that result in various clinical problems in animals as well as human beings including hepatic and renal system damages, mental retardation and degradation of basal ganglia of brain (Emongor *et al*., 2005). Water pollution due to toxic heavy metals through textile dye effluent remains a serious environmental and public problem in developing countries, (Forgacs *et al*.,2004; Hao *et al*., 2000; Saini, 2017 and Shifaw, 2018).

There are various conventional methods available for removal of toxic compounds, pigments, dyes and heavy metals from the industrial effluents. These traditional methods are less efficient, costly and time consuming. Presently bioremediation using microorganisms proved to be a revolutionary technique for the removal and degradation of these toxic compounds from soil and water (Su *et al*., 2014). Bacteria, fungi and many other microorganisms have metabolic pathways which can uptake and use toxic compounds as an energy source for their survival. Microorganisms have enzymes that can degrade the toxic contaminant into nontoxic form. Due to their characteristic derivative enzymes they have developed resistance against heavy metals in order to adapt toxic levels of heavy metals evolved diverse mechanisms for maintaining homeostasis and resistance to heavy metals, in order to adapt to toxic metals in the ecosystem (Brar *et al*., 2006; Wei *et al*., 2014). The present study was carried out to explore the heavy metal resistance and degradation capabilities of microbial diversity present into dye industry effluent.

MATERIAL AND METHODS

Study Area and Sample Collection

Effluent Samples were collected from various industrial sites in and around Faridabad, Haryana (India). Labeled polyethylene bottles previously washed with 10M HN03 and distilled water were used for sample collection and a cold chain was maintained while transferring to the laboratory in Kurukshetra University Kurukshetra. Effluent sample was filled in these bottles by making sure that there is no air space left. Collected samples were preserved at 4^0C for further experiments and analysis. Various parameters like pH, temperature, and color of effluent were documented at the sampling site using methods recommended by APHA (1992).

Isolation and Primary Screening for Heavy Metal Resistant Bacteria: For isolation of heavy metal resistant bacteria, effluent samples were diluted to obtain ten-fold serial dilutions (Azad et al., 2013). 100 µl of undiluted dye effluent sample and dilutions from 10-1 to 10-4 were spread on sterile nutrient agar plates incorporated with initial concentrations (80 µg/ml) of Ferric Chloride ($FeCl_3$), Copper Sulphate ($CuSO_4$), Zinc sulphate ($ZnSO_4$) and Nickel Sulphate ($NiSO_4$) and Lithium Sulphate ($Li2SO_4$). Heavy meatal salts used were of analytical grade and Millipore membranes with a 0.22 µm pore size used to sterilize solutions prepared. Plates were incubated at 30^oC for 48 hours and observed for bacterial colonies. To enhance the accuracy and authenticity, this screening experiment was carried out and in triplicate. Minimum inhibitory concentration (MIC) of the selected isolates against increasing concentrations of heavy metals on nutrient agar plates was evaluated until the strain unable to grow colonies even after seven days of incubation. Cultures were stored in glycerol stock solution at -20^0C.

Multiple Metal Resistance Capacity: For determining the heavy metal resistance spectrum, the bacterial strains isolated after primary screening were separately grown on nutrient agar plates supplemented with selected heavy metals (80 µg/mL) at pH 7.0 and 37 °C for 24 h. After incubation the resistance capacity of multiple heavy metals was assessed.

Determination of Minimum Inhibitory Concentration (MIC): Minimum inhibitory concentration of each heavy

metal against isolated bacterial strains was determined by growing them on heavy metal incorporated nutrient agar medium. Concentration of respective heavy metals has been increased gradually until the bacterial strain failed to grow colony. The starting concentration of the heavy metals was 80 µg/ml. The culture growing on initial concentration was then streaked on to the higher concentration of heavy metal. The concentration at which bacterial strain failed to grow colony was considered as MIC.

Determination of Optimal Growth Conditions: Growth conditions for bacterial isolate KL were optimized by growing in nutrient broth medium at different pH (5 to 10), temperature (150C to 650C) and salt concentrations (1%-10%). The optical density of the log phase growing cultures conditions was noted at 600nm to determine the growth.

Effects of Heavy Metals on Microbial Growth in Liquid Medium: Bacterial isolate KL was separately grown in nutrient broth supplemented with Nickel (Ni^{2+}), Lithium (Li^{+}), copper (Cu^{2+}), ferric (Fe^{3-}), and zinc (Zn^{2+}) (80 µg/mL) at pH 7.0 and 37 °C for 24 h. Bacterial cells in exponential phase cells were used to inoculate nutrient broth medium. The concentrations of each heavy metals were increased gradually. The cultures were incubated for 4 days at 37^{0}C and agitated at 150 rpm on an orbital shaking incubator (REMI, India). Growth rate of the strain was determined by increasing absorbance at wavelength of 600 nm [OD600] with a Spectronic 200 Spectrophotometer (Thermo Scientific, India). Experiment was done in triplicate.

Morphological and Biochemical Identification: Morphological characteristics of strain KL were examined under the microscope by using Gram Staining technique. For the analysis of biochemical characteristics bacterial culture was sent to IMTECH, Chandigarh.

Antibiotic susceptibility Test: Bacterial strain KL was tested for susceptibility to antimicrobial agent by Disc Diffusion method on Muller-Hinton Agar plates (Gupta et al, 2016). Zone of inhibition was noted after 24h incubation and susceptibility was recorded as positive. Antimicrobial agents used for the study were: Vancomycin (30 µg), Erythromycin (15 µg), Penicillin (10 µg), Minocyclin (30 µg), Gentamycin (10 µg), Rifampicin (5 µg), Ofloxacin (5 µg), Amoxycillin (25 µg), Methicillin (5 µg), Chloramphenicol (30 µg), Spectinomycin (100 µg), Metronidazole (5 µg), Nitronidazole (10 µg), Clindamycin (10 µg), Ampicillin (5 µg) and Deoxycholin (10 µg).

RESULTS AND DISCUSSION

On the basis of primary screening, carried out with an initial heavy metal concentration of 80 µg/ml on nutrient agar medium, total 15 bacterial strains were isolated. Visual observation of bacterial colonies on heavy metal containing nutrient agar medium after 3-4 days of incubation at 37^{0}C showed that the collected dye effluent sample have metal resistant diversity of bacteria. The bacterial colonies were able to grow well on solid medium supplemented with Nickel (Ni^{2+}), Lithium (Li^{+}), copper (Cu^{2+}), ferric (Fe^{3}), and zinc (Zn^{2+}) at an initial concentrations of 80 µg/ml. Out of these bacterial strains, five (KL1, KL2, KL3, KL4 and KL5) were selected for further studies. Primary screening results for KL1 are shown in figure 1.

MIC study of each heavy metal showed that all the five bacterial strains were tolerating heavy metal concentration between 130-420 µg/ml (Table 1). MIC of heavy metals showed that bacterial strain KL1 have highest tolerance to all the heavy metals. Multiple heavy metal resistance and MIC study revealed that all the five selected bacteria have high tendency to tolerate and grow under heavy metal stressed environment. On the basis of this study, potential bacterial strain KL was selected for further studies.

Growth conditions for KL were optimized for pH, tempratute and NaCl concentration in the medium.

The optimization study was carried out in nutrient brtoh medium. Bacterial strain was grown in flasks containing 200 ml sterilized nutrient broth medium at diifferent pH (5-10), temparature (15^{0}C-65^{0}C) and salt concentration (1%-10%). Results of optimization study showed that pH-7, tempratue-35^{0}C and 4% salt concentration were the optimized parameter growth of bacterial strain KL (figure 1).

After optimizing the growth parameters, bacterial strain KL was investigated for microbial growth in liquid medium (Nutrient Broth) supplemented with Nickel (Ni^{2+}), Lithium (Li^{+}), copper (Cu^{2+}), ferric (Fe^{3-}), and zinc (Zn^{2+}) (80 µg/mL) at pH 7.0 and 35°C for 24 h. Exponential phase cells (24h old) of bacterial strain were used to inoculate nutrient broth medium. The concentrations of each heavy metals were increased gradually. Incubation was given for 12hrs at 35^{0}C and agitated at 150 rpm on an orbital shaking incubator. Growth rate of the strain was determined by absorbance at wavelength of 600 nm. With increasing heavy metals of the growth of KL was declined. The most important aspect of this study was that KL can grow in nutrient broth medium containing Cu^{2+} (280), Ni^{2+} (320), Fe^{3-} (360) and Li^{2+} (400) (figure2).

KL was examined under microscope for its morphological characteristics by usnig grams staining technique. Gram staining is a technique used to distinguish two groups of bacteria (gram +ve and gram –ve) based on their different cell wall constituents. KL was found to be a rod sahpe and gram –ve bacterium. Common physiological and biochemical tests were also performed for

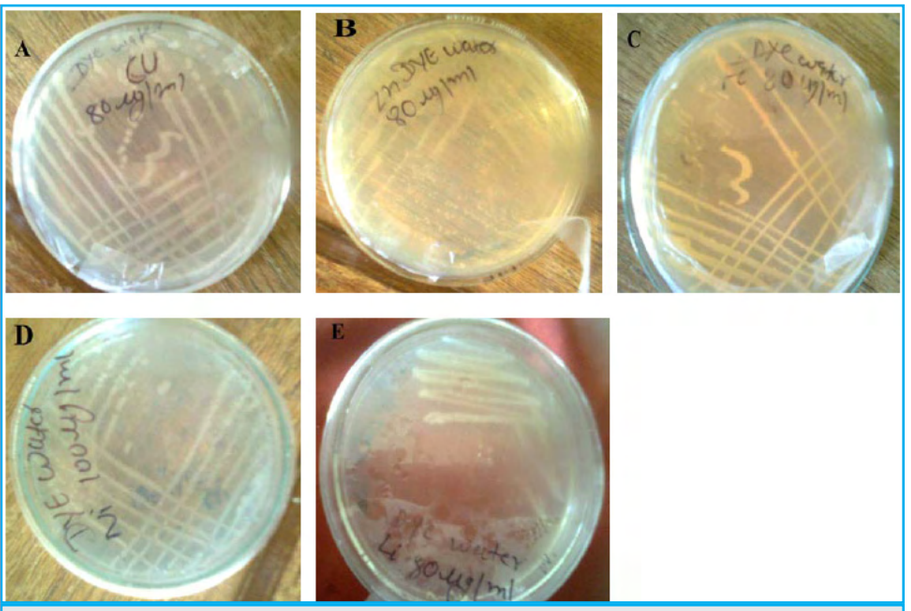

FIGURE 1. Culture plates A, B, C, D and E showing growth of Bacterial strain KL1 on Nutrient Agar medium incorporated with 80 µg/ml of different heavy metals ((Screening results).

Table 1. Multiple heavy metal resistance capacity and MIC study results of five bacterial isolates.

Metals	MIC (µg/ml)				
	KL1	KL2	KL3	KL4	KL5
Nickel (Ni^{2+})	380	220	130	160	250
Lithium (Li^{+})	420	130	170	200	170
Copper (Cu^{2+})	360	320	180	180	200
Ferric (Fe^{3+})	400	350	240	220	150
Zinc (Zn^{2+})	300	220	190	200	180

classification and identification of bacterial strain KL. Results of morphological, physiological and biochemical cahracteristics are shown in table 2.

Bacterial strain KL1 was identified as *E.Coli* on the basis of morphological, physiological and biochemical analysis report of IMTECH, Chandigarh.

KL1 was investigated for susceptibility towards different antibiotics. This study confirmed that bacterial isolate was susceptible to a wide range of antibiotics (Table 3). We need to incorporate bacterial strain to soil and water for removal of heavy metals that may be pathogenic for other living organisms. Following antibiotics available commercially can be used to treat any infection developed due to bacterial stain KL.

CONCLUSION

There are various techniques available for removal of toxic heavy metals from environment but this study revealed some significant results for metal detoxification by using microorganisms. Results of present study conclude that microorganism isolated from dye industry effluent developed the ability to tolerate heavy

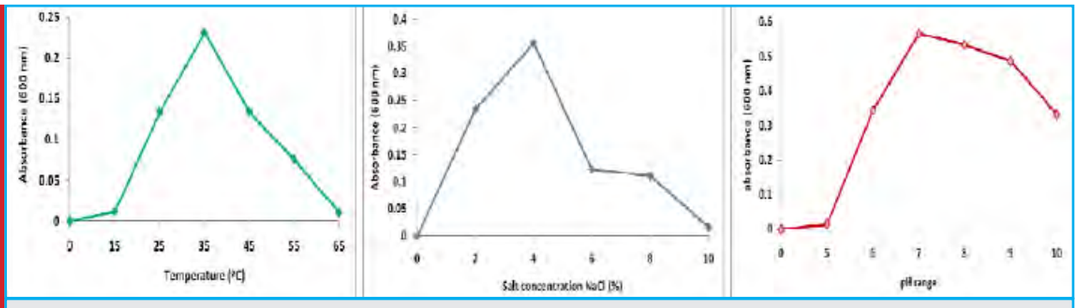

FIGURE 2. Optimized growth parameters for bactrial strain KL.

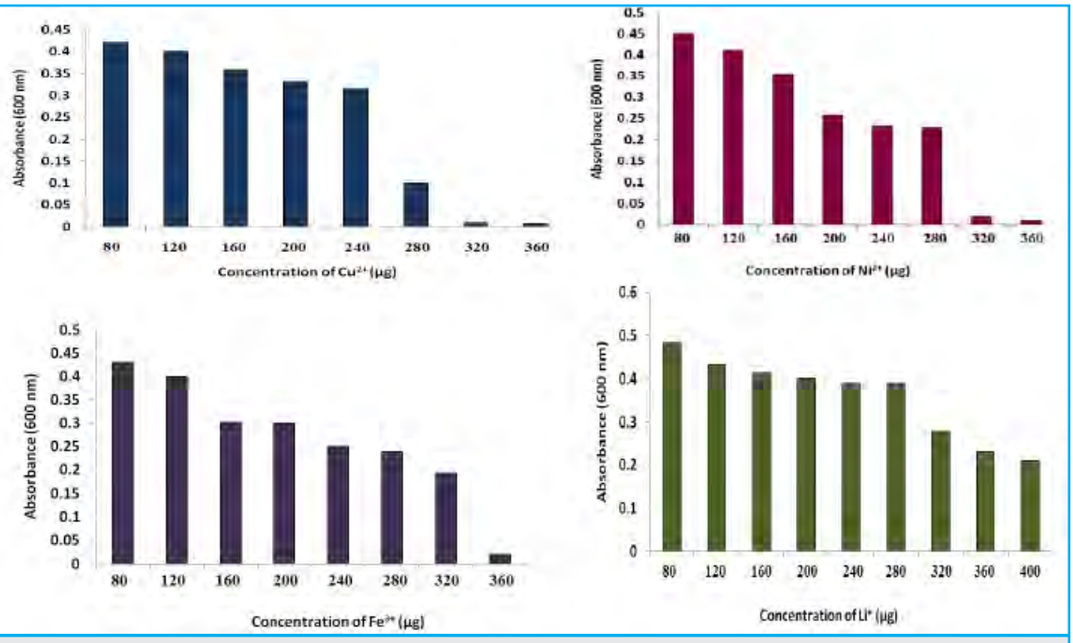

FIGURE 3. Mean (±SE) of absorbance carried out to analyze relative growth pattern of strain KL at increasing heavy metal concentrations (80-400 µg/ml).

metal stress. Bacterial strains isolated in the course of this study can be efficiently used for removal of toxic heavy metals from ecosystem and reinforce the ecological balance. Use of microbes for bioremediation is highly recommended due to its low cost and environmental friendly approach. This nature friendly technique has turned out to be the best available method which is highly efficient under heavy metal stressed environment. Further experimental study is required in the area of gene transfer for developing Genetically Modified organisms using recombinant DNA technology to make this approach more efficient and effective.

Table 2. Morphological, physiological and biochemical analysis performed on strain KL.

Morphological Test Results		Biochemical Tests Results	
Colony Configuration	Circular	Indole Test	+
Cell Shape	Rod	Voges proskauer Test	_
Colony Elevation	Flat	Citrate Utilization	_
Colony Margin	Entire	H2S Production	_
Colony Surface	Smooth	Gas production from Glucose	_
Colony Colour	Creamish	Gelatin Hydrolysis	_
Opacity	Opaque	Casein, Starch and Urea Hydrolysis	+
Gram's staining	-ve	Nitrate Reduction	_
Size	1.0	Ornithine and Lysine Decarboxylase	_
Spore	-	Catalase Test	+
Motility	Yes	Oxidase Test	_
Shape	Ellipsoidal	Acid Production from	
Position	No	Fructose, Arabinose, Galactose, Glucose, Mannitol, Xylose, Sucrose, Rhamnose	+
		Raffinose, Salicin, Mesoinositol	_

Table 3. Antibiotic susceptible profile of bacterial strain KL.

Antibiotic	Disc content (µg)	Diameter of inhibition zone (mm)	Susceptibility status
Vancomycin	30	10	Susceptible
Erythromycin	15	15	Susceptible
Penicillin	10	17	Susceptible
Minocyclin	30	20	Susceptible
Gentamycin	10	15	Susceptible
Rifampicin	5	12	Susceptible
Oflaxacin	5	13	Susceptible
Amoxycolin	25	18	Susceptible
Methicillin	5	No zone	Resistant
Chloroamphinicol	30	26	Susceptible
Spectinomycin	100	28	Susceptible
Metronidazole	5	No zone	Resistant
Nitronidazole	10	No zone	Resistant
Clindomycin	10	13	Susceptible
Ampicillin	5	15	Susceptible
Deoxycholin	10	No zone	Resistant

ACKNOWLEDGEMENTS

The authors are grateful of Director of UIET, Kurukshetra University, Kurukshetra, India, for providing instruments and facilities of this research. This research work was potentially supported by TEQIP World Bank project for providing funding.

CONFLICT OF INTEREST: The authors declare of no conflict of interest in conducting this study.

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Medical
Communication

Biosci. Biotech. Res. Comm. 12(2): 397-407 (2019)

BBRC
Bioscience Biotechnology
Research Communications

The antidiabetic activity of bioactive compounds of Indian medicinal plants: A meta data review

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ABSTRACT

Diabetes, one of the most common chronic metabolic disorders affecting a large proportion of nearly all countries is characterized by the deficiency in insulin production and resistance against insulin action. The disease ultimately leads to inappropriate and prolonged hyperglycemia which in turn affects the every system and mechanism of the body. The disorder is treated with several synthetic drugs but due to several limitations and side effects of the synthetic drugs the attention is drawn towards the employment of plant and plant product in development of herbal drugs. The ease of access, affordability and the ability of herbals to produce minimum side effects on administration have convinced a major portion of population globally to switch to this alternative approach of medicine. The plants are always been the source of immense products for the human welfare from the time immemorial. This study has mainly focused on the variety of bioactive constituents, carried out by plants, having potent medicinal properties. The article has also listed some of the famous medicinal plants of India having the potential to be used as an effective source for the herbal drug development because these plants are reservoir of many phyto-constituents for human welfare. The study concludes that if explored and studies well these plants could act the unlimited source of bioactive compounds to be used in herbal medicine development.

INTRODUCTION

Diabetes, a chronic metabolic disorder possessing a major health challenge worldwide and is very common and most prevalent diseases that affects the citizens of both developed and developing countries. Estimation gives the proportion that about 25% of the world population is affected by the disease. The current status of India revealed that there are around 40.9 million diabetic patients and the expectation are still high up to

ARTICLE INFORMATION:

Corresponding Author: anandika02@gmail.com
Received 8th April, 2019
Accepted after revision 9th June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/24

69.9 million by 2025 (Mohan *et al*., 2007). Due to this high rate of the diabetic patients India has been tagged as the diabetic capital of the world (Joshi and Parikh, 2007). Lack of an urgent preventive measure could lead it to become the major health issue among the Indian population. Number of deaths due to diabetes estimated by the Indian Diabetes Federation (IDF) is around 3.9 million that represents 6.8% of the total global mortality count (IDF, 2009). The abnormality of the carbohydrate metabolism that is due to the disturbance in the optimum level of the insulin in blood leads to the onset of the disease Diabetes (Maiti *et al*., 2004). Diabetes is also caused due to the deficiency of the insulin function or due to the insensitivity of the organ to insulin. This abnormality leads to a prolonged hyperglycemia which in turn disturbs other metabolic pathways of the human body as well (Bastaki, 2005). When left untreated this could lead to some severe damages of tissue and vascular damages that can lead to serious compilation such as retinopathy (Bearse *et al*., 2004), neuropathy (Seki *et al*., 2004), nephropathy (Looker *et al*., 2003), ulceration (Wallace *et al*., 2002) and the cardiovascular complications (Svensson *et al*., 2004). Being the most common endocrine disorder Diabetes has indirect relationship with many other diseases.

Both of the pancreatic endocrine hormones, insulin and glucagon work simultaneously to control the blood glucose level in the body to an adequate level on the basis of the body's requirement. In normal conditions insulin is secreted by the β-cells of the islets of Langerhans when there is high blood sugar level in the blood. This enzyme increases the potential ability of muscles, red blood cells, and fat cells to absorb the excess sugar out of the blood and use it for other metabolic pathways that could help to restore the optimum level of sugar in blood (Gupta, 2012). Contrast to this acts glucagon enzyme which is secreted by the β-cells of the pancreas in response to the low blood sugar level. This enzyme act to initiate the liver and other muscles cells to release the stored glucose into the blood stream for consumption by the working cells. Retention of high blood glucose level for long duration can ultimately lead to the long term damage to the organs like kidney, eyes, liver, nerves, heart and blood vessels. This type of complication in such organs may cause the death of the individual (Pari and Saravanan, 2004). The dysregulated metabolism caused due to diabetes causes certain pathophysiological changes in some organs that cause a tremendous burden on the individual. Diabetes mellitus act as the cause for several diseases like end stage renal disease, adult blind, non-traumatic lower extremity amputation, (Gupta, 2012).

The two main widely accepted types of the diabetes mellitus are type 1 and type 2 (Zimmet *et al*., 2004). The type 1 diabetes occurs in the patients with very little or no insulin secretory capacity. These types of patients are in the need for the replacement therapy in order to stay alive. The type 1 is also of two type that is type 1a conferring almost 90% of the type 1and type 1b conferring 10% of it. It is also referred as the juvenile diabetes. The type 1a results from the destruction of the pancreatic cells caused due to immunological damages and are associated with certain diseases like Addison's disease, Grave's disease and Hashimoto's thyroiditis (Atkinson and Maclaren, 1994). While type 1b is idiopathic without any etiological basis. The patients suffering from this type of diabetes possess the predominant deficiency of insulin and are susceptible to keto-acids but there is no evidence of any autoimmune disease development (MacLarty *et al*., 1990). The type 2 diabetes is the most common form of diabetes. This type is characterized by the abnormality in the insulin produced and its resistance (DeFronzo *et al*., 1992). This type is dominating in the elderly people, over 40 years. It may occur in the obese person, person with decreased body activity and this type may also be inherited from parents to offsprings (Zimmet *et al*., 1990). This type is also associated with individuals suffering from hypertension and dyslipidemia. Dietary supplements, physical activity and the oral hypoglycemic agents are responsible to enhance the disease (Zimmet *et al*., 2001, Wang *et al*., 2018; Al-Attar *et al*., 2019).

Several factors contribute to the on-set and development of diabetes in any individual. These factors are termed as the predisposing or the risk factors. Some environmental factors like diet, obesity and sedentary life style increases the risk of diabetes. Some other factors are also responsible including insulin resistance, high family aggregation, age, lifestyle, nutritional status (Deepashree and Prakash, 2007). Common symptoms of diabetes include frequent urination, excessive thirst, increased appetite, fatigue, blurred vision, slow wound healing, weight loss regardless of increased appetite (type 1), tingling, pain, or numbness of hands/feet (type 2) (Ramchandran, 2014). Diabetes mellitus is attributed to insulin inactivity or resistance as a direct result of the destruction or dysfunction of the pancreatic cells (Rezaei *et al*., 2016). The global study report states that Diabetes Mellitus is one of the non-communicable diseases based on the number of cases and its prevalence has continued to increase over the last few decades (WHO, 2016).It is evident that Diabetes mellitus leads to hyperglycemia and to many other complications such as hyperlipidemia, hypertension, atherosclerosis, retinopathy, neuropathy, and nephropathy (Wang *et al*., 2018; Al-Attar *et al*., 2019).

Importance and role of medicinal plants

The therapies available currently for the treatment of diabetes include various oral hypoglycemic agents along

with insulin like sulfonyl ureas, metformin, glucosidase inhibitors, troglitazone etc. But these agents are reported to produce several side effects on the body organs causing liver problems, lactic acidosis, and severe diarrhea (Rajalakshmi *et al*., 2009). These adverse side effects are currently affecting approx. 143 million peoples (Mentreddy *et al*., 2005) and this count down is thought to increase several folds in coming years up to 366 million (Ponnusamy *et al*., 2011). The recent encouragement is on the use of medicinal plants as alternative remedies attributed to the elevation of medication cost, synthetic medicine side influences, and lack of full recovery of diabetic patients treated with chemical hypoglycemic agents. The recently developed traditional therapies originated from medicinal plants have shown a vital role in the control of Diabetes Mellitus (Cheng *et al*., 2013).

Many plant species have been used for the prevention and management of diabetes by the leading nations like America, China, South America and Asia (Mentreddy *et al*., 2005). There are several indigenous Indian medicinal plants that have been found capable of successfully managing diabetes. The most important advantage carried out by these medicinal plants is that they are readily available and have very low or no side effects. The pharmacologically active compounds or the bioactive compounds of plants show there hypoglycemic effect by decreasing effect on α-amylase and various direct and indirect effects of the different parameters of blood that re responsible for developing diabetes (Murali, 2006).

They have an extraordinary source of drug and many of the today's generation drugs are directly or indirectly derived from the medicinal plants. The report from the ethnobotanical information covers about 800 plants that possess the antidiabetic potential in one or more of their parts (Alarcon-Aguilara *et al*., 1998). Research studies have come across several herbs with the antidiabetic property proven by the modern experimental techniques (Jafri *et al*., 2000). The study have revealed that Asian and African continents constitute up to 56% and 17% of the worldwide distribution of the therapeutic plants respectively (Chung-Hung *et al*., 2012). The plants confer the biological action due to the presence of various bioactive compounds in them like phenolics, alkaloids, flavonoids, terpenoids, coumarins and glycosides. The presence of these active lead compounds in the plant kingdom has made them as the target search by the multinational drug companies. These medicinal plants are also part of our diet as spices, vegetables, and fruits. The confer one of the most potential source for the medicine in modern society for instance, quinine, atropine, opium alkaloid and the popular hypoglycemic drug Glucophage are derived from Galega officinalis (Grover *et al*., 2002). These plants depict their effect by delaying the development of diabetic complication and also correct the metabolic abnormalities.

Virtually in all the culture the medicinal plants are used as the source of medicine (Sofowora, 1996). Treatment of diabetes mellitus using medicinal plants backs from the Ebres papyrus around 1550 BC (Kesari *et al*., 2005; Shruthi *et al*., 2012). The World Health Organization recommends the treatment of diabetes by the traditional medicines as they are effective, non-toxic, with less or no side effects and they are considered as the excellent oral therapy candidate (Khan *et al*., 2010; Singh and Koiri, 2014). Out of the 400 traditional plants discovered, only small proportion of it has received the evaluation of its efficacy by the scientists (Aruna *et al*., 2014). The report of World Health organization reveals that 90% of the populations of developed countries use plant and its products as the primary health care medicine (WHO 2002). The organization has listed around 21000 plants being used for the medicinal purpose across the world and among them 2500 plants are from India (Modak *et al*., 2007). The anti-hyperglycemic activity of these plants is due to their ability to restore the lost functions of pancreatic tissue by causing an increased output of insulin production or increases the glucose absorption and may also facilitate the metabolites in an insulin dependent process.

The most common and effective antidiabetic medicinal plants of Indian origin are Babul (*Acacia arabica*), bael (*Aegle marmelose*), church steeples (*Agrimonia eupatoria*), onion (*Allium cepa*), garlic (*Allium sativum*), ghrita kumara (*Aloe vera*), neem (*Azadirachta indica*), ash gourd (*Benincasa hispida*), Beetroot (*Beta vulgaris*), fever nut (*Caesalpinia bonducella*), bitter apple (*Citrullus colocynthis*), ivy gourd (*Coccinia indica*), eucalyptus (*Eucalyptus globules*), banyan tree (*Ficus benghalenesis*), gurmar (*Gymnema sylvestre*), gurhal (*Hibiscus rosa-sinesis*), sweetpotato (*Ipomoea batatas*), purging Nut (*Jatropha curcas*), mango (*Mangifera indica*), karela (*Momordica charantia*), mulberry (*Morus alba*), kiwach (*Mucuna pruriens*), tulsi (*Ocimum sanctum*), bisasar (*Pterocarpus marsupium*), anar (*Punica granatum*), jamun (*Syzygium cumini*), giloy (*Tinospora cordifolia*), and methi (*Trigonella foenum-graecum*); all these plants are a rich source of phytochemical compounds (Rizvi and Mishra, 2013).

Bioactive agents of the plants with hypoglycemic activity:

The Mother Nature has blessed us with the variety of plants with the medicinal properties. Survey carried out by several national and international organizations reported a diversity of the plants with the antidiabetic activity. These plants possess the ability of this activity due to the presence of the several bioactive agents

that includes alkaloids, phenols, flavonoids, steroids, glycolipid, terpenoids, saponins, amino acids, glycolpeptides etc. different plant possess these bioactive compounds in different proportion in them hence carry out some of the specific function. They carry out these effects by either increasing the level of insulin in the blood serum or they increase the production of the insulin by the β-cells of the pancreas, they can also inhibit the absorption of glucose in the gut or can increase the glucose uptake by the body for different activities (Saxena *et al*., 2004; Gupta *et al*., 2008). These bioactive compounds are reported to carry out a potent and effective hypoglycemic, anti-hyperglycemic and glucose suppressive activities (Saxena *et al*., 2006). Some of the important bioactive agents carry out the antioxidant, anti-cataract and hypolipidemic properties along with the enzymatic function restoration and repair and regeneration properties as well (Mukherjee *et al*., 2006).

Allium sativum contains sulfur amino acid called as S-allyl cysteine (Rabinkov *et al*., 1998) that could act as an alternate for insulin because the short term and long term treatment of the diabetic models with it corrects hyperglycemia (Nasim *et al*., 2009). The naturally occurring compounds with hypoglycemic and antioxidant properties are flavonoids. Flavonoids are responsible for improving the altered glucose and oxidative metabolism of the diabetic stage (Song *et al*., 2005). The phytosterols of *Aloe vera* including lophenol, 24-methyl-lophenol, 24-ethyl-lophenol, cycloartanol and 24-methylenecycloartanol carried out the antidiabetic function in type 2 diabetic mice (Tanaka *et al*., 2006). Steroids like β-Sitosterol found in *A. indica* (Prabhakar and Doble, 2008) and gymnemic acid IV from Gymnema sylvestre possess the potent hypoglycemic activity found in the animal models (Kimura, 2006). The plants like *Allium cepa* (Kumari and Augusti, 2007; Islam et al., 2007) and *Allium sativum* (Saravanan and Ponmurugan, 2010) are good source of sulphur containing amino acids like S-methyl cysteine sulfoxide and Diallyl thio-sulfinate. These amino acids activate the enzyme hexokinase, glucose-6-phosphatase and hence help in rapid consumption of glucose. The polysaccharides of *Aloe vera* are found to increase the level of insulin in blood and also show hypoglycemic properties (Yagi *et al*., 2009). Flavonoid rich extract from the seeds of *Eugenia jambolana* possess hypoglycemic properties and is experimentally reported in the rat models (Bhavana *et al*., 2008).

These many findings related with the antidiabetic role of bioactive compounds from plants have demonstrated the importance of medicinal plants and their potential use for the human welfare. The data available on the antidiabetic effects of the phytochemical compounds suggests that the plants also possess some other compounds in there different parts which acts through different pathway and have role in curing several other disease than diabetes. They may be regarded as a new type of chemotype that will help the phytochemist to offer the potential cost effective management of diabetes through cost-effective manners (Mentreddy *et al*., 2005). The antidiabetic effects of these bioactive agents have been studied in various models of animals like mice, rats and rabbits with different dosage of the plant extract and the period of incubation varied from 24 hours to 45 days. Ferulic acid and Cuminosides from the ethanolic and methanolic extracts respectively of *S. cumini* seeds have shown a significant hypoglycemic and antioxidant potential (Mandal *et al*., 2008; Farswan *et al*., 2009).

Some plants with potent antidiabetic properties:

Aegle marmelos

It is a member of family Rutaceae and is commonly called as Holy Fruit tree. The aqueous leaf extract of *Aegle marmelos* was found to be as potent as insulin in controlling the blood glucose level in STZ diabetic rats when administered orally (Grover *et al*., 2002). Alcoholic extract the plant also show effective role as the methanolic extract of the plant decreases blood glucose level in alloxan diabetic rats and also lowers the oxidative stress by reducing the peroxidation of serum and liver lipid, elevates the level of enzymes including catalase, glutathione peroxidase and superoxide dismutase (Sabu and Kuttan, 2004). Reduction in the FBG level was shown when aqueous seed extract was administered orally. This was accompanied by the decrease in total cholesterol level and triglycerides and caused a concomitant elevation in level of HDL (Keasri *et al*., 2006).

The mode of action includes the stimulation of glucose uptake or enhances the insulin secretion or both in some cases. In addition to this it helps in improving the function of beta cells and regenerates the damaged pancreatic parts (Maity *et al*., 2009). Fruit of the plant also carry out the antidiabetic function as the oral and intraperitoneal administration of aqueous fruit extract showed significant antidiabetic activity in STZ induced rats. The fruit extract caused significant reduction in blood glucose level, also glycosylated the hemoglobin and elevated the level of blood insulin and liver glycogen. A specific dose of 250 mg/kg of the fruit extract is found to be more potent than glibenclamide (Maity *et al*., 2009). The effective and relevant hypoglycemic effect shown by the plant extract is mainly due to its coumarins that are responsible for stimulation of insulin secretion from pancreatic beta cells (Maity *et al*., 2009).

Allium cepa and Allium sativum

They are member of the Liliaceae family and are one of the important dietary supplements and are mainly

involved in eastern kitchen. Ethanol extract of garlic, shown by study, regulates the blood sugar level by normalizing the activity of both liver hexokinase and glucose-6-phosphatase. The active component of *Allium cepa* is the secondary metabolite present in the form of cysteine derivative as S-alkyl cysteine sulfoxides that decompose in the presence of Allinase upon extraction into polysulfides and thio-sulfinates. The antidiabetic activity of both the plants can be conferred to the presence of these volatile decomposed products that are dominant in their oils in addition to the other non-volatile sulfur containing peptides and proteins (Augusti, 1996).Ether fractions of onion bulb are found to show hypoglycemic effect by decreasing the glucose peak in subcutaneous glucose tolerance test (Grover *et al*., 2002).

Allium cepa is responsible in increasing the fasting serum high density lipoprotein value and also exhibits the alleviation of hyperglycemia in diabetic rats. This hypoglycemic and hypolipidemic effects of onion is usually associated with its antioxidant property. The major active components present in there extracts include sulfur-containing compounds like diallyl disulfide (allicin) in garlic and allyl propyl disulfide (APDS) in onions (Dey *et al*., 2003). The ethanol extract of garlic was found to be more potent in its antidiabetic activity than the commonly known drug Glibenclamide (Eidi *et al*., 2006). The extract was found to be responsible for elevating the level of liver glycogen, serum insulin and free amino acids that causes the significant reduction in level of FBG, serum triglycerides, cholesterol, urea, creatinine, AST and ALT level (Goel *et al*., 2012).

Cinnamomum zeylanicum

Commonly known as cinnamon and is widely used in East Asia and Europe. It belongs to the family Lauraceae. It is extensively under use in the folk medicine preparation used for the treatment of diabetes. Major component of cinnamon includes the volatile oil cinnamaldehyde. It also marks the increase in the level of serum insulin, hepatic glycogen and high density lipoprotein in a dose dependent manner (Subash *et al*., 2007).

The ingestion of cinnamon decreases the total sugar level in plasma and improves the insulin sensitivity. The oral administration of the chief component cinnamaldehyde results in the significant reduction in serum glucose level, total cholesterol and triglycerides level. The aqueous extract of cinnamon is revealed to be a potent antidiabetic agent by up regulating the uncoupling protein-1 (UCP-1) and by enhancing the translocation of GLUT4 in the muscles and adipose tissues (Shen *et al*., 2010). It helps in reducing the gastric emptying and decreases the postprandial glycemic responses (Goel *et al*., 2012).

Azadirachta indica

It is the member of family Meliaceae. *A. indica* has been employed for long time in the traditional medicine for treatment of several ailments along with diabetes. Presence of characteristic high fiber content in its leaves is potent for the management of diabetes and for controlling the post-prandial hyperglycemia via delaying the gastric emptying and increasing the viscosity of the gastro-intestinal tract content. This phenomenon leads to the suppression of digestion and absorption of carbohydrate with no risk of hypoglycemia and unexpected weight gain (Atangwho *et al*., 2009). The leaves, stem, bark and seeds all are medicinally very important and all possess hypoglycemic activity by increasing the insulin secretion from the pancreatic beta cells (Tripathi *et al*., 2011).

Eugenia jambolana

It is commonly called as black plum or jamun. It is member of the family Myrtaceae. Another name for this plant is *Syzygium cumini* and this plant has been widely used over centuries for the treatment of diabetes in the traditional medicine practices. Enhancement of insulinemia, was observed resulting from the oral administration of the fruit pulp extract, through stimulating the insulin secretion and suppression of insulinase activity in liver and kidney (Grover *et al*., 2002). The seed extract of this plantwhich is flavonoid rich has shown a potent antidiabetic activity manifested by the reduction in fasting and peak blood glucose level. The mechanism of action deduced is by the upregulation of both PPARα and PPARγ and also by its ability to differentiate 3T3-L1 preadiopocytes (Sharma *et al*., 2008).

The extract of seed kernel was effective by inhibiting the function of α-glucosidase (Shinde *et al*., 2008).When alcohol extract of dried seeds was administered orally then it led to hypoglycemia and decreased glycosuria and partially restored the glycogen content as wells as hexokinase and glucose-6-phosphatse, phosphofructokinase and glucokinase enzyme of altered hepatic and skeletal muscle (Kumar *et al*., 2011).

Momordica charantia

It is a very well-known member of the family Cucurbitaceae which is commonly called as bitter melon. It is one of widely used plant in the folk medicine therapies for treatment of diabetes. The oral administration of the fruit juice or the seed powder is found to cause potent decline in the level of FBG and cause amelioration of glucose tolerance that exerts both insulin secretagogue and insulinomimetic activities (Raman, 1996). The potent antidiabetic activity of this plant is due to the presence of polypeptide similar to insulin called as pol-

ypeptide-P, and it is similar in structure to bovine insulin that reduces plasma sugar levels, into type I diabetic patients, when injected subcutaneously. Other hypoglycemic agents in the plant include sterol glucoside mixture charantin isolated from the fruit and the pyrimifine nucleoside vicine also abundant in the seeds (Raman, 1996). The polypeptide-P appears to inhibit the gluconeogenesis process. In addition to this it also improves the tolerance of glucose in type II diabetic patients (Grover *et al*., 2002; Goel *et al*., 2012).

Psidium guajava

This plant belongs to the family Myrtaceae and is commonly called as Guava. It is a reservoir of vitamins including B1, B2, B6, C, free sugars like glucose, fructose and sucrose and carotene. The aqueous extract of leaf, when administered orally as well as intraperitoneally, showed a beneficial effect on the blood glucose level in the hyperglycemic rat induced by alloxan. Beside this it also carried out potent effect on the body weight, glucose and ketone level of urine and pancreatic tissue which indicated its effect on inhibiting the tyrosine phosphatase 1B protein (Oh, *et al*., 2005). Ethanol extract of the bark of the plant have shown hypoglycemic effect by stimulating the insulin release from the pancreatic β-cells (Mukhtar *et al*., 2006). The potent constituents of this plant, includes the flavonoid glycoside exemplified by pedunculagin, isostrictinin and strictinin, widely used for the clinical treatment of diabetes to improve the insulin sensitivity (Goel *et al*., 2012).

Ocium sanctum

A member of family Labiateae is commonly known as Holy basil or Tulsi. The plant is administered as a potent medicinal agent for traditional treatment of several diseases and due to its lots of medicinal properties it is considered sacred in India. Alcohol extract of *O. sanctum* reduces glycemia and enhances the action of insulin exogenously. The presence of eugenol, the chief active constituent, is considered to be the potential reason behind its antidiabetic activity. It helps in reducing elevated serum sugar, cholesterol triglycerides levels along with lactate dehydrogenase, alanine transaminase, aspartate transaminase and alkaline phosphatase enzyme (Prakash and Gupta, 2005). Reduction in the level of FBG after 1 month administration of leaf powder was found in the diabetic rats (Tripathi *et al*., 2011).

Trigonella foenumgraecum

It is member of family Fabaceae and is commonly called as fenugreek seeds. The deflated seeds administration caused decreased fasting and posprandial blood levels of glucagon, glucose, insulin, somatostatin, total cholesterol and caused increment in the level of HDL-cholesterol levels. The chemical analysis of the seed fiber led to the conclusion that the major constituent called as galactomannan attributes to the antidiabetic activity of the seeds (Basch *et al*., 2003). The mechanism of action of the fenugreek seeds may include the enhancing of insulin synthesis and its release from the pancreatic cells. Intake of the seeds decreases absorption rate of sugar delays the gastric emptying and inhibits the blood glucose levels after meals. It also causes the stimulation of insulin receptors to burn the glucose at high-fiber diet. In case of type 1diabetes the mode of action included the reversion of lipid and glucose metabolizing enzyme activity to normal levels, and thus stabilizes the glucose homeostasis in liver and kidney (Dey *et al*., 2003). The seeds are have prominent importance due to the presence of mucilage, proteins, proteinase inhibitors, steroids saponins and saponins-peptide esters, steorls, flavonoids, nicotinic acid, coumarin, trigonelline and volatile oils (Bnouham *et al*., 2006).

Hibiscus rosa-sinesis

It is a flowering tree distributed throughout the India and is commonly known as Gudhal. Evaluation of the plant extract was carried out on both acute and subacute animal models. For the study ethanolic extract of the flower was administered to the rats at 250mg/kg and 500mg/kg doses. The administration showed a significant reduction in the blood glucose level in the animal models. The duration of reduction varied from 1, 3, 5, hours to 1, 3, 5, 7 days in acute and subacute models respectively. The result evaluated the effective applicability of the ethanol extract of the flower for the treatment of both acute and chronic diabetes (Venkatesh *et al*., 2008).

Holarrhena antidysenterica

An indigenous plant distributed throughout the Indian subcontinent and commonly called as kurchi. The seeds of the plant were evaluated for the hypoglycemic activity in the albino rat models. The study work comprises the normal and diabetic rats both treated with 1.5ml/kg normal saline and 350mg seed extract/kg. After 7 days of the extract administration the glucose level was found to be reduced and it was significant in both preprandial and postprandial level of glucose. Level of glucose also reduced in case of the normal rats. The glucose level decrement was 142.5±1.82 and 182.5±5.88 in case of fasting and feeding state respectively. Along with the hypoglycemic effect the extract treatment also improved the lipid profile of the diabetic rats while no anti-hyperlipidemic effect was observed in the normal rats. Long duration treatment with the extract also revealed some anti-hypercholesterolemic activity around 14 days of treatment, followed by effective reduction in the level of

urea nitrogen in blood after 28 days of treatment (Pankaj *et al*., 2006).

Argemone Mexicana L.

It is a member of family Papaveraceae and is commonly called as prickly poppy or Pili or Kateli and this herb is indigenously being used for medicinal purpose in several parts of Rajasthan. The plant is rich in several bioactive agent that is alkaloids including berberine, protopine, sarguinarine, optisine, chelerythrine etc. this plant is genuily very important for the primary health care as it is good source of both traditional and modern medicines. The ethanolic and aqueous extract of the whole plant have been administered on the diabetic rats induced by alloxan, and the extract have shown a good hypoglycemic effect by the single dose and multi dose treatment (Nayak *et al*., 2011).

Catharanthus roseus

A member of the family Apocynaceae and locally termed as Rose periwinkle is a plant with several medicinal activity. It is widely used in the herbal preparation of the diabetes. A major phyto-constituent of the plant is Catharanthine that shows the antidiabetic activity. This plant is a good reservoir of the phytochemical like alkaloids (Jean *et al*., 1999), flavonoids (Gilles *et al*., 1999), and steroids (Akirai, 1999). This plant is also reported to acquire many properties like anti-cancerous activity (Jean *et al*., 1999), antioxidant activity (Jaleel *et al*., 2000), beside its antidiabetic properties (Sumana *et al*., 2001), it possess hypolipidemic activity (Antia and Okokon, 2005) also.

Ficus religiosa

A member of Moraceae and coomonly known as Peepal and is considered sacred in India. This plant has been reported to be used in the traditional medicine preparation for diabetis treatment for long time (Simmonds and Howes, 2006). The bark of the tree is used to prepare the decoction which is used for treatment of diabetes. This plant contains several bioactive agents due to which carries out several active function. The major bioactive agents include tannins, polyphenolic compounds, flavonoids, saponins, and sterols. The bioactive agents that impart the hypoglycemic activity are leucocyandin 3-O-beta-d-galactosyl cellobioside, leucopelargonidin-3-O-alpha-L rhamnoside (Bonuham *et al*., 2006; Avodhya *et al*., 2010). The leaves of the plant are used in the anti-hyperglycemic activity study (Deshmukh *et al*., 2007). This plant has shown to provide a wide spectrum in vitro and in vivo activity. These activities includes antidiabetic, antitumor, antiulcer, antianxiety, hypolipidemic, anti-inflammatory, estrogenic, anti-asthmatic, apoptosis inducer, analgesic, cognitive enhancer and anti-hypertensive (Singh *et al*., 2011). Aqueous extract of the plant when administered orally showed significant result by lowering blood glucose level and elevated the level of insulin. *F. religiosa* is found to modulate the enzymes of the antioxidant defense system hence helps to combat the problem of oxidative stress.

Morus alba

It is a short lived plant belonging to the family Moraceae and locally known as white mulberry. The constituent of the plant with hypoglycemic effects are Moracin M, steppogenin-4'-O-β-D-glucoside and mulberroside A and they were isolated form the root bark plant (Zhang *et al*., 2009). The ethanol extract of leaves when administered has shown the antihyperglycemic, antioxidant and antiglycation effect in the rats suffering from chronic diabetes (Naowaboot *et al*., 2009). Freeze dried powder of the mulberry was also analyzed for the hypolipidemic activity as well (Yang *et al*., 2010). Mulberroside A is a glycosylated stilbenoid and it is found to be useful in treatment of the hyperuricemia and gout disease (Kim *et al*., 2010; Wang *et al*., 2011).

The disease Diabetes is spreading at an alarming rate throughout the world and a large population of world is affected which in tur is considered as a major cause of the high economic loss which can in turn impede the development of the nation. The uncontrolled diabetes leads to many other complications as well. For this, therapies developed along the principles of western medicine (allopathic) are often limited in efficacy, carry the risk of adverse effects, and are often too costly, especially for the developing world. Therefore the treatment of the disease with the plant derived materials which are easily accessible to most people and which do not require the laborious pharmaceuticals synthesis seems more approaching and highly attractive method.

The current study has focused on several common plants and herbs, which are easily available, for their potent antidiabetic properties along with their mode of action and some other accessory effects too. The study has revealed the importance of such common plants and herbs in effectively controlling the disease through several practical analyses. These plants have been used for many other purposes as well hence provide many other useful applications other than being antidiabetic agents. Due to their potential properties there is needed to look forward for the conservation and guidance for the effective use of these plants in daily life. According to Ayurveda there are a lot more plants and herbs available contributing to the huge collection of antidiabetic plants in the world. Not only is this there are several plants with many other important properties, nature full of potent plants with many medicinal properties against

many more disease. These plants also contribute in overcoming the complications of diabetes.

The future studies could focus on isolation, purification, characterization and analysis of such important phytochemical compounds from the plants and to study their potential in other disease treatments as well. The future studies could focus on exploring some other easily available plant and herbs with potential of treating diabetes in order to enhance the usage of plant products among world populations which indirectly will confer to conserve these important natural gifts. The further studies could focus on analyzing the individual importance and role of each phytochemical compound present such medicinal plants.

CONCLUSION

Since the time immemorial the medicinal plants are utilized widely for maintenance of the human well beings. As per Ayurveda the Mother Nature has blessed us with a huge collection of the medicinal plants with potent activities. The natural resources are still considered as the best source for the drug development programs. The medicinal plants provide us with the mine of bioactive agents that play crucial role in maintaining the human health in various aspects. The nature has provided us with lots of medicinal plants carrying out the antidiabetic activity. With the proper understanding and proper use these plants can be used as an effective resource for the treatment of this world wide disease, Diabetes. In today's generation the data on the study of the biological activity and bioactive compounds of plant is tremendously increasing. There is a need to grab the information and exploit it in a right way to obtain a better solution of the health issue. The outcome of these studies and research will help to provide the starting point for the development of the herbal drugs. The herbal drugs have gain popularity in modern time due to their effectiveness without any side effects. The plants are a rich source of variety of phytochemical that confers the disease management properties in them. The major effort should to be kept in minimizing the time for screening the plant for its antidiabetic properties and as wells as for isolation of bioactive compounds for natural drug development. If the direction of exploration is kept appropriate then these plants can act as the infinite source of the active components that can cure the disease and disorders.

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Pharmaceutical Communication

Biosci. Biotech. Res. Comm. 12(2): 408-418 (2019)

BBRC Bioscience Biotechnology Research Communications

Experimental design and characterization of nanoemulsion based topical herbal gel developed for site-specific activity of *Glycyrrhiza glabra* extract: *In vitro and Ex-vivo* studies

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ABSTRACT

Glycyrrhiza glabra contains the triterpenoid saponins which are hydrophilic therefore it is essential to provide lipid environment or these molecules should be micro or nanosized so easily diffuse within the aqueous regions near the outer surface of intracellular keratin filaments. The purpose of this study was to enhance the potential of *Glycyrrhiza glabra extract* (GGE) by preparing nanoemulsion formulation for transdermal application. Nanoemulsion system was developed with Tween 80 as surfactants and IPA as co-solvent and iso propyl myristate as oil for transdermal delivery of *Glycyrrhiza glabra* extract. Region of nanoemulsion was found in the pseudo-ternary phase diagrams developed at various Tween 80 and IPA ratios. The optimal nanoemulsion formulation consisted ofwater (14.28%), Tween 80 (38.10%), IPA (19.04%)and IPM (28.57%). *In vitro* and *Ex vivo* diffusion study shows that absorption of *Glycyrrhiza glabra extract* /(MAG) was found to be fairly rapid, as compared to aqueous solution of extract.Nanoemulsion in corporate gel (NEIG) was prepared by using Carbopol 93 4 as a gelling agent. *In vitro* diffusion study showed that there was increase in permeation of GG extract from NEIG; also improve bioavailability of the drug. Our findings suggest that such novel w/o nanoemulsion exhibited significant antimicrobial property and NEIG useful as anti-inflammatory gel and possible alternative to traditional topical formulations with bioavailability issues.

KEY WORDS: NANOEMULSION, TRANSDERMAL DRUG DELIVERY, NANOEMULSION GEL, *GLYCYRRHIZA GLABRA* EXTRACT

ARTICLE INFORMATION:

Corresponding Author: aniltatiya12171@gmail.com
Received 4th April, 2019
Accepted after revision 28th May, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/25

INTRODUCTION

Over the ancient times, natural origin compounds and their formulations recognized its biological activities and health benefits. Most of the biologically active components of plants are polar like tannins, flavonoids,terpenoids etc. on the other hand, they are weakly absorbed due to their large molecular size which cannot absorb by passive diffusion and due to their pitiable lipid solubility resulting their pitiable bioavailability (Krishna and Gejjalagere 2018). Nanoemulsion has the ability to solubilize hydrophobic as well as hydrophilic drugs in their nanostructure. As nanoemulsion contain surfactants in it's composition, the application on the skin surface usually produces an increase in the membrane permeability smooth the progress of transdermal transport (Seema 2014, Chang 2013). A Nanoemulsion is a thermodynamically or kinetically stable liquid formulation. It is a dispersion of an oil phase and a water phase, in combination with a surfactant. The dispersed phase usually consist of small particles or droplets, with a size range of 5 nm-200 nm (Devarajan 2011). The o/w nanoemulsion is used for improving delivery of hydrophobic drugs, whereas w/o nanoemulsion preferred for incorporating hydrophilic drugs (Yen, et.al., 2018).

Nanoemulsion is of great interest as pharmaceutical, drugs, nutraceuticals, and food products & cosmetics formulation. They are used for administration through various routes like parentral, oral, topical, ocular, pulmonary, mucosal, cosmetic, transdermal, controlled and target (Rachmawati et al., 2015 Eid et al., 2013). Nanoemulsions have improved transdermal permeation of many drugs over the conventional topical formulations such as emulsions and gel (Ghosh et al., 2013, Zhou et al., 2010, Sonneville-Aubrun 2018).

Glycyrrhiza glabra contains the triterpenoid saponins which are hydrophilic so it is essential to provide lipid environment or these molecules should be micro or nanosized that can easily diffuse within the aqueous regions near the outer surface of intracellular keratin filaments. The main objective of this study is to formulate nanoemulsion of *Glycyrrhiza glabra* extract which may increase the permeation through skin and its anti-inflammatory potential.

MATERIALS AND METHODS

Glycyrrhiza glabra extract was received as a gift sample from Amsar Pvt. Ltd. Indore India. Tween 80, IPA, isopropyl myristate and methanol were procured from LobaChemie, Mumbai, India. All other reagents used were of analytical grade. Solubility of extract and screening of oils and surfactants for nanoemulsion:The equilibrium solubility study was performed by adding an excess amount of *Glycyrrhiza glabra* extract in 2 mL of various oils (soybean oil, sunflower oil, coconut oil, olive oil, iso propyl myristate), surfactants (Tween 80, Tween 20, span 80, SLS, Transcutol) and cosurfactants (IPA, ethanol, n-amyl alcohol) in 5 mL capacity vials each separately vortexed using a Cyclo mixer [CM 101, REMI (INDIA)]. The solubility of *Glycyrrhiza glabra* extract in various oils and surfactants was determined using the following method. Briefly, an excess amount (approximately 200 mg) of *Glycyrrhizaglabra* was placed in a 2 mL microtube containing 1 mL of each oil. Then, the vortexed mixture was and kept for 3 days at 37° C in a shaking water bath to facilitate the solubilization. This mixture was filtered using membrane filter (Nylon, 0.45 µm, Gelman, USA). The resulting nanoemulsion were tightly sealed and stored at ambient temperature and physical stability was measured by observing periodically for the occurrence of phase separation. On basis of clarity and transparency of resultant system suitable oil, surfactant and co surfactant were used in the preparation (Rachmawati et al., 2015 Eid et al., 2013).

Construction of pseudo-ternary phase diagrams: In order to find out the concentration range of components for nanoemulsion, pseudo-ternary phase diagrams were constructed using oil titration method at ambient temperature (25 ^{0}C). On the basis of the solubility studies, IPM was selected as the oil phase. Tween 80 and TPA were selected as surfactant and co surfactant, respectively. Distilled water was used as an aqueous phase. Surfactant and co surfactant (S*mix*) were mixed at different mass ratios such as 9:1,8:2,7:3,6:4,5:5,4:6,3:7,2:8,1:9.These ratios were chosen in decreasing concentration of surfactant with respect to co surfactant for a detailed study of the phase diagrams. For each phase diagram, oil and Smix at a specific ratio was mixed thoroughly at different mass ratios from 1:1 to 2:1 in different glass vials. Different combinations of oil and Smix were made so that maximum ratios were covered for the study to define the limits of phases precisely formed in the phase diagrams. Pseudo ternary phase diagrams of oil, S*mix* and aqueous phase were developed using the aqueous titration method. Slow titration with aqueous phase was performed for each mass ratio of oil and S*mix* and visual observations were made for transparent and easily flow able o/w nanoemulsion. For convenience, the phase diagrams were constructed by drawing "water dilution lines" representing increasing water content and decreasing surfactant-co surfactant levels. The water was titrated along dilution lines drawn from the surfactant-co surfactant to the opposite oil side of the triangle. The line was arbitrarily denoted as the value of the line intersection with the oil scale (eg, 20:80, 30:70). If turbidity appeared followed by a phase separation, the samples were considered to be biphasic. If clear and

transparent mixtures were visualized after stirring, the samples were considered monophasic. The samples were marked as points in the phase diagram. The area covered by these points was considered to be the nanoemulsion region of existence (Rachmawati et al., 2015 Eid et al., 2013, Ghosh et al., 2013).

Formulation development of herbal nanoemulsion: Phase diagram demonstrate the surfactant /co surfactant in ratio 2:1 suitable for nanoemulsion formulation The liquid nanoemulsion was prepared by dissolving extract in double distilled water (0.5-1 %) to the resultant solution added drop wise the mixture of Tween 80 (38.10%), IPA (19.04%) and then IPM (28.57%) with continuous stirring on vortex mixer. No heat is supplied during formulation. The resulting nanoemulsion was tightly sealed and stored at ambient temperature and their physical stability was measured by observing periodically for the occurrence of phase separation; nanoemulsion was also subjected for characterization. Characterization of Nanoemulsion: Dilution test -Small amount of nanoemulsion was placed on a clean glass slide. A drop of water added to the nanoemulsion and was mixed with the help of glass rod and their transparency was assessed visually. If the emulsion is o/w type and it's diluted with water, it will remain stable as water is the dispersion medium; but if it is diluted with oil, the emulsion will break as oil and water are not miscible with each other, (Shivhare et al., 2009).

Dye test: Add oil soluble dye such as sudan red III in prepare a nanoemulsion with oil and surfactant Observed a drop of nanoemulsion under microscope and under motic microscope supported with Motic images version 2. Effect of dilution was observed visually and the pH of nanoemulsionmeasured directly on digital pH meter. Droplet Particle size and Polydispersity: Droplet Particle size was measured by dynamic light scattering with zetasize ZS-90 (Malvern instruments ltd., Malvern, U.K) at Temperature 25^{0}C, for 30 second duration.Data analysis was conducted using a software package (ELS-8000 software) provided by the manufacturer. Polydispersity index is the ratio of standard deviation to mean droplet size. Polydispersity index (PI) is a measure of particle homogeneity and it varies from zero to 1.0. Zeta potential: Zeta potential of nanoemulsion was determined using zetasizer ZS-90 (Malvern instrument ltd., UK). Samples were placed in clear disposable zeta cells and results were recorded. Aliquots of nanoemulsion were sampled in disposable zeta cells and zeta potentials were determined on the basis of electrophoretic mobility under an applied electrical field.

Viscosity: Low viscosity is required to make them good in appearance and easy to handle and packed. Also provide good spray ability. Determine by using Brookfield Viscometer at single mode (spindle C-15) (Stoughton, A) at 100 rpm each sample evaluated in triplicate at a temperature of 25^{0}C, and results were presented as average ± standard deviation. Stability Study: The physical stability of the nanoemulsion must be determined under different storage conditions (4, 25 and 40 °C) during study cycle. Fresh preparations that have been kept under various stress conditions for extended period of time are subjected to droplet size distribution analysis and observed for any evidences of phase separation, flocculation or precipitation. Effect of surfactant and their concentration on size of droplet is also being studied. In order to estimate metastable systems, the optimized nanoemulsion formulation was diluted with purified distilled water and was centrifuged (Remi laboratories, Mumbai, India) at 3500 rpm for 30 minute at room temperature and observed for any change in homogenecity of nanoemulsion.

Drug content of nanoemulsion: The drug content of optimized formulation was determined by UV spectrophotometer. 10 mg equivalent of extract containing nanoemulsion was dissolved in 10 ml of methanol. The concentration of solution was found to be 100µg/ml. The Mono amino glycerrhizinate content was estimated at 248nm using UV-Visible spectrophotometer UV-1700. *In vitro* diffusion studies: *In vitro* diffusion of nanoemulsion and aqueous solution of extract was carried out by Franz diffusion cell having 3.0 cm diameter and 25 ml capacity. Dialysis membrane (Hi-media) having molecular weight cut off range 12000 – 14000 kDa was used as diffusion membrane. Dialysis membrane was soaked in phosphate buffer pH 5.8 for 24 hrs prior to experiment. Diffusion cell was filled with phosphate buffer pH 5.8 and dialysis membrane was mounted on cell. The temperature was maintained at 32.5^{o}C. After a pre-incubation time of 20 minutes, the nanoemulsionand aqueous solution containing extract equivalent to 10 mg were placed in the donor chamber. Samples were periodically withdrawn from the receptor compartment for 6 hours and replaced with the same amount of fresh phosphate buffer solution, and assayed by a spectrophotometer at 248 nm.

Development of Nanoemulsion based gel :Gel base was prepared by dispersing the 1 g of the Carbopol 934 in a sufficient quantity of distilled water (100ml) after complete dispersion.Carbopol 934 solution was kept for 24 hours till complete swelling. Then the *Glycyrrhiza glabra* extract loaded nanoemulsion was slowly added to the above prepared gel baseunder magnetic stirring for the development of nanoemulsion based gel (Modi JD, Patel JK 2011)

Physicochemical Evaluation of gel: Gel was tested for homogenecity by visual inspection after the gel has been set in container they were tested for their appearance of

any aggregates. The pH of various gel formulations was determined by using digital pH meter. The measurement of pH of each formulation was done in triplicate and average values are calculated. Viscosity of the prepared gel was measured using Brookfield viscometer at single mode (spindle C-15) (Stoughton, A) at 100 rpm each sample evaluated in triplicate at a temperature of 25^{0}C, and results were presented as average ± standard deviation.One of the criteria for a gel to meet the ideal qualities is that it should possess good spread facility. It is the term expressed to denote the extent of area to which gel yarely spreads on application to skin or affected part. The therapeutic efficacy of a formulation additionally depends upon its spreading value. The spredability of gel was tenacious utilizing the following method: Gel (0.5 g) was placed within circle of 1 cm diameter remarked on a glass plate over which a second glass plate was placed. A weight of 500g was sanctioned reposing on the upper glass plate for 5 min. The incrementation in diameter due to spreading of gel was noted (Karri et al., 2015).

Extrudability study: The formulations were filled in the collapsible tubes after the gels were set in the container. The extrudability of the formulation was determined in terms of weight in grams required to extrude a 0.5 cm. ribbon of gel in 10 second. *In vitro* drug release: *In-vitro* diffusion study of nanoemulsion incorporated gel (NEIG) and extract in gel base was carried out by Franz diffusion cell having 3.0 cm diameter and 25 ml capacity. Dialysis membrane (Hi-media) having molecular weight cut off range 12000 – 14000 kDa was used as diffusion membrane. Dialysis membrane was soaked in phosphate buffer (PB) pH 5.8 for 24 hrs prior to experiment. Diffusion cell was filled with phosphate buffer pH 5.8 and dialysis membrane was mounted on cell. The temperature was maintained at 37^{o}C. After a pre-incubation time of 20 minutes, the NEIG and extract gel containing extract equivalent to 10 mg were placed in the donor chamber. Samples were periodically withdrawn from the receptor compartment for 4-6 hours and replaced with the same amount of fresh phosphate buffer solution, and assayed by a spectrophotometer at 248 nm.

Ex Vivo Skin Permeation Studies: The hairs of rat abdominal region were clipped with animal clippers. Full thickness dorsal skin was carefully excised and subcutaneous fat was removed with a dull scalpel then soaked in phosphate buffer pH 5.8. Diffusion cell was filled with phosphate buffer pH 5.8. The skin samples were mounted diffusion cell with stratum corneum side up. Diffusion cell was filled with phosphate buffer pH 5.8 and dialysis membrane was mounted on cell. The temperature was maintained at 32.5^{o}C. After a pre-incubation time of 20 minutes, the NEIG and extract in gel base equivalent to 10 mg were placed in the donor chamber. Samples were periodically withdrawn from the receptor compartment for 4-6 hours and replaced with the same amount of fresh phosphate buffer solution, and assayed by a spectrophotometer at 248 nm.

Skin Irritation studies: The skin irritation study for nanoemulsion based gel was carried out on Swiss albino mice weighing 25-35 g. The experiments were carried as protocol approved from Institutional Animal ethical committee. The animals were kept under standard laboratory conditions and housed in polypropylene cages. The animals were divided into three groups. Group I was taken as negative control received normal saline and Group II served as positive control received formalin solution and Group III applied with nanoemulsion based gel formulations. A single dose of the NEIG was applied to right ear of the mice keeping left as control in Group III. In group II formalin solution was applied; development of erythema or skin irritation was visualized after a total period of 21 days (Sonia K et al., 2011)

In-Vivo Pharmacodynamics Study Using Carrageenan-Induced Rat Paw Edema

Anti-inflammatory activity: It was evaluated on the basis of the inhibition of the carrageenan-induced hind paw edema.The rats of either sex (180-200) were divided into four groups each group containing six animals .The rats were fasted for 12 hrs prior to induction of edema however water was provided. To ensure uniform hydration, the rats receives 5ml of water by stomach tube. Thirty minutes later acute inflammation was induced by sub-planter injection of 0.1 ml of freshly prepared 1 % suspension of carrageenan in normal saline in left hind paw of the rats The NEIG formulation (0.25g) or base was applied topically with gentle rubbing to the paw of each rat of test group one hour before and one hour after the carrageenan challenge. Rats of the control groups received only the gel base and standard marketed formulation diclofenac gel BP applied in the same way as a reference standard. The paw volume was measured at 0, 1, 2, 3, 4, 5 and 6 hour using the digital Plethysmometer (Ugo Basile, Italy). The percentage inhibition of paw volume in test groups was compared with the control group (Prakash PR etal., 2010., Meshram GG et al., 2016)

Percentage inhibition of edema = (1–Vt/Vc) × 100

Where, Vt is the inflammatory increase in paw volume in test groups and V*c* is the inflammatory increase in paw volume in normal control group of rats. Percentage inhibition of edema is proportional to anti-inflammatory activity.

Antimicrobial Study*:* Broth macro dilution Assay: Broth dilution is a technique in which a suspension of bacterium of a appropriate concentration is tested against varying concentrations of an antimicrobial

agent. As nanoemulsion o/w it will break if directly mixed with broth media which was prepared in water so the 1% and 2% nanoemulsion was dissolved in DMSO so that final concentration was 2.5mg/ ml and 5mg/ml the and *Glycyrrhiza glabra* extract 5mg/ml and 10 mg/ml was prepared in DMSO. A suspension of different bacterium and fungi was poured in above test tubes. The tubes with bacterial suspension were incubated at 37 ^{0}C for 24 hours and with fungal culture were incubated at 30^{0}C for 48 hours.After incubation observed for growth or turbidity (Das K et al., 2010).

MIC by Micro titer plate method

Different concentrations of nanoemulsion and extract were prepared in DMSO. An equal volume i.e 1µl of bacterial suspension/fugal suspension and 1µl each concentration was added to the wells initial absorbance was measured at 620nm and plates were incubated at 37°C for 24 h after incubation, plates are examined for changes in turbidity as an indicator of growth. Initial absorbance and absorbance after incubation were compared. The lowest concentration of drug that reduces, by more than 50% or Concentration with sharp decline in absorbance value is the MIC (Hasan et al., 2010, Selvamohan et al., 2012, Dasari et al.,2014).

RESULTS AND DISCUSSION

Development of nanoemulsion formulation depends on physicochemical properties of drugs. The solubility of the drug is most important factor as the ability of nanoemulsion to maintain the drug in solubilised form. Solubility of the lipophilic drugs in the oil phase and hydrophilic drugs in aqueous phase is an important criterion for the selection of oils and water respectively. GGE and MAG are hydrophilic in nature. The solubility of MAG in different oils was determined since solubility of MAG was higher in aqueous phase as compared to oil phase, w/o nanoemulsion was developed for transdermal delivery of MAG. In order to select suitable oils and surfactants for good solubilizing of herbal extract of *Glycyrrhizaglabra* in various oil and surfactant were determined.

Table 1. Formulation composition of Nanoemulsion Batch 1

Ingredients	Formulations composition (% w/v)				
	FA1	FB1	FC1	FD1	FE1
Distilled Water	5.5	5.5	25.0	14.28	5.0
Tween 80	25.00	25.00	38.89	38.10	30.0
IPA	25.00	25.00	19.44	19.04	15.0
IPM	44.44	44.44	16.66	28.57	50
Extract	0.0	0.5	0.5	0.5	0.5

Table 2. Formulation composition of Nanoemulsion Batch 2

Ingredients	Formulations composition (% w/v)				
	FA2	FB2	FC2	FD2	FE2
Distilled Water	5.0	5.0	5.0	14.28	14.28
Tween 80	30.0	30.0	30.0	38.10	38.10
IPA	15.0	15.0	15.0	19.04	19.04
IPM	50	50	50	28.57	28.57
Extract	0.25	0.50	0.75	1.0	0.50

Medium and long chain triglyceride oils with different degree of saturation have been tried in the development of nanoemulsion. Oils tried were soybean oil, sunflower oil, coconut oil, olive oil, iso propyl myristate (IPM) etc. Surfactants employed were Tween 80, Tween 20, span 80, SLS, Transcutol and co-surfactant was IPA, ethanol, n-amyl alcohol.

The solubility of *Glycyrrhiza glabra* was determined indifferent oils. It was evident that *Glycyrrhiza glabra* exhibited highest solubility in isopropyl myristate (195.71±0.91 mg 2 mL-1) as polarity of the poorly soluble drugs favors their solubilization in small/medium molar volume oils. Edible oils cannot depict large micro emulsion region due to their rancid nature. IPM was selected for the preparation of nanoemulsion due to its well-known permeation enhancing property and biocompatibility. The most critical problem related in the development of nanoemulsion based drug delivery systems is the toxicity of the surfactants. Large amounts of surfactants may cause skin irritation when administered transdermally. It is therefore important to determine the surfactant concentration properly and use the minimum concentration in the development of nanoemulsion formulation (Alam et al., 2010).

Amongst various surfactants screened, Tween 80 exhibited (96.8±1.47 mg 2 mL-1) highest solubilization capacity of GGE. Whereas various co-surfactants were screened for solubility as well miscibility with surfactant, Isopropyl alcohol IPA was revealed greater solubilization capacity (101.98±1.02 mg 2 mL–1) as well as it forms transparent system (99.87 %T).The screening of surfactant and co-surfactants on the basis of solubility is difficult because all surfactant and co surfactant cannot solubilise all type of oil phase. Surfactant chosen must be able to lower the interfacial tension to a very small value to aid the dispersion process during the preparation of the nanoemulsion, provide a flexible film that can readily deform around droplets. aqueous phase, the second representing oil and the third representing a mixture of surfactant and co surfactant at a fixed mass ratio.

Figures 1 represent the pseudo ternary phase diagrams for nanoemulsion systems along with the ratios of sur-

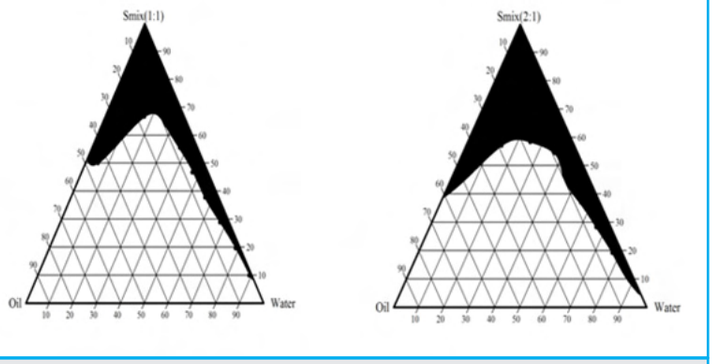

FIGURE 1. Pseudoternary phase diagram with S_{mix} (1:1) and (2:1) respectively

factant and co surfactant, as 1:1 and 2:1. The change in the area of nanoemulsion region can be very well observed in the ternary phase diagram as the ratio of surfactant to co surfactant was changed from 1:1 to 2:1. When ratio of surfactant to co surfactant was 2:1, there was increased in nanoemulsion region, because of high concentration of surfactant. Nanoemulsions are considered to be thermodynamically stable systems that are formed at a particular concentration of oil,surfactant, and water, with no phase separation, creaming, or cracking. Selected formulations from phase diagram were subjected to different stress stability testing like heating cooling cycle, centrifugation, and freeze-thaw cycle. During physical stability testing, some formulations became turbid and in some phase separation occurred. One reason of this instability in nanoemulsions may be due to the Ostwald ripening. in which molecules move as a monomer and coalescence of small droplets takes place, resulting in the formation of large droplets by diffusion processes driven by the gain in surface free energy. The other reason may be that when temperature quench occurs during stress stability study, instability of nanoemulsion occurs due to separation of oil phase and droplet distribution of smaller size is favoured by the change in curvature free energy. Only those formulations, which showed no phase separation, creaming, cracking, coalescence, and phase inversion during stress stability tests, were selected for further studies (Osanloo et al., 2018).

Novel nanoemulsion of *Glycyrrhiza glabra* extract was prepared by the spontaneous emulsification method (oil phase titration method), Isopropyl myristate was used as oil phase components. Optimized nanoemulsion consists of water (14.28%), Tween 80 (38.10%), Isopropyl alcohol (19.04%) and Isopropyl myristate (28.57%).Nanoemulsion developed was clear and transparent. From dilution and dye test it can be concluded that the system was w/o type with pH 5.59±0.01, 5.78± 0.005

As drug loading increased 0.5,1% the particle size of optimized nanoemulsion was varies, 104.2 nm 185.3 nm but almost it is in range. It has been reported that the smaller particle size of the emulsion droplets may lead to more rapid absorption and improve the bioavailability Polydispersity is the ratio of standard deviation to mean droplet size, so it indicates the uniformity of dispersity. Polydispersity index (PDI) is a measure of particle homogenicity and it varies from 0.0 to 1.0. PDI 0.186 - 0.451 found it closer to zero so the more homogenous are the particles. Polydispersity signifies the uniformity of droplet size within the formulation. The polydispersity value of the formulations was very low (<0.4) which indicated uniformity of droplet size within the formulation. The more negative zeta potential, greater the net charge of droplets and more stable the emulsion. Zeta potential values lower than -30 mv generally indicate a high degree of physical stability the zeta potential found -9.5 to -13.8.It shows little change with increase in con-

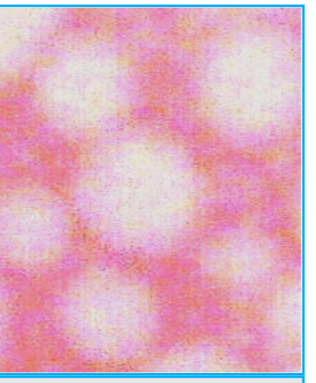

FIGURE 2. Photomicrographof W/O type of nanoemulsion.

Table 3. The influence of mixture components on characterization of prepared nanoemulsion Batch -1

Parameters	FA1		FB1		FC1		FD1		FE1	
	Initial	After 1 month	Initial	After 1 month	Initial	After1 month	Initial	After 1 month	Initial	After 1month
Zeta size	353.4	Unstable	247.1	Unstable	203.2	Unstable	245.4	206.3	236.5	183
PDI	0.568		0.339		0.26		0.249	0.384	0.249	0.204
Zeta potential	-44.7		-37.5		-44.3		-34	-16	-34.4	-27.4

Table 4. The influence of mixture components on characterization of prepared nanoemulsion batch-2

Parameters	FA2	FB2	FC2	FD2	FE2
Zeta size	208	175.1	183.5	185.1	104.2
PDI	0.202	0.198	0.234	0.451	0.186
Zeta potential	-35.3	-33.6	-19.2	-13.8	-9.5

centration of extract but all the values are in range and the nanoemulsion was found to be stable on thermodynamic stability study. The viscosity of nanoemulsion at room temperature was 67.43± 0.15cP. Generally, it was observed that the viscosity of the nanoemulsion formulations was very low. Lower viscosity is one of the characteristics of nanoemulsion formulations (Gurpreet & Singh 2018.) The drug content was found to be 97.89% (Table 3, 4)

Evaluation of prepared nanoemulsion was carried out for its physicochemical parameters. It was found that w/o nanoemulsion was thermodynamically stable and no or little effect of drug concentration on it. The formulation is safe for topical application.

In vitro diffusion through the dialysis membrane of formulation shown in Table 5, Figure 3.

After 3.5 hours drug release from the nanoemulsionwas 95.30% while drug release from aqueous solution of extract was 33.17% which is much less as compared to NE. It was observed that rate of diffusion was improved for nanoemulsion attributed to their nano range size. The significant difference in permeation between nanoemulsion formulations and aqueous solution could be due to the mean size of droplets.

Ex vivo permeation through rat skin of formulation shown as

Ex vivo permeation study results shows that drug permeation through skin in 210 min (3.5 hr) is 92.26% and through aqueous solution of extract it was only 33.61%. This proved that the nanoemulsion enhance the ability of drug to permeation through the skin.

Nano emulsion incorporated gel found to be homogeneous with pH5.65±0.02 visosity 118.66±0.30 and spreadabilty 5.36±*0.11 with good* extrudability property. Physical parameters of gel evaluation are given in Table 7.

Skin irritation studies: It was observed that nanoemulsionincorporatedgel (NEIG) application on Swiss albino mice no signs of erythema and skin irritation even on applicationfor 21 days. Thus, the developed formulation is non-sensitizing and safe for use. *In vitro* diffusion through the dialysis membrane of formulation seen in Table 8, Figure 5.

After 3.5 hours drug release from the NEIG 98.19% while drug release from extract in gel base is 36.54% which is much less as compared to NEIG. It was observed that there was increase permeation of GG extract from NEIG.It was observed that the when nanoemulsion incorporated in Carbopol gel it will enhance its permeation than nanoemulsion as such.

The nanoemulsion shows the positive susceptibility test for all the six bacterial and two fungal cultures so

Table 5. *In Vitro* Drug Diffusion study of Nanoemulsion and Extract

Time (Minutes)	% Cumulative Drug Release (Mean ± SD) of Nanoemulsion	% Cumulative Drug Release (Mean ± SD) of Extract
15	17.30 ± 0.41	8.083±0.60
30	21.57 ±0.62	10.85±0.98
60	26.22 ±1.42	13.35±0.61
90	38.64 ±1.20	16.75±0.18
120	54.66 ±0.72	20.00±0.62
150	68.80 ±1.09	24.18±0.23
180	82.58 ±0.60	28.56±2.28
210	95.30 ±0.97	33.17±1.25

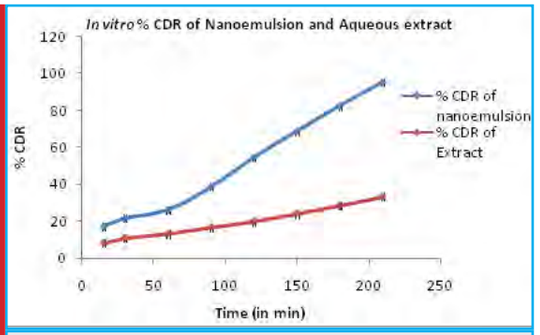

FIGURE 3. *In vitro* % CDR of Nanoemulsion and Aqueous solution of extract.

subjected for MIC determination. MIC of nanoemulsion and extract given in Table 9.

As the MIC of Nanoemulsion was less than Extract for all the bacterial and fungal cultures we can say that nanosized droplets have more energy and surfactant to destabilize the targeted microbes. The nanoemulsion particles are thermodynamically driven to fuse with lipid-containing organisms. When sufficient nanoparticles fuse with the pathogens, they release part of the energy trapped within the emulsion. Both the active ingredient and the energy released destabilize the pathogen lipid membrane, resulting in cell lysis and death.

The results of anti-inflammatory activity i.e. increase in paw volume at different time interval and % inhibition after topical administration of Diclofenac gel, NEIG, Extract in gel are given in Table 10 and Figure 6

All the values expressed mean±S.E.M. (percent inhibition) n=6. Data was analyzed byone way ANOVA followedby Dunnet test ** *P<0.05* ,*** *P< 0.001*

Statistical analysis showed that the topical preparation has significant inhibition of carrageenan induced rat paw edema when compared with control group. It was also observed that the % inhibition of edema by NEIG was greater than standard diclofenac gel and Extract in gel base. Glycyrrhizaglabra extract has anti-inflammatory activity so it reduces the paw edema but when we use NEIG activity increases two fold which could be due to enhanced permeation of MAG through skinSo it was concluded that the nanosize of extract in NEIG responsible for better absorption so as to increase its bioavailability and therapeutic effect (Mahboobian 2017, Rai et al., 2018).

Table 6. *Ex-vivo* Drug permeation study data

Time (Minutes)	% Cumulative Drug Release (Mean ± SD) of Nanoemulsion	% Cumulative Drug Release (Mean ± SD) of Extract
15	16.77±0.12	9.25±0.72
30	20.12±0.29	11.11±0.53
60	23.85±0.25	13.31±0.27
90	36.60±0.98	14.96±0.23
120	48.23±0.40	21.37±0.54
150	65.36±0.95	25.23±0.19
180	83.68±0.19	30.17±0.78
210	92.26±0.37	33.61±0.71

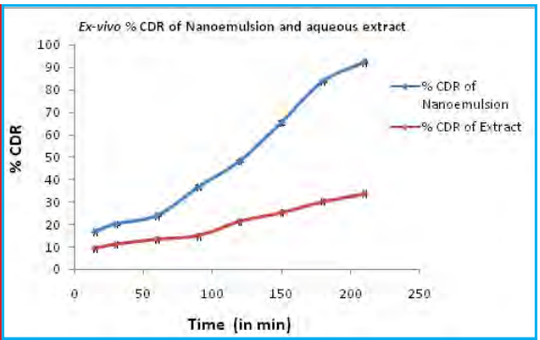

FIGURE 4. *Ex-vivo* % CDR of Nanoemulsion and Aqueous Solution of extract.

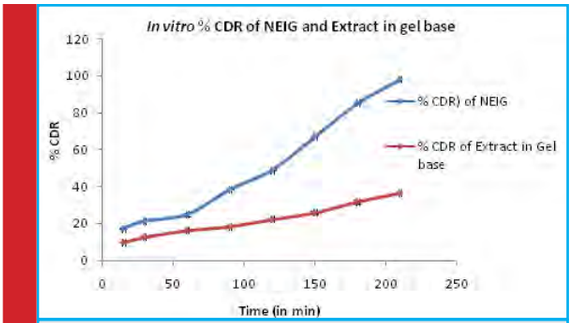

FIGURE 5. *In vitro* % CDR of NEIG and Extract in gel base.

Table 7. Evaluation parametersof nanoemulsion incorporated gel

Formulation	Homogenecity	pH	Viscosity	Spreadability	Extrudability
NEIG	Homogeneous	5.65±0.02	118.66±0.30	5.36±0.11	Good

Table 8. *In vitro* Drug Diffusion data of NEIG

Time (Minutes)	% Cumulative Drug Release (Mean ± SD) of NEIG	% Cumulative Drug Release (Mean ± SD) of Extract in Gel base
15	17.30±0.10	9.75±0.08
30	21.32±0.42	12.75±0.09
60	24.88±0.26	16.31±0.31
90	38.43±1.02	18.48±0.13
120	49.10±0.15	22.31+0.43
150	67.22±0.19	25.97+0.42
180	85.24±0.21	31.92+1.07
210	98.19±0.22	36.54+1.25

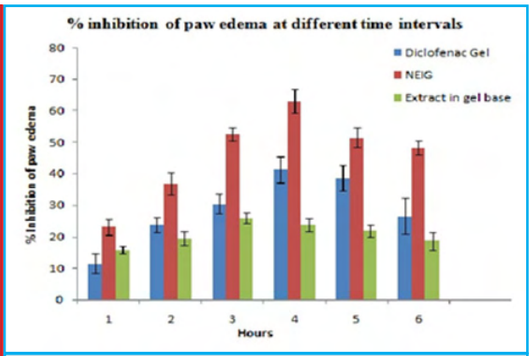

FIGURE 6. % inhibition ofpaw edemaby a) Diclofenac sodium gel (marketed gel).

CONCLUSION

In-vitro drug diffusion and *Ex-vivo* permeation study was concluded that permeation rate was faster in nanoemulsion as compared to solution of extract; also MIC value of nanoemulsion was very less as compared to extract it means better antimicrobial activity. Nanoemulsion further incorporated in Carbopol 934 (NEIG) with good homogenisity. *In-vitro* diffusion study revealed that the nanoemulsion incorporated in Carbopol gel enhances its permeation than nanoemulsion alone.Drug delivery through the skin to the systemic circulation is convenient for a number of clinical conditions due to which there has been a considerable interest in this area.NEIG has significant anti-inflammatory and antimicrobial effect attributed to nanosize of extract in responsible for rapid and complete absorption improving its therapeutic effect. Use of nanoemulsion in transdermal drug delivery represents an important area of research in drug delivery, which enhances the therapeutic efficacy and also the bioavailability of the drugs without any adverse effects. It is also regarded as a promising technique with many advantages including, high storage stability, low preparation cost, thermodynamic stability, absence of organic solvents, and good production feasibility.

Table 9. Minimum inhibitory concentration (MIC) of Extract and nanoemulsion for different micro-organisms

Micro-organism	MIC of Extract mg/ml	MIC of nanoemulsion mg/ml
Escherichia coli	5+0.3	2.5
Staphylococcus aureus,	10+1.00	2.5
Pseudomonas aeruginosa,	15+1.74	2.0
BascillusSubstalis	15+1.52	2.5
Proteous species	20+1.98	1.25
Shigellasoni	15+1.02	3.75
Aspergillus niger	5+0.25	3.75
Candida Albicans	20+1.06	3.75

Table 10. Mean paw volume and % inhibition at different time interval

Paw volume at different time after carrageenan injection							
Treatment	**Initial**	**1h**	**2h**	**3h**	**4h**	**5h**	**6h**
Group I (Control)	1.19±0.020**	1.43±0.021***	1.62±0.018***	1.67±0.017***	1.71±0.015***	1.63±0.013***	1.61±0.010***
GroupII Standard (Diclofenac)	1.17±0.003** ---	1.23±0.009*** (13.80)	1.29±0.008*** (20.43)	1.31±0.002*** (21.28)	1.39±0.01*** (18.74)	1.36±0.013*** (16.55)	1.32±0.019*** (17.67)
Group III Test1 (NEIG)	1.08±0.021** ---	1.18±0.02*** (16.85)	1.26±0.01*** (22.55)	1.286±0.01*** (23.04)	1.345±0.01*** (21.52)	1.33±0.01*** (18.26)	1.32±0.001*** (17.88)
Group IV Test2 (Extract gel)	1.12±0.025** ---	1.29±0.039*** (9.42)	1.36±0.038*** (15.90)	1.44±0.04*** (13.78)	1.50±0.04*** (11.92)	1.48±0.04*** (8.60)	1.48±0.038*** (8.01)

ACKNOWLEDGMENTS

Authors are thankful to Amsar Pvt Ltd Indore, India for providing gift samples of *Glycyrrhiza glabra* extract. The authors are also thankful to the Department of Pharmaceutics, (R. C. Patel Institute of Pharmaceutical Education and Research, Shirpur, for providing necessary facilities to carry out this work.

Disclosure: The authors report no conflict of interest in this work

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Parasitological Communication

Biosci. Biotech. Res. Comm. 12(2): 419-424 (2019)

BBRC Bioscience Biotechnology Research Communications

Characterization of a new plant parasitic nematode isolated from rhizospheric soil of rice-plants

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ABSTRACT

Survey for soil and plant parasitic nematodes associated with paddy (*Oryza sativa* L.) in Purba Bardhaman district of West Bengal, India revealed the presence of a new plant parasitic species belonging to the genus *Tylenchus*. The new species *Tylenchus scythecaudus* sp. nov. comes close to *Tylenchus aquilonios* Wu, 1969, *Tylenchus cylindricaudus* Wu, 1969, *Tylenchus hazanensis* Wu, 1969, *Tylenchus helenae* Szczygiel, 1969, *Tylenchus quartus* Szczygiel, 1969 in having continuous lip region and filliform tail with pointed terminus, those differs from others. The present species differs from all existing species of *Tylenchus* in having different values of a, b, c, V, short gradually tapering tail with scythe-shaped tail terminus and short post-uterine sac. *Tylenchus scythecaudus* sp. nov. is described and illustrated here.

KEY WORDS: NEMATODE . NEW SPECIES. PADDY. *TYLENCHUS*. WEST BENGAL

INTRODUCTION

West Bengal is contributing a significant part in India as far as rice production is concerned. Purba Bardhaman district is known as the 'Grainery of West Bengal' as it produces maximum amount of rice within the state. Nematodes are tiny creatures causing huge damages to the crop. Species belonging to the order Tylenchida are plant parasitic in nature. A survey for soil and plant parasitic nematodes associated with paddy in Purba Bardhaman district of West Bengal was conducted in view to find out the plant parasitic nematodes associated with this crop. The study encountered a new species of plant parasitic nematode *Tylenchus scythecaudus* sp.

ARTICLE INFORMATION:

Corresponding Author: soumen.microbiology@gmail.com
Received 2nd April, 2019
Accepted after revision 25th May, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/26

nov from the rhizospheric region of paddy which is an added information of Tylenchid nematode species of this important crop.

The genus *Tylenchus* was proposed by Bastian (1865) originally and then it was re-established by Filipjev in 1936. The species of this genus are characterized by having smallto medium sized body about 0.4–1.3 mm in length, ventrally curved after fixation, moderately thick cuticle around 1–2 µm, distinctly annulated. Lateral fields having four incisures. Dorso-sublateral, postmedian phasmids present just behind the vulva. Annulated cephalic region continuous with the body. Stylet length varies between 8–21 µm, conus is more than one-third of the total stylet length. Median oesophageal bulb oval shaped which is present anterior to the mid-oesophageal position. Cardia is distinct. In most of them excretory pore is present opposite to the basal bulb. Vulva, a transverse slit like, reproductive organ occupying 60–70% of the total body length. Post-uterine sac generally a body width or often a less long. Round to oval, offset spermatheca is present. Ovary outstretched. Ventrally arcuate tail, sometimes hooked, gradually tapering to a pointed or minutely rounded terminus. Review of literatures of all existing species of *Tylenchus* revealed that *Tylenchus scythecaudus* sp. nov. encountered during the survey distinctly differed from others. The species is illustrated here with morphometric details, line diagrams and photomicrographs.

MATERIALS AND METHODS

Soil sample collection

The rhizospheric soil samples were collected from paddy field of Kalna II block of Purba Bardhaman district of West Bengal, India.

Processing of the soil Sample: Nematodes were extracted from the samples following 'Cobb's Sieving Technique' (Cobb, 1918). For this at first 250g soil sample was mixed with water with gentle stirring by hand in a medium sized bucket. The mixing was done in such a way that there was no soil lumps present. The mixture was allowed to rest for few seconds (15-20s) so that the nematodes can float on the upper surface of the water and then sieved. In the final step of extraction, decanting was done by following 'Modified Baerman's Funnel Method (Christie & Perry, 1951)

Killing and fixation

The nematode samples were killed as well as fixed in hot F.A (Formaldehyde-Acetic acid) solution.

Post fixation process

The nematode samples were further processed by 'Seinhorst's Slow Dehydration Method' (Seinhorst, 1959) in which fixed nematodes were picked in cavity block and allowed them to dehydrate in Glycerin alcohol solution for thirty days. Desiccators were used to keep the cavity blocks. After that the specimens were mounted on slides in glycerin (anhydrous) and sealed. Ocular micrometer of Olympus Research Microscope (Model No. BX 41, with drawing tube attachment) was used for taking the measurements. Dimensions were tabulated following De Man's Formula (De Man, 1884). Diagrams were drawn using camera lucida. Photomicrographs were captured using Leica Research Microscope (Model No. Leica DM 1000). The species was identified following the keys, made by Siddiqi (2000)

RESULTS AND DISCUSSION

Systematics

The following classification is given by Siddiqi (2000)

Tylenchus scythecaudus **sp. nov.** (Figs. 1-10)

Measurements

All measurements are provided in Table 1.

The following abbreviations are used in the text and table a= body length/maximum body diameter, b= body length/oesophageal length, c= body length/tail length, c'= tail length/body width, V= (position of vulva from anterior end/body length from) × 100, V'= (position of vulva from head tip/distance of anus from head end) × 100, G_1= (length of anterior female gonad /body length) × 100, G_2= (length of posterior female gonad / body length) × 100, m= length of conus/total stylet length, O= (distance between orifice of dorsal oesophageal gland and stylet/stylet length) × 100, VL/VB= distance of vulva to posterior end of the body/vulval body width.

Descriptions

Female

Body slender, small about 0.450-0.472 (0.461 ± 0.008) mm long, uniformly tapering; ventrally curved at vulval region, tail end dorsally bended, scythe shaped (Fig.1). Cuticle with transverse annulations; lateral fields with four incisures. weakly developed deirids, located near end of oesophagous. Lip amalgamated, continuous with the body i.e. not set off. Stylet is well developed about 9-10 (9.6 ± 0.380) µm, conus about 37% of stylet length; basal knobs prominent. Orifice of dorsal oesophageal gland nuclei present in very close proximity to the stylet base. Median bulb well developed, more or less oval in shape (4.2×3.4) µm, located at the position of 38% of total oesophageal length from anterior extremity. The terminal oesophageal bulb about 17.2-20.2

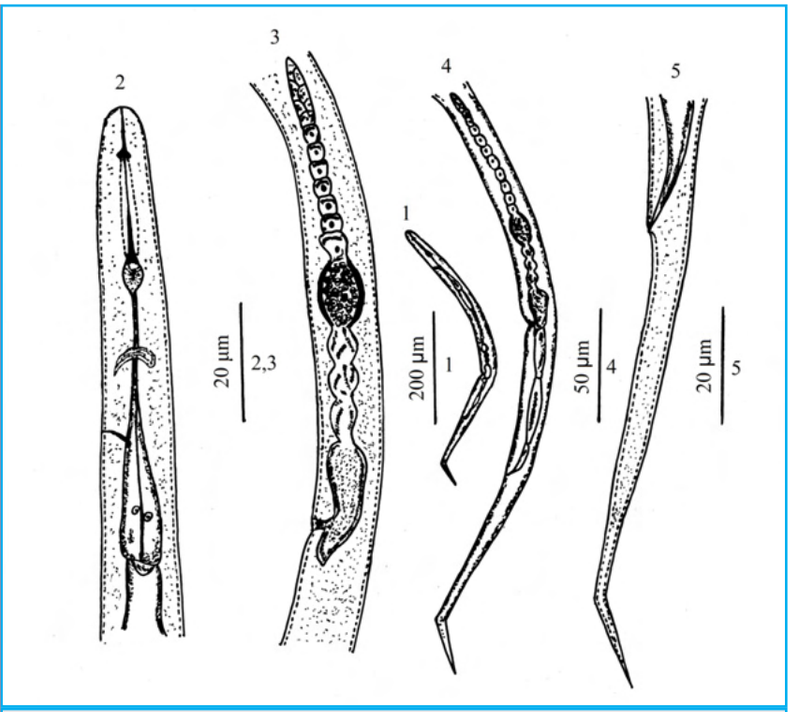

FIGURE 1. Figure captions of *Tylenchusscythecaudus* sp. nov.in Camera Lucida
1. Entire female
2. Anterior region showing stylet; mediun bulb; nerve ring; terminal bulb and cardia
3. Genital system showing vulva; ovary; receptaculumseminis
4. Posterior region showing gonad, rectum, anus
5. Scythe-shaped tail terminus

(18.05 ± 1.252) µm, sac like, occupying about 20% of total oesophageal length; two oesophageal gland nuclei prominent. Excretory pore present at the beginning of terminal oesophageal bulb.

Hemizonid present at 76.3% of oesophageal length from head end and anterior to excretory pore, cardia small about 1.2-2.2(1.55 ± 0.3728) µm, cone shaped, about 0.46 times of lip region width and occupying 9.6% of corresponding body width. Reproductive system prodelphic. Vagina thin walled, present at right angle to the body, occupying about half of corresponding body width. Anterior genital branch about 140.5-145 (142.88 ± 1.710) µm in length, occupying 98% of total gonadal length. The post-uterine sac rudimentary with pointed tip, only about 0.23 times of corresponding body width. *Receptaculum seminis* (spermatheca) distinct, well set off and filled with sperms. Ovary with a single row of oocytes, except terminal region. Rectum about 17-18.5 (17.56 ± 0.513) µm, 2.3 times anal body width long. Tail filliform, 85-90.5 (87.6 ± 2.22) µm long with pointed terminus, 0.77 times of vulva-anal distance; tail terminus scythe-shaped and bended dorsally.

Male: Male not found.Type habitat and locality Specimens were collected from the rhizospheric soil of *Oryza sativa* L. by the first author in 20th October 2016 from Kalna II block (23.168391° N, 88.245036° E) of Purba Bardhaman district, West Bengal, India.Type materials: Specimens were deposited to the National Zoological Collections of Zoological Survey of India, Kolkata, West Bengal, India under the Registration No. WN 1948 (Slide with Holotype female and two Paratype females).

Etymology :The new species is named Tylenchus scythecaudus as it bears scythe shaped tail terminus.

Differential diagnosis and relationships

Tylenchus scythecaudus sp. nov. differs from all existing species of *Tylenchus* in having short, gradually tapering tail with scythe-shaped tail terminus and short post-uterine sac.

Tylenchus scythecaudus sp. nov. comes close to *T. aquilonios* Wu, 1969, *T. cylindricaudus* Wu, 1969, *T. hazanensis* Wu, 1969, *T. helenae* Szczygiel, 1969, *T. quartus* Szczygiel, 1969 in having continuous lip region and filliform tail with pointed terminus, those differs from others.

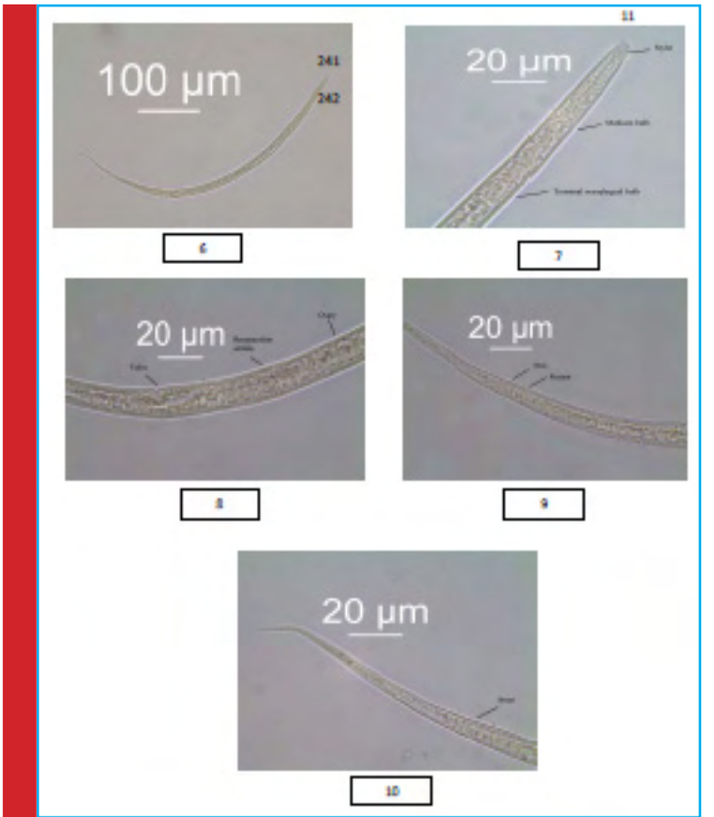

FIGURE 2. Figure captions of *Tylenchusscythecaudus* sp. nov in Photomicrographs
6. Entire female
7. Anterior region showing stylet; mediun bulb; nerve ring; terminal bulb
8. Genital system showing vulva; ovary; receptaculumseminis
9. Posterior region showing rectum; anus
10. Scythe-shaped tail terminus and anus

The species is similar to *Tylenchus aquilonios* Wu, 1969 in having continuous lip region, filliform tail but the present species differs from it in having small body size, smaller values of a, b, c, V, shorter oesophageal length, lesser vulval body width, shorter post-uterine branch, lesser vulva-anal distance and short tail with scythe-shaped terminus, (L= 0.803 mm, a= 35, b= 6.7, c= 6.9, V= 68, oesophageal length= 144-153 µm, post-uterine branch= 13-15 µm, body width at vulval region= 19-26 µm, vulva-anal distance= 115-149 µm, tail length and shape= 116 µm; tapering gradually to a pointed terminus in *Tylenchus aquilonios* Wu, 1969).

The present species is similar with *Tylenchus cylindricaudus* Wu, 1969 in having slender, gradually tapering body with continuous lip region, filliform tail with pointed terminus but differs from it in having smaller body size, lesser values of a, b, c, V, slightly shorter stylet, short post-uterine branch, lesser body width at vulva, reduced vulva-anal distance, shorter tail and different shape of tail terminus (L= 0.95mm, a= 41, b= 6.9, c= 6.6, V= 66, stylet length= 11-13 µm, oesophageal length= 127-137 µm, post-uterine branch= 11-14 µm, body width at vulval region= 19-21 µm, vulva-anal distance=183 µm, tail length= 147 µm, shape of tail terminus is straight filliform in *Tylenchus cylindricaudus* Wu, 1969). *T. scythecaudus* sp. nov. is similar to *Tylenchus hazanensis* Wu, 1969 in having rounded head with continuous lip region and filliform tail with needle like pointed terminus but varies from it in smaller body size, smaller values of a, b, c, V, smaller stylet, shorter oesophageal length, presence of annules at mid-tail region, lesser vulval body width, shorter post-uterine branch, lesser vulva-anal distance and short tail with scythe-shaped tail terminus (L= 02 mm, a= 38, b=

Table 1. Measurements of *Tylenchus scythecaudus*. sp. nov. (in µm, except L in mm)

Morphometric characters	Holotype female	Paratype females (n=4)
L	0.45	0.450-0.472 (0.461 ±0.008)
A	31.2	31.2-31.7(31.4 ± 0.194)
B	5.2	5.2-5.3(5.24 ± 0.054)
c	5.2	5.2(0)
c'	11.6	11.4-12.5(11.8 ± 0.418)
V	56.7	55.6-56.8(56.44 ± 0.482)
V'	70	69.6-70.0(69.9 ± 0.173)
G1	31.4	30.7-31.4(31.06 ± 0.270)
G2	0.55	0.55-0.63(0.574 ± 0.033)
Width of lip	2.6	2.2-2.8(2.4 ±0.189)
Total Stylet length	9.7	9-10(9.6 ±0.380)
Median bulb from anterior end	32.3	38.5-40(32.96 ± 0.996)
Median bulb diameter	4.2×3.4	-
Oesophageal length	85.75	85.5-88.6 (86.67 ± 1.18)
Nerve ring from anterior end	44.5	44.2-48.4(45.4 ± 1.60)
Hemizonid from anterior end	65.5	65.3-67.8(66.34 ± 1.00)
Length of terminal bulb	17.15	17.2-20.2(18.05 ± 1.252)
Length of cardia	1.2	1.2-2.2(1.55 ± 0.3728)
Maximum body width	14.5	14.3-15(14.6 ± 0.264)
Vulva from anterior end	257.3	256-262.8(259.8 ± 3.05)
Body width at vulva	11.2	11-11.7(11.26 ± 0.219)
Anterior genital branch	142	140.5-145 (142.88 ± 1.710)
Posterior genital branch	2.5	2.5-3 (2.66 ± 0.207)
Body width at anus	7.4	7-7.9 (7.46 ± 0.320)
Rectum	17.3	17-18.5 (17.56 ± 0.513)
Tail length	85.8	85-90.5 (87.6 ± 2.22)
Vulva-anal distance	110.2	109.3-114.2 (111.9 ± 2.11)
m	37.4	33.3-38.7 (36.84 ± 2.100)
O	72	72 (0)
VL/VB	17.78	17.5-17.9 (17.7 ± 0.158)

6.8, c= 4.7, V= 62, stylet length= 15.5 µm, oesophageal length= 149-165 µm, mid-tail annulation absent, vulva-anal distance= 178 µm, tail length= 224 µm and tail terminus shape is straight filliform, needle like in case of *Tylenchus hazanensis* Wu, 1969).

The present species is again similar with *Tylenchus helenae* Szczygiel, 1969 in having small slender body, continuous lip region with body, filliform pointed tail, needle like tail terminus but differs from it in having larger body length, larger values of a, b, c, V, larger stylet, more anterior position of median bulb in oesophagous, smaller post-uterine sac, presence of spermatheca, distinct anus and scythe- shaped tail end (L= 0.43 mm, a= 37, b= 4.6, c= 3.6, V= 57.5, position of median bulb in pharynx at 43%, size of post-uterine sac is large, more than one body diameter long, spermatheca absent, anus obscure, long filliform tail ending with a needle like terminus in *Tylenchus helenae* Szczygiel, 1969). *Tylenchus scythecaudus* sp. nov. shows similarities with *Tylenchus quartus* Szczygiel, 1969 in having similar body contour *viz*. delicate, slender, gradually tapering body with continuous lip region and filliform tail but the present species differs from it in having shorter body length, smaller value of a, c, V, but larger values of b, lesser value of G1, shorter stylet length, presence of distinct knobs, oval shaped median bulb, dorsally bended scythe-shaped tail (L= 0.56 mm, a= 39.5, b= 4.7, c= 5.3, V= 65.2, stylet length= 11.8 µm, conspicuous basal

knobs, elongate fusiform median bulb, uniformly tapering tail with very sharp pointed terminus in *Tylenchus quartus* Szczygiel, 1969).

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Compliance with Ethical Standard: Ethical standard has been maintained, no toxic or harmful chemical is used during the experiment.

Author contribution: Author Paromita Roy carried out the work *viz*. collection of the specimen, slide preparation, and preparation of the manuscript. Author Viswa Venkat Gantait helped to identify the specimen. Author Soumendranath Chatterjee helped to prepare the manuscript. All the authors agreed and approved the final draft of manuscript.

Funding: No funding

Conflict of interest: None

Biotechnological Communication

Biosci. Biotech. Res. Comm. 12(2): 425-434 (2019)

BBRC Bioscience Biotechnology Research Communications

Efficiency assessment of pure Fe oxidizing microorganisms in iron supplemented and non-supplemented medium and pure S oxidizing microorganisms for bioleaching of mobile phone printed circuit boards

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ABSTRACT

The microbial culture as well as the components of the bioleaching medium affects the efficiency of microbial leaching. The present study on mobile phone printed circuit boards (MPPCBs) was conducted to know that whether the addition of energy source is required during the microbial leaching or microorganisms can utilize the iron (Fe) content of the MPPCB. The study was conducted with pure Fe oxidizers in Fe supplemented (9g/L, pure Fe 9K), non-supplemented (0g/L, pure Fe 0K) medium and pure sulfur (S) oxidizers supplemented with 3g/L of elemental sulfur (Pure S 3g/L S^0). The copper (Cu) content of the feed material was 26.3% (w/w) by X-Ray Fluorescence (XRF) spectroscopy. The Cu recovery in pure Fe 0K and pure Fe 9K was 100% while with pure S oxidizers it was 39.41%. The acid consumption in pure Fe 0K and pure Fe 9K was 579.65kg/ton and 559.05kg/ton respectively. The bioleaching rate of Cu was 0.128g/L/h, 0.075g/L/h and 0.023g/l/h in order of pure Fe 9K> pure Fe 0K>pure S. Bioleaching with pure Fe oxidizers in 0K medium was found to be efficient and economical in terms of metal recovery and acid consumption, whereas, bioleaching with pure Fe 9K showed the maximum Cu leaching rate. The Cu recovery was same in both Fe supplemented and non-supplemented medium and microorganisms can utilize the Fe present in feed (PCB) itself.

KEY WORDS: BIOLEACHING, COPPER, 9K MEDIUM, 0K MEDIUM

ARTICLE INFORMATION:

Corresponding Author: csgahan_mbio@curaj.ac.in
Received 8th April, 2019
Accepted after revision 7th June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
Crossref Clarivate Analytics
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/27

INTRODUCTION

A significant amount of valuable metals resides in a printed circuit board (PCB) of an electronic device. There are various established technologies for the extraction of metals from PCBs such as mechanical separation, pyrometallurgy, hydrometallurgy, etc. (Shah, Tipre and Dave, 2014). The application of these techniques for treatment of the waste costs more in comparison to the final product. In that respect, bioleaching has been a promising alternative for metal recovery from low-grade ores and the waste (Cui and Zhang, 2008; Mishra and Rhee, 2010; Erüst *et al.*, 2013; Johnson, 2014). The microorganisms which are primarily used for bioleaching belong to genus *Acidithiobacillus* due to their robust nature and ability to oxidize the inorganic ferrous (Fe^{2+}) and elemental sulfur (S^0) (Rawlings, 2005; Arshadi and Mousavi, 2014; L. Wang *et al.*, 2018; Quatrini and Johnson, 2019).

The 100% Cu recovery by cell-free extract of *Leptospirillum ferriphilum* and *Sulfobacillus thermosulfidooxidans* from 5g/L of PCB indicates the indirect bioleaching (Wu *et al.*, 2018). However, the regeneration of the reagent (Fe^{3+}) is necessary for the continuation of the bioleaching process at higher pulp densities. Initial pH and Fe^{2+} ion concentration influence the rate of Cu recovery in the bioleaching medium (Xiang *et al.*, 2010). Few studies suggest that Fe content in PCBs can be utilized by bacteria and addition of extra Fe source leads to jarosite precipitation hence, loss of Fe from the bioleaching system (Wang *et al.*, 2018). The bioleaching of PCBs is an indirect non-contact mechanism where the significant role of the bacteria is oxidation of ferrous (Fe^{2+}) into ferric (Fe^{3+}), (Silva *et al.*, 2015; Mostafavi *et al.*, 2018). Low pH environment can prevent the jarosite formation; it favors the microbial growth and enhances the Cu recovery (Wang *et al.*, 2018). The pH values also influence the bioleaching kinetics of *Acidithiobacillus ferrooxidans* to mediate Cu recovery (Yang *et al.*, 2014). Previous studies on bioleaching emphasize that the recovery and rate of Cu bioleaching from PCBs mainly depends on Fe^{2+} ion concentration and pH. The parameters such as temperature, pulp density, etc. influence these two factors and consequently affects the bioleaching (Wang *et al.*, 2018; Arinanda *et al.*, 2019).

The present study aimed to investigate bioleaching efficiency of pure Fe oxidizing microorganisms in two different bioleaching mediums, i.e., with and without Fe supplements. The hypothesis for using medium without external energy source is 1. The feed material itself has Fe which can be utilized by the Fe oxidizing microorganisms for bio-oxidation 2. The external Fe added to the medium might be an additional amount and not be used completely by the microorganism 3. Fe precipitation and jarosite formation is a common factor which influences the bioleaching yield 4. The extra Fe in bioleaching medium tends to enhance the possibility of jarosite formation which consequently increases the process cost due to the input of additional energy source, post residue treatment as well as the negative impact on Cu bioleaching efficiency. Most of the research suggests that addition of Fe might not be required during bioleaching of PCBs as the Fe which dissolves into the bioleaching medium from PCB can be utilized by the microorganisms. (Bryan *et al.*, 2015; Wang *et al.*, 2018).

The research on bioleaching of PCBs are on laboratory scale due few limitations of its largescale operations such as toxicity, time requirement as well as the cost. The present paper focuses on the current needs for the commercialization of the bioleaching system by comparing the bioleaching yield of Cu from waste MPPCBs in Fe free and supplemented growth medium for Fe oxidizing microorganisms. The paper also studied the bioleaching efficiency of only S oxidizing microorganisms. The bioleaching experiments were compared in respect of acid consumption, Cu recovery and time required for complete bio-oxidation of Cu.

MATERIAL AND METHODS

Mobile phone printed circuit boards (MPPCBs):

Printed circuit boards were separated by manual sorting of the waste mobile phones collected from the mobile phone repair shops in Alwar, Rajasthan, India. The crushing of MPPCBs was carried out at the Mineral Processing department of CSIR-Institute of Minerals and Materials Technology, Bhubaneswar, India. The PCBs were ground to a particle size below 250 µm in an impact crusher followed by sieving. The elemental composition of this grounded material (feed) was analyzed by X-Ray Fluorescence analyzer (Bruker) (Table 1).

The XRF analysis revealed that Cu was 26.3% of the total metal constituents in the feed material. The recovery of Cu was then focused along with Ni (1.08%) and Zn (1.08%). The X-ray diffraction (XRD) and Scanning electron microscopy with energy dispersive spectroscopy (SEM-EDS) (Nova Nano FE-SEM 450 (FEI) analysis was done to determine the mineralogical phases and elemental weight percentage (%) of the feed material (Figure 1 and 2).

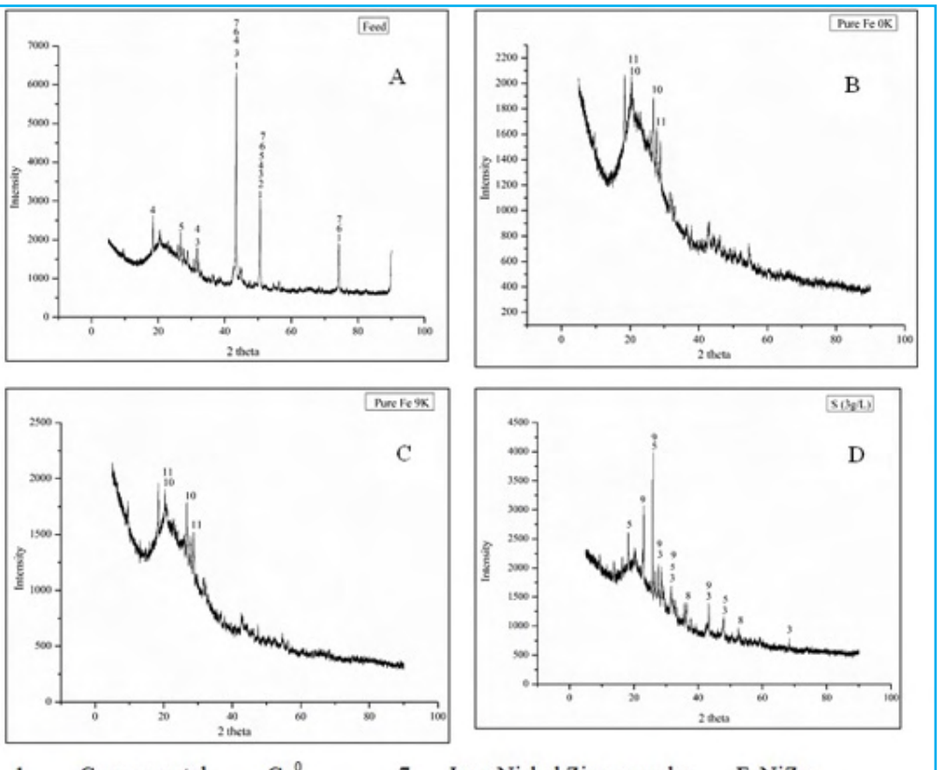
The small dotted arrangement patterns The same pattern of arrangement found in the Sulphur (S), Copper (Cu) and Iron (Fe) indicates the presence of the copper mineral complex with iron and sulfur, which was also observed in the mineralogy obtained from the XRD studies (Figure 1and 2)

Table 1. Metal content (%) in mobile phone printed circuit boards (MPPCBs).

MPPCB Metal content (%)	Cu	Si	Ca	Br	Al	Fe	Sn	Ni	P	Zn	S	Ti	Pb
	26.3	21.7	14.7	9.6	6.1	5.3	3.1	1.1	1.2	1.1	0.8	0.8	0.7
	Ag	Mg	Sb	Cr	Ta	Sr	Cl	Nd	Mn	K	Au	Ga	Co Nb
	0.4	0.4	0.3	0.3	0.3	0.3	0.2	0.1	0.1	0.1	0.1	0.01	0.01 0.01

Bioleaching experiments

The bioleaching of crushed MPPCBs was done in three sets of experiments. Among three two bioleaching experiments namely pure Fe 0K and pure Fe 9K were done with pure culture of *Leptospirillum* dominated Fe oxidizing bacteria in bioleaching medium without Fe supplement (0g/L, Fe) and with Fe supplement (9g/L, Fe) respectively. Another bioleaching experiment Pure S (3g/L) was done with *At. thiooxidans* dominated pure S oxidizing bacterial culture supplemented with 3g/L of



1.	Copper metal	Cu^0	7.	Iron-Nickel Zinc complex	$FeNiZn_{6.5}$
2.	Calcium aluminosilicate	$Ca_2Al_2SiO_7$	8.	Copper Oxide	Cu_2O
3.	Copper Sulfide	CuS	9.	Potassium Iron oxide	$K_2(Fe_2O_4)$
4.	Nickel Zinc complex	$NiZn_3$	10.	Potassium aluminium silicate hydroxide	$Al_2H_2KO_{12}Si_3$
5.	Silicate	SiO_2	11.	Potassium Jarosite	$KFe[SO_4]_2[H_2O]$
6.	Nickel	Ni			

FIGURE 1. XRD Diffractogram of feed and bioleached residues of pure Fe 0K, pure Fe 9K and pure S(3g/L).

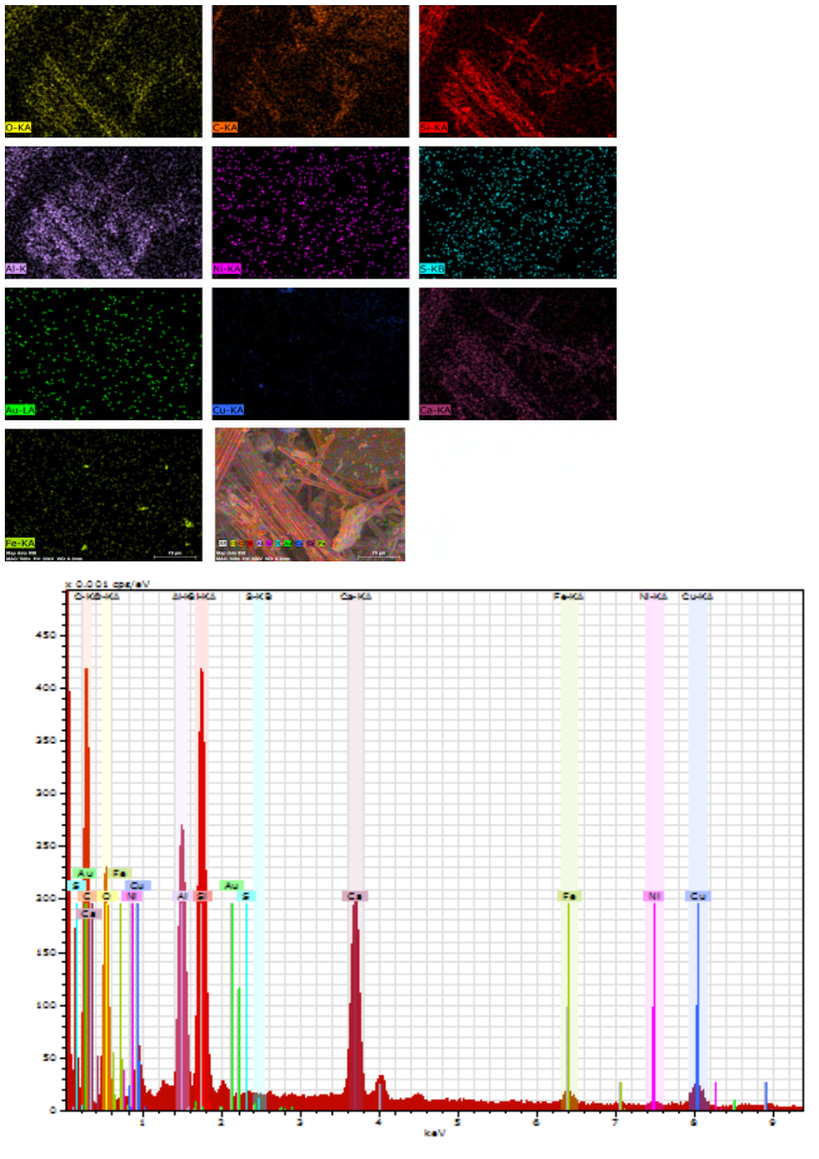


FIGURE 2. SEM-EDS mapping profiles (point scan) and EDS micrograph of feed.

elemental sulfur (S^0).The inoculums used for bioleaching experiments were the activated microbial cultures grown in their respective mediums. The Fe oxidizing microbial culture was grown in a 0K medium [$(NH_4)_2SO_4$, 3.0 g/L; KCl, 0.1 g/L; K_2HPO_4, 0.5 g/L; $MgSO_4 \cdot 7H_2O$, 0.5 g/L; $Ca(NO_3)_2 \cdot 4H_2O$, 0.01 g/L] (Silverman and Lundgren, 1959), supplemented with 9g/L of ferrous (Fe^{2+}) in the form of ferrous sulfate ($FeSO_4.7H_2O$) as an energy source. The pH was controlled at 1.5 and the temperature at 30°C. On the other hand, *At. thiooxidans* was grown in a X-Umea basal salt medium ($Na_2SO_4.10H_2O$, 3.2g/L; $(NH_4)_2SO_4$, 3.0 g/L; KCl, 0.1 g/L; K_2HPO_4, 0.5 g/L; $MgSO_4 \cdot 7H_2O$, 0.5 g/L; $Ca(NO_3)_2 \cdot 4H_2O$, 0.01 g/L) supplemented with 3g/L of elemental sulfur (S^0) as the energy source. The growth of S oxidizing microorganisms was started at 3 pH and 30°C. The growth of Fe oxidizing microorganisms was analyzed by the regular measurement of pH, redox potential, ferrous (Fe^{2+}) and total iron. The maximum value of oxidation-reduction potential (ORP) and zero residual Fe^{2+} ion was indicated activated Fe oxidizers. The increasing sulfate ion (SO_4^{2-}) concentration and decreasing trend in pH val-

ues were marked in the activated S oxidizing microbial culture.

The bioleaching experiments were done with a working volume of 1L (v/v), 90% (v/v) of growth medium and 10% (v/v) of the inoculums. The pH of the bioleaching medium was maintained at pH 1.5 by addition of 5M H_2SO_4 for the activity of Fe oxidizing microorganisms. The bioleaching medium composition was the same as the growth medium for pure S oxidizing microorganisms. No pH adjustments were made during the bioleaching of S-oxidizing microorganisms. Temperature for both the bioleaching experiments was maintained at 35°C. 50g/L of the PCB powder was added in the bioleaching medium (growth medium + activated culture) in the starting of all the three experiments. The daily addition of H_2O compensated the evaporation loss of water (H_2O). The changes in pH during bioleaching experiments were regularly monitored by a pH meter (Eutech). The changes in Fe^{2+} concentration and ferrous/ferric (Fe^{2+}/Fe^{3+}) couple during bioleaching with pure Fe oxidizing microorganisms was monitored by a titrimetric method using cerium sulfate with 1,10-Phenanthroline as an indicator and an ORP meter having platinum electrode with Ag, AgCl reference electrode. The concentration of SO_4^{2-} was analyzed by using a turbidimetric method by visible spectrophotometric method (420 nm) described in the American Public Health Association, 1975 (APHA) (Kolmert, Wikström and Hallberg, 2000).

The metal ion concentration in all the three experiments was determined by atomic absorption spectrophotometer (AAS) (Thermo Scientific iCE 3000 SERIES). A bright field microscope did the planktonic viable microbial cell count at 100X magnification on a Neubauer hemocytometer. After constant values of metal ion concentration and other parameters, the bioleaching experiments were harvested by solid-liquid separation. Bioleached residue was washed thoroughly using 1.5 pH water and air dried. The mineralogical analysis of both feed and the bioleached residue was determined by "PANanalytical Powder XRD." The samples for XRD were pulverized first to ensure the homogeneity then the diffraction patterns were measured at angles between 10° to 90°, the step size was 0.02 angle/sec. The crystalline phases were identified by using the joint committee for powder diffraction standards (JCPDS) file.

RESULTS AND DISCUSSION

The pH of without Fe supplement (0K) bioleaching medium was high during the initial three days of the experiment due to the alkaline nature of the PCBs and bio-oxidation of Fe^{2+} into Fe^{3+}(Figure 3).

After that, no significant increase in pH was observed. The highest concentration of Fe^{3+} was marked on the 4th day of the experiment with a notable rise in ORP values after that the ORP was in an increasing trend along with microbial cell count. The bioleaching experiment in 0K medium was initiated with a total iron concentration of 0.92g/L and reached 3.51g/L on the 3rd day (Figure 4) which indicates complete dissolution of Fe presented in the feed (2.66g/L).

The decreasing trend in total Fe concentration with a notable low pH value after the 3rd day of the experiment

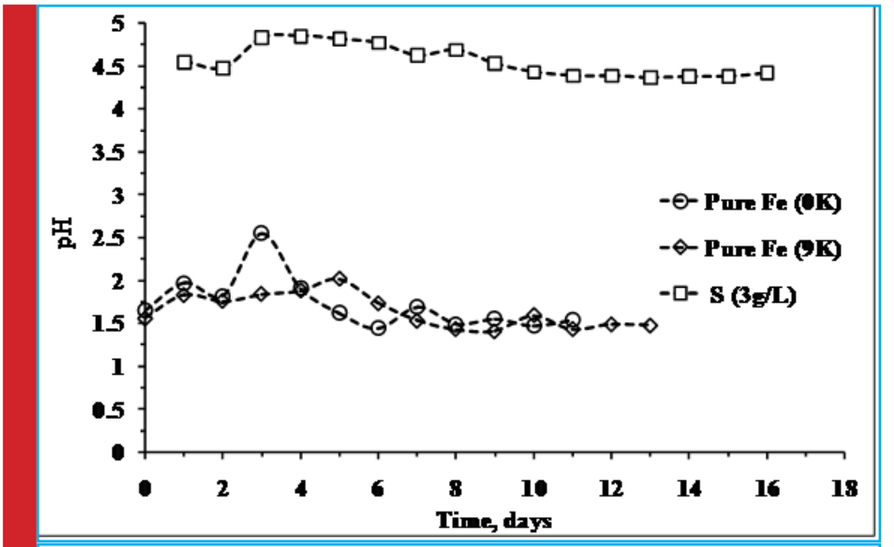


FIGURE 3. Change in pH profilewith time during the bioleaching experiments.

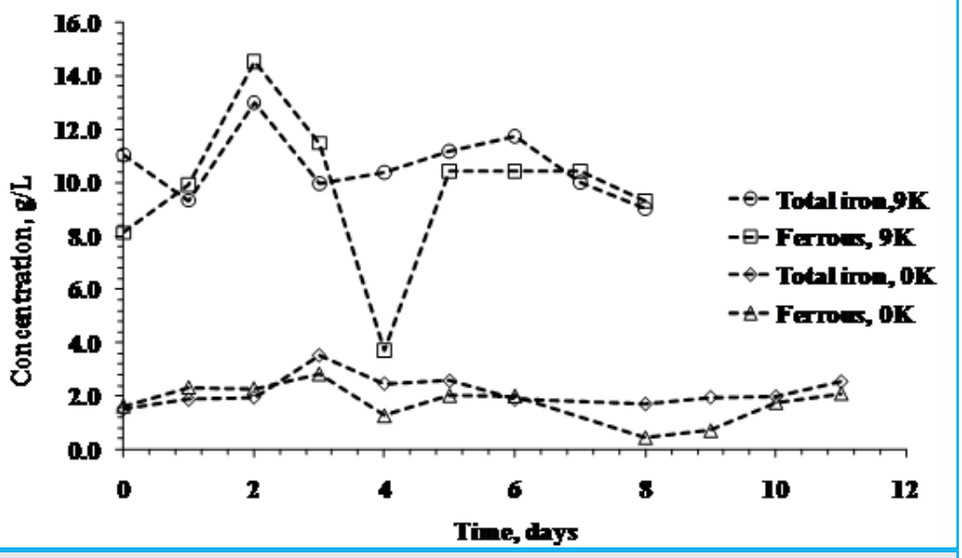


FIGURE 4. Ferrous concentration profiles of pure Fe 0K (Ferrous,0K) and pure Fe 9K (Ferrous,9K) and total iron concentration profiles of pure Fe 0K (Total iron, 0K) and pure Fe 9K (Total iron, 9K).

was due to formation jarosite. The presence of potassium jarosite peaks (characteristic in a Fe oxidizing microbial bioleaching system) in XRD data of bioleached residue confirms jarosite formation (Figure 1B). The significant increase in pH values in Fe supplemented medium (9K) was observed from the 4th day with increasing values of ORP and microbial cell count. The acid was mainly added during initial days to maintain a pH value of 1.5 which is accounted for the growth of *Leptospirillum ferriphilum* and highest Cu recovery (Wang *et al.*, 2018).

The pH values in both the experiments were low during the last days, and no more acid was added. The ORP values in both the systems were unstable after addition of PCBs in the bioleaching medium; hence, hard to evaluate. The stable ORP values from the 1st day of the experiments were low and started increasing from 4th and 5th day respectively. At the end of the operations, the ORP value of 0K bioleaching medium was 406mV while, 438mV in the 9K bioleaching medium (Figure 5).

As soon as the PCB powder was added in bioleaching medium the initial microbial cell count of the 0K and 9K bioleaching medium was decreased from a value of 2.80E+08 to 7.20E+06 and 4.80E+06 respectively (Figure 6).

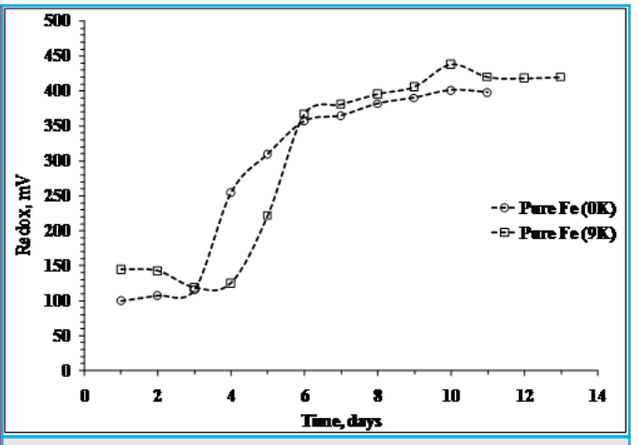


FIGURE 5. Redox profile with time of pure Fe 0K and pure Fe 9K.

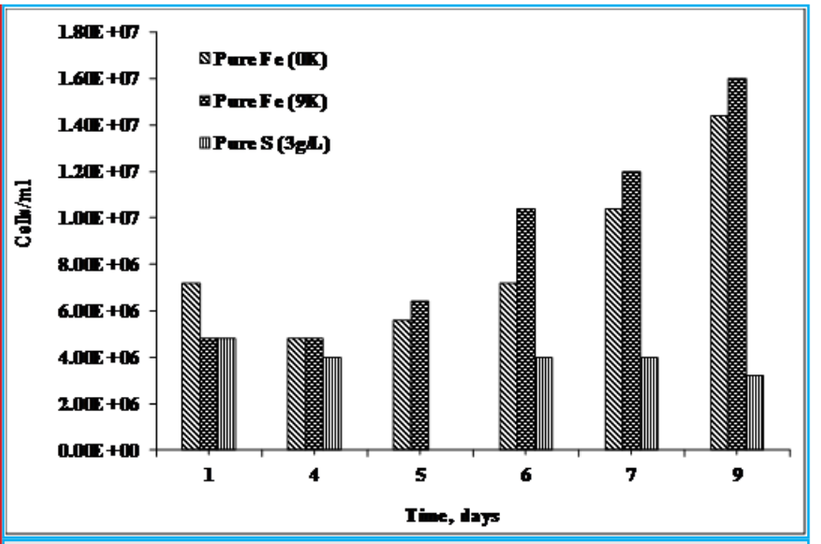


FIGURE 6. Cell count profile of pure Fe 0K, pure Fe 9k and pure S (3g/L)

The microbial cell count was recovered after the 5th day in the amount of 1.60E+07 cells/mL of bioleaching medium. The total amount of acid consumption was 559.05 kg/ton and 579.65kg/ton in 0K and 9K bioleaching medium respectively. Complete recovery of Cu (100% recovery) took place in 8 days and six days of Pure Fe 0K medium and pure Fe 9K medium respectively. The recovery of Zn and Ni in pure Fe 0K and 9K systems was 75.57%, 98.14%, 75.12%, and 22.10% respectively (Figure 7).

The Cu dissolution rate was 0.128g/L/h in pure Fe 9K system while it was 0.075g/L/h in pure Fe 0K system (Figure 8).

The 50g/L PCB was added in X-Umea salt medium at pH 1 and initial sulfate (SO_4^{2-}) concentration of 6.14g/L (inoculum+ media). The initial cell count of the bioleaching media was decreased from 6.64E+08 to 4.80E+06 as soon as PCB was added in the medium (Figure 6). The microbial cell count, pH as well as the sulfate concentration was not recovered with time. The acid was also added in initial few days to maintain the pH at three optimized for the growth of S oxidizing microorganisms. At pH 3, Fe does not remain in the soluble form which ensures only biogenic proton leaching of PCB. The SO_4^{2-} contributed from 18.19M of H_2SO_4 with a purity of 96% and a density of 1.84g/mL was 6.93g/L. The total sulfate detected at the end of the experiment was 11.20g/L which was lesser than the total added SO_4^{2-} concentration. The Cu recovery in pure S oxidizing system was only 39.41% which quite lower in comparison to the

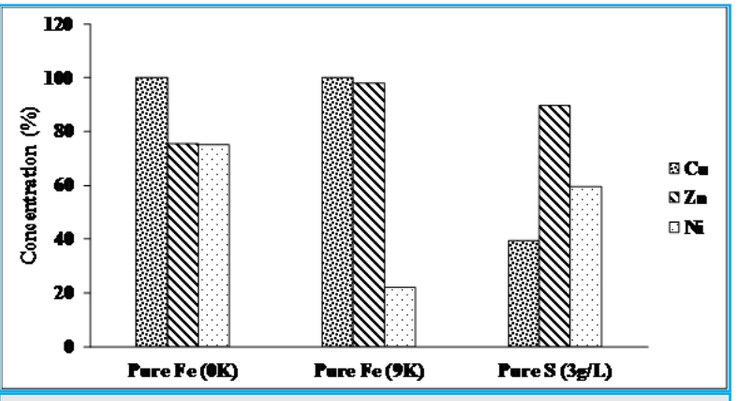


FIGURE 7. Comparative bar graph representing leaching yeild % of bioleaching experiments.

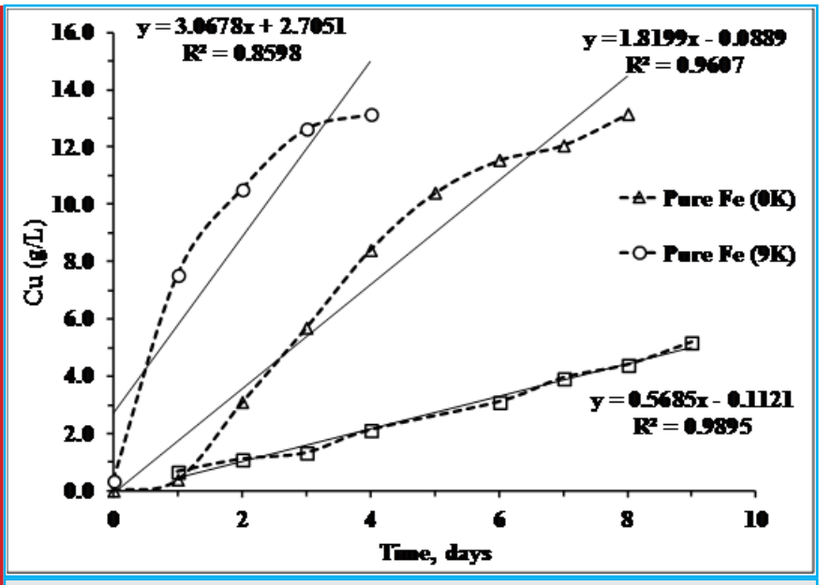


FIGURE 8. Cu dissolution rate with time during bioleaching

other two experiments with a slower dissolution rate of 0.023g/L/h. Recovery of the other two metals *viz* Ni and Zn was 59.38% and 89.68% respectively (Figure 7 and 8).

The low pH and redox values of 9K bioleaching medium during initial days indicate that as soon as PCB was added in the bioleaching medium the Fe^{3+} ion attacked on it and converted into Fe^{2+}. The total Fe profile of 9K bioleaching medium shows that high Fe^{3+} ion concertation on the 0^{th} hour was immediately reduced into Fe^{2+}. The Fe^{2+} to Fe^{3+} bio-oxidation was then marked with an increasing ORP after a microbial growth lag phase of 4 days. The Fe^{2+} to Fe^{3+} bio-oxidation thus started in later days of the experiment. The results are consistent with a previous study stated that the Fe^{2+} to Fe^{3+} bio-oxidation is the rate-limiting step during bioleaching (Wu *et al.*, 2018). The significant portion of the acid was added during the initial four days of the bioleaching without Fe supplements while in 9K medium bioleaching the acid addition was done during the experiment. This acid was required for the bio-oxidation of Fe^{2+} into Fe^{3+} which was high in the 9K medium. The more acid consumption by Fe oxidizing microorganisms in 9K medium shows that addition of Fe puts an extra burden on microbes as the Cu recovery in both the bioleaching mediums was similar with less consumption of acid in 0K (Table 2).

A study on PCBs found that 0.25g/L of Fe_2SO_4 is sufficient to mobilize Cu from 50g/L of PCBs in a multistage system, (S. Wang *et al.*, 2018). The Fe^{2+} to Fe^{3+} bio-oxidation and jarosite formation are the two main reasons behind low pH values during the last days in 0K medium which can be confirmed by an increasing trend of redox potential, viable cell count, decrease in Fe^{2+} ion concentration. Hence, the Fe content in PCBs itself was sufficient to be utilized by the microorganisms for bioleaching.

The changes in pH, SO_4^{2-} as well as the cell count profile of bioleaching in the presence of pure S oxidizing bacteria, was not significant. The bioleaching experiment was initiated with an SO_4^{2-} concentration of 6.14g/L. The increased SO_4^{2-} in the bioleaching system was contributed by the acid addition. The acid production by the S oxidizing microorganisms was insignificant. According to the previous study, the S oxidizers are more sensitive for the higher metal concentration as well as the toxicity related to PCBs (Wang *et al.*, 2009). A previous study on bioleaching of PCBs also concluded the Cu recovery

Table 2. Summary of bioleaching experiments.

	Pure Fe 0K	Pure Fe 9K	Pure S (3g/L)
Feed weight, g	50	50	50
Bioleach residue, g	35.44	35.06	48.9
Acid Consumption, kg Conc. H_2SO_4/ton PCBs	579.65	559.05	-

in presence to *At. thiooxidans* was less in comparison to the abiotic chemical leaching (Hong and Valix, 2014). The results were persistent with our study also where Ni and Zn dissolution were high in comparison to Cu. The XRD data of pure S oxidizers bioleached residue reveals the presence of CuS and Cu_2O which according to a previous study tends to precipitate Cu, forms a passivation layer and results in an incomplete Cu dissolution (Hong and Valix, 2014). The faster Cu recovery rate in 9K medium shows that the presence of Fe^{3+} during first few hours helped in Cu dissolution. However, the bio-oxidation of Fe^{2+} into Fe^{3+} took a time of three days, and an increased amount of Fe^{3+} was again noted on the 4^{th} day of the experiment. The metal dissolution in pure S oxidizers was due to proton leaching by addition of H_2SO_4 and not by the biogenic acid.

CONCLUSION

The present study was done to check the feasibility of MPPCBs bioleaching by using pure Fe oxidizing microorganisms in with and without Fe supplemented medium and by pure S oxidizing microorganisms. The Cu recovery was 100% in both the experiments with a faster dissolution rate of Cu in the 9K medium. The Cu dissolution rate was 0.1278g/L/h and 0.075g/L/h in pure Fe 9K and 0K medium whereas 0.023g/L/h in S oxidizers. The complete recovery of Cu took six days and 8days in pure Fe 9K and 0K medium respectively. The acid consumption in 0K medium was less in comparisons to 9K medium. The recovery of Ni and Zn was also satisfactory in bioleaching pure Fe 0K medium. The bioleaching with pure Fe oxidizers in 0K medium was found to be more feasible in terms of metal recovery, acid consumption, leaching time and total cost of the process. Whereas, 9g/L Fe supplemented medium was efficient to enhance the Cu recovery rate. The pure S oxidizing microorganisms supplemented with 3g/L of S^0 were found inefficient for the bioleaching of 50g/L of MPPCBs.

ACKNOWLEDGMENTS

The authors want to acknowledge DST SERB for providing funds for the research. The authors also wish to thank DST INSPIRE for offering fellowship to Ph.D. The authors want to pay their gratitude to the Central University of Rajasthan for providing infrastructure facility. Last but not the least the author thanks to IMMT, CSIR, Bhubaneswar India and Material research center (MRC), MNIT, Jaipur for availing the instrumentation facilities.

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Microbiological
Communication

Biosci. Biotech. Res. Comm. 12(2): 435-439 (2019)

BBRC
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Antimicrobial activity of *Rhizobium japonicum and Bradyrhizobium japonicum* on different plant pathogenic fungal strains

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ABSTRACT

Symbiotic nitrogen fixation is a key to the nitrogen nutrition to the legumes. The most important agents for the symbiotic nitrogen fixation are the bacteria of the genus *Rhizobium*, which invade the root hairs of leguminous plant and develop nodules on the roots, where nitrogen fixation occurs. *Rhizobium* promotes growth of plants by fixing nitrogen from the atmosphere and is also a biocontrol agent which inhibits growth of pathogens. The biocontrol effect is due to the secretion of secondary metabolites.The present study describes the physiological, biochemical characterization and antagonistic activity of Rhizobium species were isolated from root nodules of leguminous plant. The *Rhizhobium spp*. were rod shaped, gram negative and mucous producing. Antifungal activity of *Rhizobium spp*. isolates were tested against three fungi which are potential phytopathogens on legumes. Inhibition zones were observed, hence *Rhizobium spp*. can be used as biocontrol agent.

KEY WORDS: ANTAGONISTIC ACTIVITY, ANTIFUNGAL ACTIVITY, BIOCONTROL EFFECT, INHIBITION ZONE, *RHIZOBIUM SPP*

INTRODUCTION

Chemicals used to control plant diseases contaminate the soil environment, degrade its fertility and also defile underground water, causing health risk. Thus, biocontrol agents emerge as an alternate to those antifungal chemicals, these are inexpensive, eco-friendly and have no harmful effects on human, animals and plants (Deshwal *et al.*, 2003). Legumes establish a symbiotic interaction with soil bacteria termed as Rhizobia. These bacteria in association with legumes can fix atmospheric N and through this feature. Hence, they are introduced

ARTICLE INFORMATION:

Corresponding Author: orjitnandi@gmail.com
Received 15th April, 2019
Accepted after revision 25th June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/28

into agricultural systems to improve soil fertility, plant growth and limit the use of chemical fertilizers (Ouma *et al*., 2016 Srinivasan, 2017 Yassine, 2018).

Rhizobia not only fix nitrogen from atmosphere and supply it to plants but also promote the growth of plants. Rhizobia synthesize phyto-hormones and solubilization of minerals act as a biocontrol agent and can inhibit the growth of pathogens. Due to the secretion of secondary metabolites such as antibiotics and HCN by rhizobia, they have the capacity to restrict the growth of fungal pathogen. In iron stress conditions in rhizobia, siderophore production provides an added advantage, resulting in the exclusion of pathogen due to iron starvation. Rhizobacteria possessing all these features are also referred as plant growth promoting rhizobacteria (PGPR).Not only this but these bacteria also protect plants against flooding, drought, salt, flower wilting, metals, organic contaminants, and both bacterial and fungal pathogens, (Glick, 2014, Subramanium *et al*., 2015, Srinivasan, 2017 and Yassine, 2018).

Legumes fix atmospheric nitrogen only in association with a bacterial symbiont of the genus *Rhizobium*. Rhizobia have been arbitrarily divided into two classes: fast growing and slow growing. Normally, commercial cultivars of the soybean *Glycine max* are nodulated only by slow-growing *Rhizobium japonicum* (Keyser *et al*., 1982). The isolate appeared to be effectively nodulate only the wild soybean *Glycine soja* and an unbred soybean cultivar from China (*G. max* cv. Peking) *Rhizobium japonicum* 191 is a member of a salt-tolerant group of *R. japonicum* recently described by (Keyser *et al*., 1982).

Bradyrhizobium japonicum is a Gram negative bacterium belonging to rhizobia group associated with roots of soybean and have the capacity to fix N2 in the presence of nitrogenase enzyme (Baoling *et al*., 2007). This bacterium and nitrogenase enzyme both are very sensitive for the environmental conditions. The commercially introduced strains must compete with highly adapted indigenous rhizobia for legume nodulation under specific physiological, biological and environmental soil conditions. Soil acidity limits symbiotic nitrogen fixation by limiting Rhizobium survival in soils, as well as reducing nodulation (Ibekwe *et al*, 1995). Chakraborty and Purkayastha, (1984) reported that some strains of *Bradyrhizobium japonicum* produce rhizobitoxine which can protect soybean crops from the infection of *Macrophomina phaseolina*, which is a charcoal rot fungus of leguminous crops. *Rhizobium* has shown to reduce root-rot of soybeans caused by *Phytophthora megasperma*. Rhizobial mechanism such as improvement in intake of plant nutrients by altering root morphology, production of siderophore (Antoun *et al*., 1998; Arora *et al*., 2001; Chabot *et al*., 1996; Srinivasan, 2017) to meet the iron requirement of the plant under iron stressed conditions and lowering of ethylene through ACC deaminase enzyme are some example with direct positive effects on non leguminious plant growth. *B. japonicum* strain inhibit the growth of seven pathogenic microorganisms causing disease in soybean was studied by (Balasundaram and Sarbhoy 1998) The fast growing rhizobial strains were found to completely inhibit the growth of white sclerotia of *S. rolfsii*. Different strains of Rhizobium and *Bradyrhizobium* have been reported to inhibit the growth of *M. phaseolina* (Deshwal *et al*., 2003; Arora *et al*., 1998). The rhizobia having antagonistic property showed more competency in root hair infection in host plants as compared to non- biocontrol rhizobia. Rhizobia also appear to influence the plant defense mechanism by stimulating the production of phytoalexins by plants. Antibiotics produced by rhizobia have been found to play an important role in disease control. HCN, a secondary metabolite produced by several microorganisms, has deleterious effect on the growth of some microbes (Knowles, 1996).

Studies conducted on numerous plant microbe interaction have shown that such antagonistic rhizobacteria could function by competition and antibiosis i.e. by producing antimicrobial compounds like bacteriocin (Rodelas *et al.,* 1998; Joseph *et al.,* 1983) but also indirectly induces systemic resistance against plant diseases. The enzyme system of bacteria during nodulation in the roots supplies constant source of reduced nitrogen to the host plant and the plant in turn provides nutrients and energy for the activities of the bacteria (Singh *et al.,* 2008). It has also been evaluated that *Rhizobium* increases plant growth by various ways such as production of plant growth hormones, vitamins, siderophores, by solubilisation of insoluble phosphates, induction of systemic disease resistance and enhancement in stress resistance (Hussain *et al.,* 2009; Yassine, 2018).

Some *Rhizobium spp*. have shown antimicrobial activities towards *Pseudomonas spp*. (Kacem *et al.,* 2009) *Aspergillus niger* (Yuttavanichakul *et al.,* 2012). In the present study *Rhizobium* was isolated from the root nodules of soybean and its antagonistic activity was studied against pathogenic fungi such as *Aspergillus niger* and *Fusarium oxysporum*.

MATERIAL AND METHODS

a) Isolation of nitrogen fixing bacteria from soybean root nodules: Soybean plants were uprooted carefully from the soil so that intact roots can be obtained without destroying the nodules. Healthy soybean nodules were detached from the root and further isolation of root nodulating rhizobia was carried out (Vincent, 1970).

The separated root nodules were washed in tap water to remove the adhering soil particles from nodule sur-

face. Fresh root nodules from soybean were collected and surface sterilized with 70% ethanol and 0.1% mercuric chloride and washed thrice with sterile distilled water. Root nodules were crushed in saline solution. Test isolate was isolated by spreading 0.1ml crushed root nodule suspension on YEM (Yeast extract mannitol, pH.7.0) agar plate and incubated at 36°C.Colonies of test isolate were observed in 2-3 days (Singh *et al.*, 2008) were further used for morphological and biochemical characterization. To check the antibacterial activity, rhizospheric bacteria were isolated by serial dilution of soil. All the experiments were carried out in triplicates. *Bradyrhizobium japonicum* (RJ(s)TAL102) was collected from M.P. State Agro Industries, Bhopal.

b) Morphological characterization: Morphological characters such as shape, colour, size elevation, margin, opacity and gram staining were performed for identification of the bacteria (Gachande and Khansole, 2011) and for further biochemical test.

Biochemical characterization: All the tests were carried out with triplicates. For characterization of bacteria, the acid production test (Jordan, 1984), oxidase test (Kovaks, 1956), catalase test (Graham and Parker, 1964), methylene blue test, starch hydrolysis test (Oliveria *et al.,* 2007), glucose peptone agar test (Kleczkowska *et al.*, 1968), gelatin hydrolysis test (Sadowsky *et al.,* 1983), growth in NaCl (Sadowsky *et al.,* 1983), citrate utilization test gram staining and motility were performed.

c) Temperature tolerance: Effect of different physical parameters on the growth of test isolate was studied by keeping plates at different temperatures. Differences in the range of growth temperatures were investigated by incubating bacterial cultures in YEM agar at 32°,34°,36°,38° and 40°C.

d) Antifungal activity: The antifungal effect of test isolate were evaluated against pathogenic fungi (*Aspergillus niger*, *Alternaria alternate* and *Fusarium oxysporum*) by filter paper method. Fluconazole antifungal was used as a positive control and saline as negative control. Bacterial suspension was made and filter paper was dipped into it and placed on the surface of assay plates labelled as Control. The plates were incubated for 24 - 48 hour at 28°C to observe antifungal activity and the zone of inhibition were recorded (Arfaoui *et al.*,2006).

RESULTS AND DISCUSSION

The colonies isolated from roots of soybean were entire, opaque with regular margin, milky white, translucent, circular in shape, shiny, raised (convex), sticky consistency, musky colour of the colony and 2-4mm in diameter. The isolated bacterium was aerobic, non spore forming, pink coloured gram negative rods and motile. *Rhizobium* showed positive results for acid production test, catalase test, oxidase test, starch hydrolysis test, glucose peptone agar test and NaCl test. Also the nega-

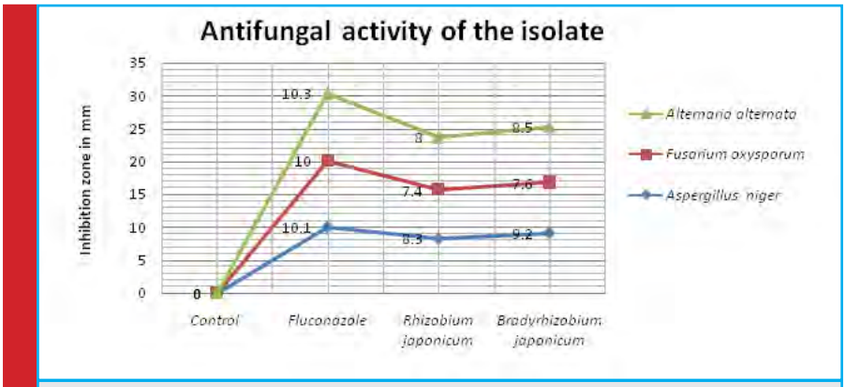
Table 1. Biochemical characteristics of the isolate

TEST	REMARK	
	Rhizobium japonicum	Bradyrhizobium japonicum
Acid production test	+ve	+ve
Catalase test	+ve	+ve
Oxidase test	+ve	+ve
Methylene blue test	-ve	+ve
Starch hydrolysis test	+ve	+ve
Glucose peptone agar test	+ve	+ve
Gelatin hydrolysis test	-ve	+ve
NaCl test	+ve	+ve
Gram staining	Gram negative rod shaped	Gram negative rod shaped

Table 2. Antifungal activity of the isolate

S. no.	Treatments	Aspergillus niger	Fusarium oxysporum	Alternaria alternata
1.	Rhizobium japonicum	8.3	7.4	8.0
2.	Bradyrhizobium japonicum	9.2	7.6	8.5
3.	Fluconazole	10.1	10.0	10.3
4.	Control	0	0	0

*Note: Inhibition zone measured in mm



GRAPH 1.

tive result were seen for gelatin hydrolysis and methylene blue test.The optimum temperature was 28°C. There is a gradual decrease in a colony count was observed after 40°C and growth was totally absent at temperature 44°C in broth.

Both the isolates of *Rhizobium* inhibited the growth of *Aspergillus niger*, *Alternaria alternata* and *Fusarium oxysporum* which is pathogenic fungi and affects on the yield of crop plants. The zone of inhibition (in mm) was recorded and measured.

The test isolates were identified as *Rhizobium japonicum* and *Bradyrhizobium japonicum* by morphological and biochemical characterization. These characteristics were found to be similar with the standard characteristics of the *Rhizobium* and thus this indicates that the isolated microorganisms are *Rhizobium japonicum* and *Bradyrhizobium japonicum*.

The zone of inhibition (in mm) recorded was 8.3 for *Aspergillus niger*, 7.4 for *Fusarium oxysporum* and 8 for *Alternaria alternata* from *Rhizobium japonicum* and for *Bradyrhizobium japonicum* the zone of inhibition was recorded 9.2 for *Aspergillus niger*, 7.6 for *Fusarium oxysporum* and for 8.5 *Alternaria alternata*. Rhizobia have been reported to inhibit significantly the growth of *Fusarium spp*. and *Aspergillus spp*. (Srinivasan, 2107). Antifungal activity of *Bradyrhizobium japonicum* against *Fusarium oxysporum* has been reported by Mariastuti *et al*., (2018) that inhibition of *Fusarium oxysporum* by *Bradyrhizobium japonicum* ranged from 19% to 54.9%. Thus, indicating *Rhizobium japonicum* and *Bradyrhizobium japonicum* as a biocontrol agent.

CONCLUSION

The aim of the study was screening of *Rhizobium spp*. (*Rhizobium japonicum* and *Bradyrhizobium japonicum*) and determine its antifungal activity against *Aspergillus niger, Alternaria alternata* and *Fusarium oxysporum* causing various root rot diseases. In our investigation the antifungal activity of *Rhizobium spp*. were found to inhibit fungal growth showing inhibition zone, suggesting production of certain antifungal metabolites. *Rhizobium spp*. could be effectively used as a biocontrol agent in the form of bio-inoculant against fungal pathogen but enhancement in its antifungal properties would prove to be more efficacious. Therefore efforts are required to understand biocontrol mechanism of rhizobia against fungi. Genetic engineering approach can also be used to introduce the genes coding for the synthesis of antifungal and antimicrobial metabolites into rhizobial strains selected for use in biocontrol.

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Biotechnology
Communication

Biosci. Biotech. Res. Comm. 12(2): 440-445 (2019)

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Analysis of electroplating industry effluent and bioprospecting of heavy metal resistant microbial diversity

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ABSTRACT

Electroplating industry effluent is discharging heavy metals like Ni, Zn, Mg, Mn, Pb, Cu and Cr into soil and water bodies. The challenge is to properly tackle the waste disposal so that the industrial solid wastes do not contribute any type of pollution. Keeping all these perspectives in view, analysis of Industrial effluent was done by ICP-MS results from which showed that the concentration of lead (4.09mg/L) and zinc (376.28mg/L) were beyond the standard limits. Further heavy metal resistant microbes isolated from contaminated soil were identified as *Providencia sp*. and *Enterobacter sp*., which could be potential remedial measure for bioremediation of heavy metal polluted sites.

KEY WORDS: POLLUTION, ELECTROPLATING, BIOREMEDIATION AND ICP-MS

INTRODUCTION

Population has been increasing exponentially and industrialization activities are polluting our environment by depositing heavy metals (Marzan *et al.*, 2017; Shifaw, 2018). The need to control toxic materials particularly, heavy metals discharged from industrial effluent is currently increasing in our environment. These days most of the rivers receive millions of gallons of domestic waste and industrial effluent containing pollutants of varying characteristics from simple nutrient to highly toxic substances (Nivruti *et al.*, 2013). Although industries in India abide by the guidelines of Central Pollution Control Board (CPCB) but environment situation is still

ARTICLE INFORMATION:

Corresponding Author: amitakuk@gmail.com
Received 17th May, 2019
Accepted after revision 30th June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/29

far from satisfactory (Lokhande *et al.,* 2011). Most of these defaulting industries are petrochemical industries, distilleries, sugar mills, leather processing industries, agrochemicals, paper mill, pesticides and pharmaceutical industries. Heavy metals are accumulated in soils and plants, interfere with physiological activities of plants such as nutrient absorption, photosynthesis and cause reductions in plant growth. Dietary intake of heavy metals through consumption of plants has long term detrimental effects on human health (Saikia *et al.,* 2015).

A quandary before the scientists is how to tackle the toxic contaminants that endanger the environmental. Many novel approaches to clean-up the environment are being developed and many are already in practice such as conventional physico-chemical methods like electrochemical treatment, ion exchange, precipitation, reverse osmosis, evaporation and sorption (Dixit *et al.,* 2015). Bioremediation is an environmentally friendly and potentially very effective alternative to physical remediation methods. Bioaccumulation a process dependent upon metabolic potential of microorganisms whereas, biosorption or bioaccumulation is a metabolically passive process, meaning it does not require energy, amount of contaminant a sorbent can remove is dependent on kinetic equilibrium and the composition of the sorbents cellular surface, (Hansda *et al.,* 2016).

MATERIAL AND METHODS

Collection of sample: Industrial waste water samples and nearby landfill soil was collected from two different battery recycling industries. A cold chain was maintained while transporting the samples to laboratory at Kurukshetra University, Kurukshetra. Samples were stored at 4˚C for analysis of electroplating industry effluent and screening for heavy metal resistant bacterial strains.

Determination of physicochemical properties of effluent samples: Analysis of physiochemical parameters in the industrial effluent samples was carried out. Different parameters such as pH, COD, BOD, chloride estimation, Total residual chlorine, conductivity and salinity, TDS and oil and Grease were estimated as per the Indian Standard Methods and American Public Health Association (APHA., 1981). Heavy metal contents were determined with the help of Inductive Coupled Plasma-Mass Spectroscopy using APHA 3125B (APHA., 1992).

Isolation and Identification of Bacterial Strains: Isolation of heavy metal resistant bacteria was done by using spread plate method on sterile nutrient agar. Plates were incubated at 37˚C for 48 hrs. After 48 hrs, plates were observed for growing bacterial colonies. On the basis of morphological differences (e.g. shape, size and color) different bacterial colonies were picked up and repeatedly streaked on nutrient agar plates to get pure culture (Raja *et al.,* 2009). Identification of isolated bacterial cultures was done on the basis of morphological, physiological and biochemical characteristics. Different tests were used to identify the isolates according to Bergey's Manual of Systematic Bacteriology (Bergey *et al.,* 2005). Isolated colonies of purified strains grown on solidified agar plates were observed and data was recorded regarding the form (circular, filamentous and irregular); elevation (flat, convex and umbonate); margin (entire, undulate, erose and filamentous) and optical feature (opaque, translucent and transparent) of the colonies (Pelczar *et al.,* 1958). Cells were observed after Gram staining under the microscope (oil immersion, 100 X) (Duguid *et al.,* 1989). For biochemical characterization Hi25™ Enterobacteriaceae Identification Kit was used. The kit contains a combination of 25 biochemical tests.

Determination of Minimum Inhibitory Concentration (MIC) of metals for growth of bacterial colonies: The MIC of the heavy metals (Pb, Cd, Cu, Ni, Zn and Mn) for different isolated strains (VX1, VX2, VX3, VX4 and VX5) was determined according to Summers and Silver with some modifications (Summers and Silver., 1972).Maximum resistance of the selected isolates against increasing concentrations of heavy metals was evaluated by agar plating techniques until the strains unable to grow colonies on the agar plates (Xin *et al.,* 2006). The lowest concentration of heavy metals that completely prevent the growth is known as MIC (Yilmaz., 2003).

Antibiotic Resistance Test: The isolated bacterial strains were checked for antibiotic resistivity and sensitivity using single disc diffusion method (Bauer *et al.,* 1966).

RESULTS AND DISCUSSION

Data given in the table 1 shows analytical data of various physiochemical parameters set according to General standards for Discharge of Environment Pollutants, Part A: Effluent as per Schedule VI of the Environment (Protection) Rules 1986.

A total of 5 morphologically different bacterial colonies were picked and streaked on nutrient agar plates. These bacterial strains were isolated from lead battery manufacture and recycling small scale industries and named as VX1, VX2, VX3, VX4 and VX5. After that all these bacterial strains were tested for tolerance level against six heavy metal salts (Pb, Cd, Cu, Zn, Mn and Ni) as shown in table 2.

Gram staining was performed with all the isolated bacteria to characterize their morphological features. VX1 and VX2 was found gram negative rod. VX3, VX4 and VX5 were found gram positive rod. Results of physiological characterization are given in the table 3.

Table 1. Analysis of industrial effluent of electroplating industry.

No	Characteristics	Lead-battery industrial effluent	EPA standards
1.	Color and odor	Turbid	-
2.	TDS	1750mg/L	2100
3.	pH value	5.5	5.5 to 9
4.	Temperature	27˚C	45˚C
5.	Oxidation and Reduction Potential (ORP)	1570mV	
6.	Conductivity	4200µS	3000 µS
7.	Salinity	3.5ppm	-
8.	Oil and grease mg/L	2.403	10
9.	Dissolved oxygen mg/L	10.3	-
10.	Cadmium (as Cd) mg/L	0.024	2
11.	Copper (as Cu) mg/L	2.9	3
12.	Iron (as Fe) mg/L	0.144	3
13.	Lead (as Pb) mg/L	4.09	0.1
14.	Manganese (as Mn) mg/L	1.98	2
15.	Nickel (as Ni) mg/L	0.328	3
16.	Zinc (as Zn) mg/L	376.28	5

VX3 and VX5 were grown at temperatures ranging from 20 to 45˚C. Optimal temperature for both the strains was 30 to 35. Bacterial strain VX3 and VX5 have shown the ability to grow at salt concentration as high as 6%. Both the isolates have the capability to grow at pH ranging from 6-10. Their optimum pH range was found to be 6 to 8. Two best bacterial strains VX3 and VX5 were analyzed for biochemical characteristics. For this a total of 25 biochemical tests were performed on the Hi25™ enterobacteriaceae Identification Kit. This kit contains two test strips. Strip 1 contains 12 biochemical tests and strip 2 contains 12 carbohydrate metabolism tests. The results of biochemical tests are shown in table 4.

Minimum inhibitory concentration (MIC) of various strains showed minimum concentration at which bacterial strain is able to survive efficiently. From this study it was conclude that at initial VX3 and VX5 showed promising results against maximum heavy metals. VX4 showed moderate growth against Mn, Zn and Pb. VX2 and VX1 were only resistant to Mn.

Table 5 shows that bacterial strains VX3 and VX5 showed resistance against kanamycin but sensitive towards other antibiotics.

Table 2. Multiple heavy metal resistance and MIC against five bacterial isolates.

Metal	MIC (µg/ml) concentration of metals in mM				
	VX1	VX2	VX3	VX4	VX5
Nickel (Ni2+)	1	1	4	1	12
Lead (Pb)	1	3	15	4	15
Cadmium (Cd)	1	1	4	1	1
Copper (Cu)	1	2	6	1	4
Zinc (Zn)	3	4	6	4	6
Manganese (Mn)	4	6	25	10	30

Table 3. Physiological characteristics of bacterial strains (VX1, VX2, VX3, VX4 and VX5)

Characteristic		Name of bacterial strain	
		VX3	VX5
Temperature	20	+	-
	25	++	+
	30	+++	+++
	35	+++	+++
	40	++	+
	45	-	-
Salt Tolerance	2	+++	+++
	4	++	++
	6	+	+
	8	+	-
	10	-	-
pH	4	-	-
	6	+	+
	7	+++	+++
	8	+++	+++
	10	+	+
	12	-	-

(+, scant growth) (++, moderate growth) (+++, abundant growth) (-, no growth)

Table 4. Biochemical tests of bacterial strains (VX3 and VX5)

S. No.	Names of biochemical test	Name of bacterial strain	
		VX3	VX5
1.	ONPG	-	+
2.	Lysine utilization	-	-
3.	Orinthin utilization	-	+
4.	Urease detection	-	-
5.	Phenylalanine Deamination	+	V
6.	Nitrate reduction	+	+
7.	H2S production	-	-
8.	Citrate utilization	V	+
9.	Voges Proskauer's	-	+
10.	Methyl Red	V	-
11.	Indole test	+	V
12.	Malonate utilization	-	V
13.	Esculin hydrolysis	-	+
14.	Arabinose	-	+
15.	Xylose	-	+
16.	Adonitol	+	-
17.	Rhamnose	-	+
18.	Cellobiose	-	+
19.	Melibiose	-	+
20.	Saccharose	V	+
21.	Raffinose	-	+
22.	Trehalose	-	+
23.	Glucose	+	+
24.	Lactose	-	+
25.	Oxidase test	-	+

+ Positive (more than 90%), - Negative (more than 90%) and V= 11-89% Positive.

Our study deals with two main aspects, firstly, analysis of untreated electroplating industrial effluent according to IOS methods set under EPA norms and secondly, biologically remediating this problem caused due to this waste to the environment.Electroplating industry effluent is discharging heavy metals like Ni, Zn, Mg, Mn, Pb, Cu and Cr into soil and water bodies. Lead is one of the most abundant heavy metals in nature. Maximum permissible limit for lead in drinking water is 0.01 mg/L and for industries is 0.1 mg/L. But the industrial sample analysed in our studies contained extremely might amount of lead after that is (4.09 mg/L) after analysis was extremely high. In our study extremely high concentration (376.28 mg/L) of zinc was present in the industrial effluent samples which are very high as compared to the EPA standards (5 mg/L). The concentration value of 2.9 mg/L obtained for Cu was on the line of accepted standard. However, rest of the metal concentrations were within the acceptance limits (Ni 0.328mg/L, Fe 0.144mg/L, Cd 0.024mg/L, Mn 1.98mg/L). Similar studies have also been done by (Khan *et al.,* 2008). Khan studied the effect of heavy metals in contaminated crops irrigated with waste water and its associated health risks. Results indicate that there is a substantial buildup of heavy metals in waste water-irrigated soils, collected from Beijing (China).

Table 5. Antigram study of isolated bacterial strains against different antibiotics.

Antibiotic	Disc content (mcg)	Diameter of zone against bacterial isolates (mm)	
		VX3	VX5
Neomycin	30	25	29
Ciprofloxacin	5	30	27
Amoxicillin	10	15	12
Kanamycin	5	8	9
Ceftazidime	30	16	21

(mcg- microgram)

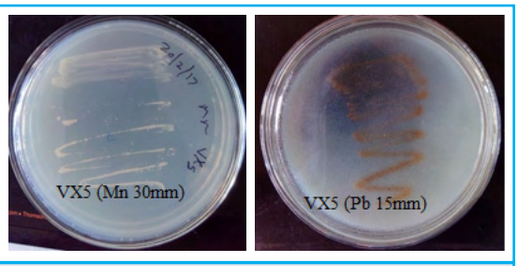

FIGURE 1. Bacterial strain VX5 growing at various metal concentrations Mn 30mm and Pb 15mm

High oxidation and reduction potential (ORP) is convenient for quickly disinfecting the water but leads to decrease in pH and increase in dissolved solids. Increase in the number of free ions present in water body leads to progressive rise in conductivity and salinity. Sample analysis revealed values of ORP (1570mV), conductivity (4200mS), TDS (1750) and salinity (3.5ppm) which are intolerant to most of the microbial species. Dissolved oxygen is necessary for aquatic ecosystem. Dissolved oxygen level when drops below 5mg/L cause stress to aquatic life. Oil and grease reduces the availability of dissolved oxygen affecting the aquatic system. Sample analysed had oil and grease at a permissible limit of 2.403mg/L. DO value of our sample was found to be 10.3mg/L. Similar study was carried out by Sugumaran (2014) for the analysis of electroplating industry effluent which showed high concentration of suspended solids (200 or 300 mg/L), heavy metals (300 to 600 mg/L) and cyanide.

Metals exert toxic effect on microorganisms through various mechanisms resulting into development of metal resistant microbial diversity in these habitats. Their potential skills could be used for bioremediation in heavy metal industries. In the present study, we have isolated 12 bacterial isolates from two different samples. Six heavy metal salts ($MnSO_4$, $C_4H_6O_4Pb$, $CuSO_4$, $ZnSO_4$, $CdCl_2$ and $NiSO_4$) which were screened for heavy metal tolerance against bacterial strains VX1, VX2, VX3, VX4 and VX5 were selected after primary screening at conc. of 1mM for further study.The presence of Pb, Mn, Cu, Cd, Zn and Ni

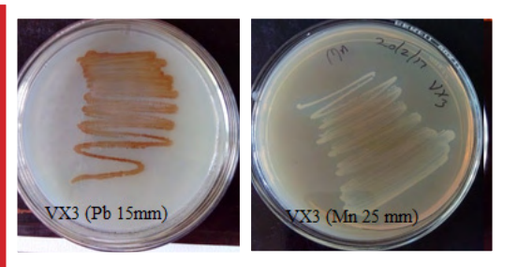

FIGURE 2. Bacterial strain VX3 growing at various metal concentrations Mn 25mm and Pb 15mm.

in growth medium had direct effect on colonial morphology and growth of isolated bacterial strains. The isolates showed different tolerance level for these metals.

Bacterial strain VX3 and VX5 have shown high resistance towards lead. Both the bacterial strains were identified as *Providencia sp*. and *Enterobacter sp*. These species could tolerate as high as 15mM concentration of lead. This study is quite significant because very limited species of bacteria show so much resistance for lead till now.Roane (1999) carried out study in which the overall mechanisms of a lead-resistant *Pseudomonas marginalis* and *Bacillus megaterium*. These bacterial strains tolerated lead concentration up to 2.5mM which is significantly lower than the concentration tolerated by VX3 and VX5. In addition, VX3 has also shown good resistance against Pb (15mM), Cd (4mM), Cu (6mM), Zn (6mM), Ni (4mM) and Mn (25mM). Similarly VX5 showed high resistance against Pb (15mM), Cd (1mM), Cu (4mM), Zn (6mM), Ni (12mM) and Mn (30mM).

CONCLUSION

Heavy metals are important to human beings in many aspects, especially in the manufacturing of important products, such as accumulators (Pb), utensils (Al), mercury-arch lamps and a wide range of other products. But the toxic effects, when unduly exposed, could be potentially life threatening hence, cannot be neglected. Heavy metals possess high threat to mankind and other living organisms. The effluent collected from the contaminated site contained high amount of heavy metal contamination, particularly Zinc which was found to be 70 times more than the permissible limit. This critical situation leads to an urgent call for remediation without further affecting our environment. Further study is needed to resolve the issue of heavy metal contaminants from the soil. Considering above situation we have isolated heavy metal resistant bacteria which could survive even at higher concentrations of heavy metals. Their ability to attain life in such hostile conditions makes them ideal for bioremediation purposes. Bacterial strains VX3 and VX5 identified as *Providencia sp*. and *Enterobacter sp*. were found to be specifically resistant to lead. The isolated microbes could be potential remedial measure used for bioremediation of heavy metals polluted sites.

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Agricultural Communication

Biosci. Biotech. Res. Comm. 12(2): 446-455 (2019)

BBRC Bioscience Biotechnology Research Communications

Studies on interaction of rice and bacterial leaf blight causing *Xanthomonas oryzae* pv. *oryzae*

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ABSTRACT

Bacterial leaf blight pathogen, *Xanthomonas oryzae* pv. *oryzae* is a major biotic constraint in rice production which eventually make the global food grain sustainability under stress. Host resistance being the only strategy of management of the disease, the present study focused on screening of 28 near isogenic lines and/or varieties under field condition which revealed resistant reaction of the following lines *viz*., IRBB 21, IRBB 57, IRBB 58 and IRBB 61 against *Xanthomonas oryzae* pv. *oryzae* (NCBI Accession No. MH464904). Besides, R genes *viz*., *Xa21*, *xa5* and *xa13* were identified in these near isogenic lines where different combinations of genes have been identified. With the intention of understanding the kind of avr genes present in X. o. pv. oryzae isolate and thereby elucidating the kind of interaction persists among rice and bacterial leaf blight pathogen, gene specific amplification and identification of avr genes were performed. The results revealed that *avr* genes *viz*., *PthXo1*, *avrxa3*, *AvrXa10*, *Avr/pthC8b* and *Avr/pth56B* were positively amplified whereas *Avr/pth3* was absent in the isolate even at repeated attempts. Interaction of *X.* o. *pv*. oryzae against ADT 38 rice variety through SDS PAGE analysis of the pathogen and abiotic agents (Biotin (0.1mM), Riboflavin (0.5mM), Chloramphenicol (0.1mM), Ethrel (1µl/ml)) applied samples showed that defense related proteins were induced alike in all the samples treated irrespective of the treatment when compared to untreated control.

KEY WORDS: *AVR* GENE BACTERIAL LEAF BLIGHT, R GENE, RICE

INTRODUCTION

Plants are confronted consistently with pathogens and based on the molecular determinants in plants (Pathogen recognition receptor (PRRs), Resistance genes) and in pathogen (Pathogen associated molecular patterns (PAMPs), *Avr* genes) both interacts to build defense responses where PRRs and PAMPs interacts to provide broad spectrum resistance (horizontal resistance) whereas R genes and *Avr* genes interaction may result in gene

ARTICLE INFORMATION:

Corresponding Author: pn40785@gmail.com
Received 7th April, 2019
Accepted after revision 6th June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/30

specific resistance (vertical resistance) (Vander Plank, 1984). Rice is one of the most significant cultivated food crops which feeds half of the population worldwide where the demand for rice is progressively increasing in developing countries (Khush and Jena, 2009) whereas bacterial leaf blight pathogen, *Xanthomonas oryxae* pv. *oryzae* (*Xoo*) becoming one among the important delimiting biotic factor that reduces rice production up to 81 per cent (Kumar *et al.* 2012). Host resistance is the only management strategy that could strongly dependent to contain the disease as the practice of application of chemicals in management of disease fronting adverse backlashes (Rajpurohit *et al.* 2011, Dokku *et al.* 2013, Suh *et al.* 2013; Sundaram *et al.* 2014; Arunakumari *et al.* 2016; Hajira *et al.* 2016; Gao *et al.* 2018).

Hence, most favorable strategies for crop improvement program for the disease resistance is either selection of donor source of resistance against *Xoo* and thereby using for resistance breeding or by introduction of resistance genes into desired variety or cultivars. Since understanding of interaction between rice and bacterial leaf blight pathogen is still remain ambiguous, researches on elucidating the mechanism of interaction and defense response has significant role. Interaction between rice and bacterial leaf blight pathogen is tend to follow the classical gene-for-gene relationship (Flor 1971), however, there exists distinctive differences for the interaction of R genes of bacterial leaf blight disease from characterized R genes of other crops where researchers also identified closely linked molecular markers (Yoshimura *et al.* 1995, Sonti 1998; Rao *et al.* 2002, Gu *et al.* 2008; Khan *et al.* 2015; Arunakumari *et al.* 2016; Hajira *et al.* 2016; Chukwu *et al.* 2019).

Exploration, identification, and utilization of new resistant germplasms in rice breeding are the strategical steps to control the bacterial blight disease of rice. The *Xa21* gene has been successfully introgressed into several elite rice varieties and hybrid rice parental lines all over the world either singly or in combination with other major resistance genes such as *Xa4*, *xa5*, and *xa13* (Singh et al. 2001; Joseph et al. 2004; Sundaram et al. 2008; Sundaram et al. 2009; Perumalsamy et al. 2010; Pandey et al. 2013).

Most of the reported R genes of other crop-pathogen systems are dominant in nature, unlike in rice where one-third of the R-genes conferring resistance to bacterial leaf blight disease have been reported as recessive (Verdier *et al.* 2012). Forty resistant genes, *Xa1* to *Xa39* have been identified in rice system which were against bacterial leaf blight pathogen (Kim *et al.* 2015).

Such R genes correspondingly recognize *Avr* gene products of the bacterial leaf blight pathogen directly or indirectly which could mediate defense response. However, such responses to contain the disease never ensue often in a host-pathogen system as the pathogen could evolve and evoke the virulence to cause the disease with distinct avirulence and virulence factor. Hence, screening of rice lines against various strains or pathotypes of bacterial leaf blight pathogen has prominent role to play. TAL effector dependent R genes induces downstream expression of R genes, for example, *Xa10* contains a binding element for the TAL effector, *AvrXa10* (*EBEAvrXa10*) in its promoter, and which induces *Xa10* expression (Tian *et al.* 2014). Hence, *Avr* and R gene plays imperative changes in host system to combat bacterial leaf blight disease in rice. Therefore, identification of the presence of *Avr* genes in the pathotypes prevailing in each location and its virulence nature against the cultivars and Near Isogenic Lines (NILs) could help in developing resistant donor parent for the crop improvement program through resistant breeding. Moreover, for successful deployment of stable resistance genes, their characterization and availability of tightly linked markers will greatly facilitate the development of new versions of cultivars (Vikal and Bhattia, 2017).

The present study is focused on identification of R genes present in NILs which confer downstream defense response after recognition (directly or indirectly) of *Avr* genes and also the marker assisted genotyping of major R genes of bacterial leaf blight of rice.

MATERIAL AND METHODS

Bacterial leaf blight symptom of rice was isolated from various locations *viz*., Coimbatore, Aduthurai, Gudalur, Krishnagiri, Vellur, Theni, Wayanad, Hyderabad and New Delhi during the survey and were brought into laboratory for isolation of the pathogen under *in vitro* condition. Briefly, under *in vitro,* the diseased samples collected were sliced off into leaf bits containing both the healthy and infected portions were transferred into an Eppendorf tube containing sterile water. Later, the leaf bits were crushed using sterile rod to release bacterial colonies as ooze and the loopful of suspension was streaked onto the autoclaved, solidified Peptone sucrose agar media ($CaNO_3$-0.5g, $FeSO_4$. $7H_2O$- 0.5g, sucrose-15g, peptone-5g, Na_2HPO_4. $7H_2O$-2g, agar-15g, distilled water-1000ml) poured into the Petri dish. Triplicates of samples were maintained for isolation and were incubated at 25°C for the formation of yellow, mucoid, doom shaped colonies with entire margins were developed and were sub cultured for further studies. Pathogenicity of the isolate was proved in bacterial leaf blight susceptible varieties *viz.,* ADT 38 and TN1. Seeds of the susceptible varieties were collected in a jute bag and immersed overnight in water and on next day, the soaked seeds in jute bag were kept overnight, air tightened inside the hay to sprout. Sprouted seeds were collected on the

next day for sowing in pots filled with clay loamy soil. Seedlings emerged from the pots were transplanted to another one after 15 days of sowing which were maintained in a growth chamber of temperature of 25^{0}C and 85-90% relative humidity. Forty eight hours old bacterial suspension prepared in nutrient broth conditioned to 0.1 value in spectrophotometer at 600nm were employed for testing pathogenicity nature of the isolate through clip inoculation method (Kauffman *et al.* 1973).

Concisely, clip inoculation method was performed into the seedlings with maximum tillering stage where surface sterilized scissors were used to nick out the top 5cm of leaves of the seedling after dipping into the bacterial suspension prepared earlier in the nutrient broth. Remaining bacterial suspension was sprayed onto the cut ends and margins of leaves to permeate the entry of bacterial colonies into the leaves and cause the infection. Observation for symptom appearance from the day of inoculation was recorded and the organism was re-isolated from the lesion and compared with that of the original isolate and hence proved the pathogenicity. Characterization of bacterial leaf blight pathogen was instigated by isolation of total genomic DNA from the bacteria which was performed using lysis buffer method (Chen and Kuo, 1993) where the bacterial culture was multiplied in 100 ml nutrient broth kept for 48hr in rotary shaker at 180rpm. Saturated culture was harvested in 1.5 ml of Eppendorf tube and allowed for centrifugation for 3 min at 12,000 rpm. 200 µl of lysis buffer (40 mM Tris-acetate pH 7.8, 20 mM sodium-acetate, 1 mM EDTA, 1% SDS) was suspended in the cell pellet and lysed by vigorous pipetting. 66 µl of 5M NaCl was added and mixed well which remove most proteins and cell debris and then the viscous mixture was centrifuged at 12,000 rpm for 10 min at 4°C.

Clear supernatant obtained after centrifugation was transferred into new vial and an equal volume of chloroform was added followed by vortex until the solution turned milky. Subsequently, centrifuged the solution at 12,000 rpm for 3 minutes, and the supernatant extracted was transferred to another vial and the DNA was precipitated with 100% ethanol, washed twice with 70% ethanol, dried, and redissolved in 50 µl of 1 x TE buffer. Later, the quality of the DNA was assessed by 1.2% agarose gel electrophoresis. To assess the genetic identity of the pathogen, PCR was performed using 16s rRNA primers (27F: 5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') which shows the amplicon size of 1537bp. The PCR product was outsourced for sequencing to identify upto the species level of bacteria. Hence, the bacteria identified as *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) through molecular tools were used for screening of bacterial leaf blight pathogen against rice cultures in field. Thirty one rice lines were sown in rice beds and were transplanted to fields after 20 days of sowing such that each lines had a minimum of ten hills in a row. Integrated nutrient management practices were followed as per the package of practices for rice crop. Maximum tillering was obtained at 45 days old crop and in which clip inoculation was carried out following the method of Kauffman *et al.* (1973) described above. As the bacterial infection spread from the cut ends of the leaves, the lesion length was recorded at 7 and 14 days after inoculation of bacterial suspension and the lesion length was compared with that of the resistance reaction to bacterial leaf blight mentioned in Standard Evaluation System of Rice.

Screening of these culture lines were carried out twice during the period of 2017-18 and 2018-19 and confirmed the resistant reaction nature of the culture lines in comparison with the existing susceptible cultivars. To understand the interaction between host and pathogen, presence of resistance genes and *avr* genes in rice cultures and *X. o.* pv. *oryzae* respectively were identified using polymerase chain reaction amplification through the gene specific primers. Genomic DNA of thirty one rice lines were isolated using conventional CTAB method in which 0.5g of leaf samples were ground with 500µl of CTAB buffer using pestle and mortar. The contents were transferred to a vial and kept in water bath at 65^{o}C for 15 minutes followed by cooled at room temperature. Chloroform and Isoamyl alcohol mixture at 24:1 ratio were prepared and equal volume of the solution was added into vial and centrifuged at 12,000 rpm for 15 minutes. Aqueous phase thus formed was transferred into a new vial and poured equal volume of ice cold isopropanol and incubated overnight at -20^{o}C. Pellet was separated from the solution by centrifugation at 12,000rpm for 15 minutes and added 200µl of 70% of ethanol. Air dried the pellet and kept the pellet dissolved in sterile water at -20^{o}C for further studies.

Genomic DNA in each rice lines were checked at 0.8% of agarose gel electrophoresis. A total of 25µl of reaction mixture for polymerase chain reaction amplification of genomic DNA of 31 rice lines were performed. Primers *viz., xa5* (F: 5' GCACTGCAACCATCAATGAATC 3'; R: 5' CCTAGGAGAAACTAGCCGTCCA 3'), *xa13* (F: 5' CCTGATATGTGAGGTAGT 3'); R: 5' GAGAAAGGCTTAAGTGC 3') and *PTA 248* (F: 5' AGACGCGGGAAGGGTGGTTCCCGGA 3'); (R: 5' AGACGCGGGTAATCGAAAGATGAAA 3') (Robert *et al.* 1992) each at 100pm/µl were used to identify the presence of R genes (*xa5, xa13* and *Xa21*).

Initial denaturation of 5 min at 94°C followed by 40 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 55°C for *xa5 and xa13* gene primers and 59^{o}C for *Xa21* gene primer, and 1 min of extension at 72°C and final extension was 10 min at 72°C. PCR amplification of genomic DNA of *X. o.* pv. *oryzae* was performed using

Avr gene primers specific to *avrxa3 gene, Avr/pth3 gene, Avr/pth56B, Avr/pthC8b* and *AvrXa10, Pth/Xo1* (Table 4.). PCR conditions followed were initial denaturation of 5 minutes at 94°C followed by 40 cycles of 1 minute of denaturation at 94°C, 1 minute of annealing at 55°C and 1 minute of extension at 72°C and final extension was 10 minutes at 72°C. Resistance genes in host and *avr* genes in the pathogen are the important molecular determinants that downstream the defense mechanism in host system by induction of secretion of several proteins.

An experiment was laid out to detect the presence of proteins secreted after bacterial leaf blight susceptible rice variety, ADT 38 was confronted with the pathogen and abiotic agents *viz.,* Biotin (0.1mM), Riboflavin (0.5mM), Chloramphenicol (0.1mM), Ethrel (1µl/ml). Virulent bacterial colonies were inoculated into nutrient broth and incubated for 48hr in BOD incubator with 180rpm till the spectrophotometer read 0.1 at 600nm. Bacterial suspension was clip inoculated into the 40 days old plant followed by abiotic agents were applied 24 hr after the inoculation. Leaves were collected 24hr after application of abiotic agents separately from each and were subjected to protein extraction for SDS PAGE analysis to detect the presence of protein fractions secreted.

RESULTS AND DISCUSSION

Organism causing bacterial leaf blight disease in rice was isolated from the leaf samples collected during the survey in Coimbatore, Aduthurai, Gudalur, Krishnagiri, Vellur, Theni, Wayanad, Hyderabad and New Delhi under *in vitro* condition. The virulent strains of the bacterial leaf blight isolates were demarcated with that of the non-virulent one as the former appeared yellow, round, mucoid, convex colonies after 72 hr of incubation whereas the latter remained white, slimy, mucoid and convex as detailed by Webster and Gunnell (1992). Pathogenicity tests for 9 isolates were proved for all the 9 isolates in ADT 38 and TN1 which showed initial symptom of the disease after 7 days of inoculation. Virulence spectrum of *X. o.* pv. *oryzae* isolates were compared after inoculating in ADT 38 and TN1 varieties revealed that Coimbatore isolate imparted highest virulence than any other. While comparing the lesion size formed by each isolate, highest lesion size was observed in the variety inoculated with Coimbatore isolate whereas the least was observed with New Delhi isolate.

Genomic DNA of nine bacterial leaf blight pathogens were extracted and the molecular characterization of the nucleic acids using 16S rRNA primers showed amplification at 1200 bp and partial sequencing of the isolates revealed the identity of the organism as *Xanthomonas oryzae* pv. *oryzae*. Screening of 29 rice lines along with improved samba mahsuri (resistant check) and TN1 (Susceptible check) against bacterial leaf blight pathogen (Coimbatore) showed formation of typical bacterial leaf blight symptom after 7 days of inoculation. Lesion size were recorded during 7th and 14th day of inoculation, revealed the resistant reaction offered against the pathogen by the rice cultures. When compared to the resistant check, improved samba mahsuri, rice cultures showed better resistive potential. Three rice cultures *viz.,* IRBB 57, IRBB 58, IRBB 61 were categorized as resistant among the 29 culture lines according to the Standard Rice Evaluation System of IRRI, Philippines whereas 11 were moderately resistant (IRBB 7, IRBB 8, IRBB 11, IRBB 13, IRBB 14, IRBB 21, IRBB 50, IRBB 56, IRBB 63, IRBB 64 and IRBB 66) and 13 culture lines (IRBB 1, IRBB 3, IRBB 4, IRBB 5, IRBB 10, IRBB 51, IRBB 52, IRBB 53, IRBB 54, IRBB 55, IRBB 59, IRBB 60 and IRBB 62) were moderately resistant (Table 1, Table 2).

Similarly, Bharathkumar *et al.* (2014) also tested the resistance reaction of rice and bacterial leaf blight pathogen and categorized pathotypes of *X. o.* pv. *oryzae* isolates prevailing in different states of India. Incongruence with the present study, Rajappan and Ravi (2015) evaluated twenty two gene pyramided rice cultures against bacterial leaf blight disease to estimate the resistant reactions against the disease during 2012-2015. These rice cultures were gene pyramided with resistance genes (*Xa1* to *Xa21*) to bacterial leaf blight and with the combinations of the same which in turn give resistive nature to the lines. Identification of such combinations of R genes in the lines are advantageous for the identification of donor parent intended to development of a resistant varieties. Gene specific primers pertaining to *xa5, xa13* and a functional marker of *Xa21* gene (*PTA 248*) were amplified using rice genomes of twenty eight rice cultures and three cultivars where the results showed that polymorphic genotypes attained after gel electrophoresis were directly related to the resistant nature of the lines (Table 3).

Gene specific amplification of *Xa21* marker in 31 rice lines/varieties, 8 lines showed (IRBB 11, IRBB 55, IRBB 56, IRBB 57, IRBB 58, IRBB 59, IRBB 60, IRBB 61, IRBB 64, IRBB 65 and IRBB 66) amplification at 900 bp thereby confirmed the presence of *Xa21* gene (Figure 1). Similarly, presence of *xa5* gene amplification established after the comparison of gene amplification pattern similar to that of IRBB 5. The results revealed that IRBB 50, IRBB 59, IRBB 60, IRBB 61, IRBB 63, IRBB 64, IRBB 66 showed similar *xa5* gene marker amplification at 270 bp (Figure 2). In the case of *xa13* gene, rice lines *viz.,* IRBB 51, IRBB 53, IRBB 54, IRBB 56, IRBB 58, IRBB 60, IRBB 61, IRBB 63, IRBB 65 were showed similar banding pattern of that of IRBB 13 which remarked the presence of *xa13* gene (Figure 3).

Table 1. Field screening of rice cultures against bacterial leaf blight pathogen during 2017-18

Sl. No	Rice lines	Average lesion size (cm) 7th day	Average lesion size (cm) 14th day	Resistant reaction
1.	IRBB 1	7.5^{l}	8.2^{ijk}	MS
2.	IRBB 3	5.6^{f}	6.6^{efgh}	MS
3.	IRBB 4	7.8^{l}	8.2^{ijk}	MS
4.	IRBB 5	6.8^{ijk}	7.0^{ghi}	MS
5.	IRBB 7	3.9^{c}	4.2^{bc}	MR
6.	IRBB 8	4.8^{d}	5.0^{cde}	MR
7.	IRBB 10	5.1^{e}	5.5^{def}	MR
8.	IRBB 11	6.1^{hi}	6.6^{efgh}	MS
9.	IRBB 13	3.6^{c}	4.2^{bc}	MR
10.	IRBB 14	3.6^{c}	4.3^{bc}	MR
11.	IRBB 21	1.8^{a}	2.2^{a}	R
12.	IRBB 50	4.6^{d}	5.0^{cde}	MR
13.	IRBB 51	6^{gh}	6.6^{efgh}	MS
14.	IRBB 52	6.7^{ij}	7.0^{ghi}	MS
15.	IRBB 53	6.7^{ij}	7.0^{ghi}	MS
16.	IRBB 54	5.7^{fg}	6.6^{fghi}	MS
17.	IRBB 55	6.7^{ij}	7.0^{ghi}	MS
18.	IRBB 56	6.8^{k}	7.2^{ghif}	MS
19.	IRBB 57	2.6^{b}	2.9^{a}	R
20.	IRBB 58	2.5^{b}	2.8^{a}	R
21.	IRBB 59	6^{fg}	6.5^{efg}	MS
22.	IRBB 60	7^{k}	7.3^{hij}	MS
23.	IRBB 61	2.7^{b}	3.2^{ab}	MR
24.	IRBB 62	6.7^{ij}	7.5^{hij}	MS
25.	IRBB 63	3.7^{c}	4.3^{bcd}	MR
26.	IRBB 64	5.8^{fg}	6.6^{fghi}	MS
27.	IRBB 65	6^{fg}	6.7^{fghi}	MS
28.	IRBB 66	4.6^{d}	5.2^{cdef}	MR
29.	DV-85	2.8^{b}	3^{ab}	MS
30	ISM	5.9^{fg}	5.6^{def}	MR
31.	TN1	8^{m}	8.8^{jk}	MS
	CD (0.05)	0.311	1.37	

HR: Highly Resistant (<1cm); R: Resistant (1-3cm); MR: Moderately Resistant (3-6cm); Moderately Susceptible (6-10cm); Susceptible (>10cm)

Genotyping of *Xa21, xa5* and *xa13* genes showed that all the three genes were present in IRBB 60 and IRBB 61. *Xa21* and *xa5* genes were present in IRBB 59 alone whereas *xa5* and *xa13* genes were amplified in IRBB 63 and IRBB 65 lines. To contain the bacterial leaf blight of rice, researchers paved major attention in introgression of multiple resistance genes through breeding program. Guvvala *et al.* (2013) have experimented gene pyramiding of R genes *viz., Xa4, xa5, xa13* and *Xa21* into bacterial leaf blight susceptible mahsuri variety and various such related works are in progress (Pinta *et al.* 2013, Pradhan *et al.* 2015, Chukwu *et al.* 2019).

Hence, the present study to identify the donor parents carrying R genes for resistance breeding program

Table 2. Field screening of rice cultures against bacterial leaf blight pathogen during 2018-19

Sl. No	Rice lines	Average lesion size (cm) 7th day	Average lesion size (cm) 14th day	Resistant reaction
1.	IRBB 1	6^{def}	8^{f}	MS
2.	IRBB 3	6^{def}	8^{f}	MS
3.	IRBB 4	6^{def}	8^{f}	MS
4.	IRBB 5	7^{f}	7^{f}	MS
5.	IRBB 7	2^{a}	4^{abc}	MR
6.	IRBB 8	5^{bcd}	5^{bcd}	MR
7.	IRBB 10	6^{def}	8^{f}	MS
8.	IRBB 11	4^{abc}	6^{def}	MR
9.	IRBB 13	4^{abc}	6^{def}	MR
10.	IRBB 14	4^{abc}	6^{def}	MR
11.	IRBB 21	2^{a}	4^{abc}	MR
12.	IRBB 50	5^{bcd}	6^{def}	MR
13.	IRBB 51	6^{def}	8^{f}	MS
14.	IRBB 52	7^{ef}	7^{f}	MS
15.	IRBB 53	7^{ef}	7^{f}	MS
16.	IRBB 54	6^{def}	8^{f}	MS
17.	IRBB 55	7^{ef}	7^{f}	MS
18.	IRBB 56	4^{abc}	6^{def}	MR
19.	IRBB 57	3^{ab}	3^{ab}	R
20.	IRBB 58	3^{ab}	3^{ab}	R
21.	IRBB 59	5^{bcd}	5^{bcd}	MS
22.	IRBB 60	7^{ef}	7^{ef}	MS
23.	IRBB 61	3^{ab}	3^{ab}	R
24.	IRBB 62	7.66^{ef}	7.66^{ef}	MS
25.	IRBB 63	4^{abc}	6^{def}	MR
26.	IRBB 64	4^{abc}	6^{def}	MR
27.	IRBB 65	6^{def}	6^{def}	MR
28.	IRBB 66	5^{bcd}	5^{bcd}	MR
29.	DV-85	3^{ab}	3^{ab}	R
30	ISM	5^{bcd}	5^{bcd}	MR
31.	TN1	8.8^{f}	7.66^{f}	MS
	CD (0.05)		1.78	1.85

HR: Highly Resistant (<1cm); R: Resistant (1-3cm); MR: Moderately Resistant (3-6cm); Moderately Susceptible (6-10cm); Susceptible (>10cm)

has pursued its importance. In the bacterial leaf blight pathogen, *Xanthomonas oryzae* pv. *oryzae* (NCBI Accession No. MH464904) after gene specific amplification of *avr* genes *viz.*, *pthXo1* (433 bp), *avrxa3* (623 bp), *AvrXa10* (562), *Avr/pthC8b* (420 bp) and *Avr/pth56B* (623) were obtained whereas *Avr/pth3* gene was absent in the strain (Figure 4). As a testimonial to the above results, Wu *et al.* (2007) came into conclusion that *avrXa3* gene containing three nuclear localization signal (NLS) motifs which is consistently present in all members of the *avrBs3/pthA* family was identified in JXOIII strain of *X. o.* pv. *oryzae*. Numbers of the *avr/pth* genes vary among different strains of *X. o.* pv. *oryzae*.

Table 3. List of primers

S. No.	Genes	Nucleotides	Annealing temperature
1.	16S rRNA	27F: AGAGTTTGATCCTGGCTCAG 1492R: GGTTACCTTGTTACGACTT	55
2.	PTA248	F: AGACGCGGGAAGGGTGGTTCCCGGA R: AGACGCGGGTAATCGAAAGATGAAA	59
3.	xa13	F: CCTGATATGTGAGGTAGT R: GAGAAAGGCTTAAGTGC	55
4.	xa5	F: GCACTGCAACCATCAATGAATC R: CCTAGGAGAAACTAGCCGTCCA	55
5.	Avrxa3	F: CATCTTGTTCCCACATCACG F: GCCGGAATTGATCAGAAGAG	55
6.	Avr/pth3	F: AGGACATAATCAGGGCGTTG R: CCAATACGGCGATTGACTCT	55
7.	Avr/pth56b	F: GCCGGAATTGATCAGAAGAA R: CATCTTGTTCCCACATCACG	55
8.	Avr/pthC8b	F: GCCGGAATTGATCAGAAGAA R: CATCTTGTTCCCACATCACG	55
9.	AvrXa10	F: ATCTGCTCCGTCAGTTCGAT R: TGGCCTGTGTCCAACTGTAA	55
10.	PthXo1	F: GAGAGCATTGTTGCCCAGTT R: CTGAAGTAGGGGACGGTTTG	55

Similar to the identification of *avr* genes in individual strain, researchers have identified presence of 15 *avr3/pth* genes in Korean race 1 (KACC10331) (Lee *et al.* 2005) whereas Yang and White (2004) demonstrated 25–32 *avr/pth* genes in different Philippine strains. After inoculation of *X. o.* pv. *oryzae* suspension into rice lines carrying R genes, *avr* gene products interact correspondingly with that of R gene product and based on the downstream action of the corresponding gene interaction compatibility or incompatibility were established

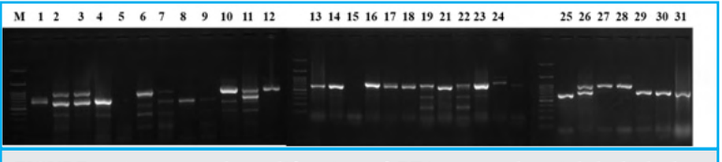

FIGURE 1. Agarose gel amplification of *PTA248* marker tightly linked to *Xa21* gene in 31 rice lines

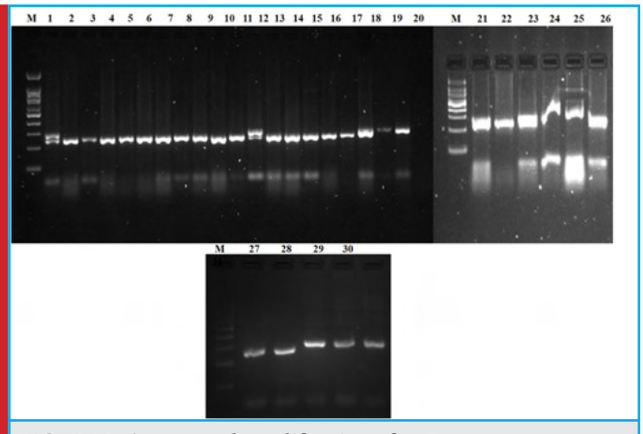

FIGURE 2. Agarose gel amplification of *xa5* gene

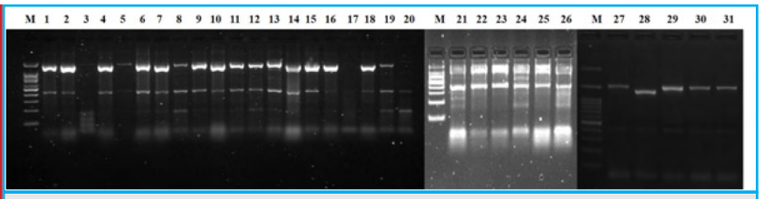

FIGURE 3. Agarose gel amplification of *xa13* genes

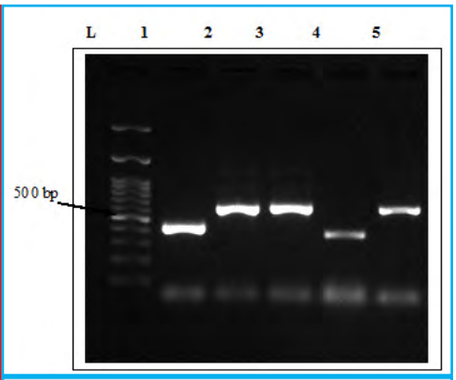

FIGURE 4. Agarose gel amplification of Avr genes (1. *PthXo1*, 2. *avrxa3*, 3. *AvrXa10*, 4. *Avr/pthC8b*,5. *Avr/pth56B*,) in *X.* o. pv. *Oryzae*

to cause disease susceptibility or resistance. Disease susceptibility gene, *Os8N3* or susceptible allele of recessive R gene *xa13* were found to be specifically induced by TAL effectors, *PthXo1* (Romer *et al.* 2010, Yin *et al.* 2017).

Hence, as *PthXo1* gene was present in the Coimbatore strain of *X. o.* pv. *oryzae,* it could induce the expression of disease susceptibility allele, *Xa 13* gene which thereby results in susceptibility of the line. *Avr/pth56B* and *Avr/pthC8b* were also identified in *X. o.* pv. *oryzae* isolate of Coimbatore, whereas there are ambiguity over the detailed evidence regarding the interaction with the R genes and those *avr* genes. In an extension to the identification type of R genes and *avr* genes present in the host and pathogen, to analyze the PR protein or other defense protein induction, SDS PAGE analysis was performed in rice variety, ADT 38, after inoculation of the pathogen followed by abiotic chemical agents (Biotin (0.1mM), Riboflavin (0.5mM), Chloramphenicol (0.1mM), Ethrel (1µl/ml)). The results revealed that comparing to the untreated control, all the treatments has induced the defense proteins consistently at 66.4 kDa thereby demonstrated that the susceptible rice variety could induce defense proteins with the application of abiotic agents (Figure 5). SERK2 protein is a 69 kDa proteins which is secreted as immune response mediated by the LRR receptor kinases, *Xa21* and the bacterial pathogen interaction. Hence, the apparent molecular weight of the protein observed in SDS-PAGE are consistent with that of SERK2 protein. In congruence to the above study, Wu et al. (2011) has conducted an experiment to compare the expression of PR proteins in compatible and incompatible interactions and results showed that in both the case the induction was noticed. Six out of ten PR proteins (PR1, PR2, PR3, PR4b, PR8, and PR-pha) showed enhanced expression in Xa21-mediated resist-

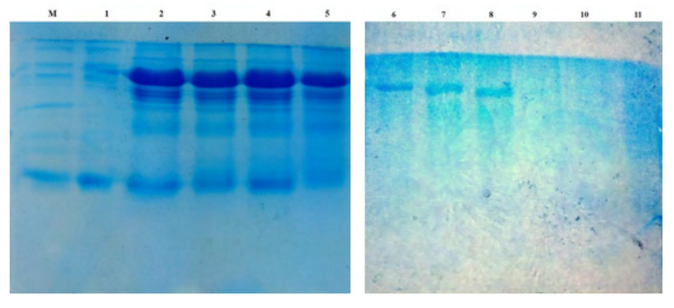

FIGURE 5. Defense protein induction in rice-*X.o.pv.oryzae* interaction by SDS PAGE analysis M- Protein ladder (14-96 kDa),1-control, 2-Biotin (0.1mM), 3-Riboflavin (0.5mM), 4-Chloramphenicol (0.1mM), 5-Ethrel (1µl/ml), 6-8- *Xoo* treated ADT 38, 9-10-Sterile water (control) treated ADT 38

ance responses at late stages after inoculation with *X. o.* pv. *oryzae* (Hou *et al.* 2011).

CONCLUSION

Interaction between rice and bacterial leaf blight pathogen, *Xanthomonas oryzae* pv. *oryzae* resulted in induction of defense related proteins which were observed through SDS PAGE analysis. The defense related proteins synthesized were mediated by the direct and/or indirect interaction of resistance genes and corresponding avirulence genes of rice cultures and *X. o.* pv. *oryzae* respectively. *PthXo1, avrxa3, AvrXa10, Avr/pthC8b* and *Avr/pth56B* were the A*vr* genes identified from *X. o.* pv. *oryzae,* Coimbatore isolate.

ACKNOWLEDGEMENTS

We acknowledge the help and cooperation of Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore, India. Department of Rice, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore, India

CONFLICT OF INTEREST

All authors have declared no conflicts of interest in this communication

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Technological Communication

Biosci. Biotech. Res. Comm. 12(2): 456-463 (2019)

BBRC Bioscience Biotechnology Research Communications

A comparative analysis between conventional profit making business and corporate social responsibility from information technology perspective

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ABSTRACT

This papers major aim is to determine the exclusivity of the concept "social business" with not just explaining the concept but also comparing it with other concepts seemingly that are similar to it such as social corporate responsibility and conventional profit-making businesses from information technology perspective. Through this paper we have given an in-depth understanding on how social business adds superior value, addresses social problems, and may motivate the business community to dedicate their talents and energy and invest money in this business.

KEY WORDS: SOCIAL BUSINESS, PROFIT MAKING BUSINESS, SOCIAL RESPONSIBILITY, CORPORATE SOCIAL RESPONSIBILITYTRADITIONAL BUSINESS, SOCIAL

INTRODUCTION

Gas (2012) reported that about 81 billionaires gave to charity close to about 50 percent of their fortune, while about 128 individuals and couple billionaires signed various contracts in theirdeath with donating their large portion of their wealth to a charitable cause. The first phases of the lives of successful business tycoons such as bill gates and Warren buffet where lived in building in a successful business structures while they are now using the second phases to generate more wealth and invest in businesses that will have a huge impact in the lives of the masses. The combination of the these two concepts which are business in generating profit for one's satisfaction and business for the satisfaction of the masses or community is what is referred to as social business, (Irene 2015, Ghaderi 2019).

Social Business can be said to be to inception of a firm whose main goal is centered in creation of benefit for a whole ecosystem which consist of the CEOs, share-

ARTICLE INFORMATION:

Corresponding Author: mzibari1@gmail.com
Received 9th April, 2019
Accepted after revision 15th June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/31

holders, customers and employees by making sure that the operations and culture of the business is integrated with collaborating, engaging and information sharing tools .The concept of social business begins with analyzing the concept of deciding if to be major person making most of the profit in the market system or if you will want a business which there are equal or more opportunities to everyone involved including the poor members of the community. It is more like a selfless cause with the creation of social wealth with the aim of making sure not only the firm benefits but also the community. The introduction of Social business concept was brought about in 2007 by professor Dr. Muhammad Yunus in who is a Nobel laureate making it an area of interest for various academicians and authors. Many of companies that are interested in the developing field of social businessare focuses on using business methods and practices to achieve progressive social revolution, which lead to Connecting financial to social performance, (Margolis and Walsh 2001 Hausmann 2018).

Just like every other normal business, products, employees, revenue, customers and so on are included in social business operations with the business incurring no loss or dividend but it refunds back the initial investment of the owner. A social business is completely different from a charity work in the sense that there is benefit or profit for everyone. The managers of social business also reasons and acts like the managers of normal profit making business company because it needs to recover back its capital to back the owner and also profit to sustain itself. The return of profits made back into the business makes social business have potential of growing and increasing in worth and value. Social business concept is one that combines the profit making business concept (which major aim is based on making and increasing profit) and also non-profit making organization (whose main aim is focused on charitable works). A social business the arrival of social business is one that is meant to occupy the gap created by rapid declining rate in human development and also lack of government intervention in the provision of social and basic amenities for its people, (Huda 2015).

RESEARCH QUESTIONS AND METHODOLOGY

Research Questions

The introduction part of this project has given an elaboration on what social business, corporate social responsibility and also traditional or conventional profit-making business. This project will help in giving elaborate answers to the following questions:

- What is the concept behind social business?
- What are the difference and similarities between social business and CSR?
- What are the difference and similarities between social business and Conventional profit making businesses?

Research Methodology

This research was carried out based on anintegrative review methodology where relevant articles based on the research scope with key words such as business, social business, corporate social responsibility and profit making organizations were used.An integrative review methodology is a unique technique that outlines relevant papers both past and present for review in other to give better understanding to the topic of discussion Souza & Silva (2010). With the use of this technique, this project was divided into four (4) stages:

1. Problem Identification and Formulation Stage
2. Literature Search/Data Collection
3. Data Evaluation and Analysis
4. Presentation.

Problem Identification and Formulation Stage

There are a lot of similarities on the concept of social businesses and corporate social responsibility and also social businesses with profit making businesses. Despite the fact that all these three topics share common characteristics but there still exist mega differences between them which we will highlight and discourse in more details, (Cornelius et. al 2008).

Literature Review

SOCIAL BUSINESS

Despite the fact that that social business concept is gradually becoming a trend in the academic line, there are several opinions to what the exact concept of social business is. A lot of authors have brought out their ideas to what social business is. Yunus has made elaborations o the concepts of social business some of which was centered on social business been a social problem solving financially fit organization in which the initial investment can be removed while the remaining profit can be reinvested into the business in other to increase its impact in the society, (Yunus, 2014).

Muhammed Yunus came up with this concept due to the obvious reason of money-centric firms and also charitable acts not been able to solve the increasing issues of poverty in the world. He saw that social business could be a medium in making all parties benefit from business. He was of the view that in solving the problems of humans using a business approach is usually a very effective means. New companies should see the way their business will also be of benefit to others and not just only themselves. The profit realized from

social business companies should be returned back into the business inn other to increase the company's growth and reach thereby helping more. He also made argument-elaborating entrepreneurs should not limit their scope in business to just selfish interest but also to put the benefit of the community in mind, (Muktadir-Al-Mukit 2016).

It also allows for the foundation of Low-Profit Limited Liability Corporations and outlines its benefits and latent weaknesses to social entrepreneurs. Galpin and Bell (2010). Additionally, we can organize some of social business benefits in brief as enhanced customer satisfaction, improving products and services by applying their input and affects more invested and engaged part of your business. Competitive advantage often comes from innovate usage which is a practice that is reinforced by the support from institutional environment, (Roy et al 2015).

The present needs of social enterprises are not compatible by the present delivery for such organizations since such endowment miscarries to statement the planned tension that be existent between social and business purpose. Settles with recommendation for instruction to the social enterprise sector and business support providers. By investing in social business, companies have better chance situated to grab the open doors in present unstable, virtual business environment, (Prusak et al 2001).

TRADITIONAL PROFIT-MAKING BUSINESS

This is an organization whose sole aim for all their operation is for the purpose of making profit. The normal operations in traditional profit-making business is for the purpose of maximizing the wealth of the organization and its owners. Profits that are gained in this type of business are either used to improve the organization and its operations or shared by the owners as dividend. The structure of a traditional profit making organization can be either based on sole proprietorship, partnering, Hindu united partnership or even a jointventure. The activities that take place in this type of business focuses on minimizing business input and maximizing the business profit which basically means been efficient and effective, (Sigurjonsson et al (2018).

Differences between social businesses traditional profit making business

Yunnus set clear differences between social business and conventional business where he stated that the evaluation between the two should be made by their objectives in which is shown in table 2.1. After Grove and Berg (2014).

As we will discussed next, another worldview for innovation is rising, an association between private endeavor and public interest that generate beneficial and feasible change for the two sides. There are several of companies or corporations moving further than corporate social responsibility to corporate social innovation to accomplish the future.

Corporate Social Responsibility

There is a strong culture of corporate social responsibilities in a lot of organizations especially in the past decade when people began to pick up interest in it because it stood for positive impact in the society. A lot of reasons such as sense of obligation, economic interest and so on have made lots of organizations partake in corporate social responsibilities. CSR can be said to be a hard on contract that involves having social responsibility or obligation to a community. The rate and extent of the agreement is not certain when dealing with CSR and it has been in use by organizations since in the 1950 in the North. CSR have been seen in the light of various meanings and interpretations based on the ideas of several authors and companies. The several perspectives and notions to what CSR is brings about several issues especially to companies that want to invest in certain communities, (Spieth et al 2018)

An author caller Weber gave an explanation on CSR as a selfless social operation in handling the societal and environmental needs. Elaborating that CSR should

Social Business	Traditional Profit Making Business
Its focusses on the lives it touches by providing social benefits for them	Its focus is based on making profit for its owner
Profits made by the company is not taken by the owner but it is reinvested in the company	Business owners are not selfless and can take out profit for personal use
The drive of a social business is it's cause and not benefit	Profit making is its drive
It deals with solving social related problems especially those related to poverty	It deals with profit maximization instead of social benefit
Positive Societal change is the dividend of stakeholders	Stake holder dividend is either is the profit they get
It has a long term vision of growing and increasing its benefit to the community	Its might have short or long term vision depending on the perspective of the owner

be seen as a form of corporate selfless act or activity that goes beyond the law Weber, (2008).

Another author by the name Milton made his own elaboration by stating that when In business, an organizations responsibility to a society is by operating and making profit within the boundaries of the law in the country or state in which they operate in. Social responsibility should be left for people while organizations main concern should revolve around sticking to the rules and making profits, (Baden 2014).

Other ethical authors have disputed the fact that companies have no role to play in social responsibility in the sense that social responsibility is far beyond obedience to law, guidance and contracts. Everyone who is impacted by the activities of an organization (stakeholders) also has should also be seen as part of the organization. As shareholders believe that an organization is meant to go after theirgoals, which is usually maximizing profit in a short and long run, the companies' activity is what attracts or repels investors and putting the society in mind is a aroma that paints an organization in a good light attracting more investors.

Another researcher by the name Parnell explained that recent view of CSR is intertwined with competitive advantage manages strategy.

Despite the fact that CSR is been seen as a charitable set aside donation or act by a firm in other to positively impact or influence the society in which she operates in. Some of the CSR implemented by firms includes building of affordable schools for children, giving scholarshipsopportunities, building hospitals that is affordable for the community and so on Xinhua (2018).

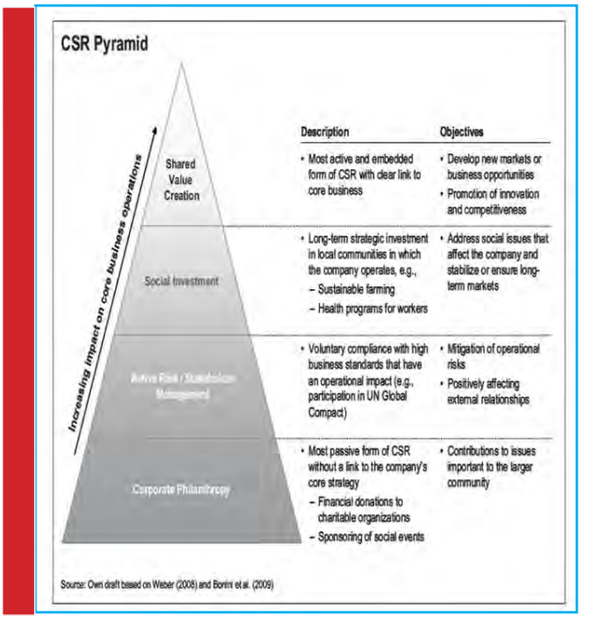
The Multinational enterprises possibly try in being not only part of the problem, but yet additionally maybe part of the solution, is progressively acknowledge and took the forward of the stage in research concentration in corporate social responsibility (CSR) activities and supportable development inferences of IB. kolk et al (2010).

Types of CSR

1. There are several types of social responsibilities which are
2. Corporate Philanthropy: This is seen as the most compliant form of social responsibility. It deals with giving of charities to the less privilege, helping sponsor events for the betterment of the society, assisting in handling matters bigger than the community etc. Fritscher & Pigneur (2014)
3. Active Risk Management: This is a social responsibility that deals with the relationships of an organization. It deals with social activities that is of more benefit to the face of the stakeholders Abdul (2018).
4. Social Investment: This deals with an organization making long term investment in the community in which they operate an example is creation of schools

Shared Value Creation: This deals with operations to help the society and also bring more profit to the organization. An example is the development of profitable business opportunity that the society will stand to benefit from, (Ghaderi 2019).

We can take a short window and mention some of CSR benefits.One, it inspires businesses to act morally and to think through the social and environmental effects of their industry. Two, it prop the outcomes value of public. Three, it empowers both expert and self-improvement, and Four it improves associations with customers.



Different levels of CSR

CORPORATE SOCIAL RESPONSIBILITY VERSUS SOCIALBUSINESS

There is a lot of similarities between Social business and CorporateSocial Responsibility. The two of them have the stakeholders in mind and want them to benefit from business and at the same time addressing and handling societal problems. This doesn't dispute the fact that the both of them are still different in a lot of aspects such as aim, policies relating to profit, issues with compliance etc. table shows lighter on this differences, Renck (2014)

Projects that involves sustainable CRS has a financial impact on shareholders of the company.Despie the fact that people value the concept or benefit from CSR, the cost is solely beard by the organization. Sharehold-

Social Business	CSR
There is no dividend	There is sharing of Profit
Maximization of profit sharing	Maximization of profit
It is company based	It is project based
The projects handled is pioneered based on the different social issues	The projects handled is pioneered based on the business strategy of the company
Its focus is on the poor and less privilege	Its focus is on satisfying the expectations of stakeholders
The result brings about lasting or permanent solutions	The result brings about temporary solutions
Generating its own financial profit	Donating just a little portion of profit
Yielding to the "selflessness" in other to solve societal problems	Yielding to ethical obligations in other to solve societal problems
All the owners and shareholders are in the same page of reinvesting the profit into expanding the business	All the owners and shareholders have bias on reinvesting the profit into expanding the business

ers always kick against corporate society responsibility when the see that the profit that will be generated from the investment will be given back to the society Chao et al (2018). In CSR the business handles all the cost in incurring the project in other to solve a problem affecting the society, this makes the business selfless which will give them the satisfaction of having to touch, impact and improve lives. The efforts of CSR are normally aimed at little aspects of the problems in a society , that is CSR basic focus in on the reduction or suppression of the problems that the society is facing whereby social business has an objective of fixing a large portion of the societies issues such as poverty, child abuse etc. The individuals that venture into social businesses are far betterthan large firms in identifying societal problems and the strategy in solving it. Ferrell (2019)

Data Evaluation and Analysis

1. From the various data gotten from the various articles it is seen that social businesses have unique features such as:
2. In the Social Business social business concept, beneficiaries are not been considered as object, they are just seen as independent business partners. The owner doesn't also see the business as a charity but he sees it as a normal business that is growing making profit and impacting the society and the poor people are seen as employees, managers and workers that can think, act make decisions and also earn salary making them confident and independent. The poor people can then get independent and get the ability to employ and empower others.
3. Charities and donation does not initiate the concept of social business, the initial investment can come inform of charity or donations, but when the investment is made, the entity becomes a business that runs and generates profit which is then recycled to generate more and more profit that will be used to expand the business. The making of profit is similar or the same to that of profit making business where there is production of goods or services and then sold for profit. Muhammad Yunus, made it clear that the startup of a social business can be from charity income at first but the returns can be reinvested for as long as possible. A social business has an obligation to be competitive like any other profit making business in the aspect of their product quality, customer service, product creation etc.
4. Social businesses has a fundamental aim of sorting out social problems and solving social needs. Despite the fact that social business is a business in all ramification, it cant put profit making strategy over that of the societal needs. The fact of been of major service to the community as been the vision of a social business its what makes it different from a profit making enterprise. Socialbusiness can thrive to make enormous profit, but not at the expense of ignoring the societal needs of the society it was meant to cater for.
5. A lot of opportunities are created by Social business in assisting the masses that have not been giving the opportunity to put their diverse talent and skills into good use due to financial constraints. Social businesses generates a professional platform in which investments can bemade in form of charity or donations to bring a lasting solution to societal problems. These poor masses will have the opportunity to be under successful entrepreneurs with the opportunity of meeting and learning from their experiences in business. Investors get the opportunity of implanting a structure that will continuously sort out the need of the poor masses without losing money.
6. I the hierarchy need of Maslow, it was stated that self-actualization is the paramount interest of those that are at the hierarchy top. The urge of Self-fulfillment is been achieved by those who has already sorted out their materialistic needs when

Table 1. Effect of operations between Social Business, Profit Making Business & CSR

	Profit making	Community Benefit	Professionalism
Social Business	✓	✓	✓
Profit making Business	✓		✓
CSR		✓	

they establish momentous projects that makes their legacy to live on and on in ages making them immortals. A typical example are billionaires that have donated a huge part of their wealth to the society such as Bill Gates and Warren Buffet. Self-actualization can be achieved by those who invest in social businesses because their service to humanity is one that will bring in a continuous change in the lives of people withoutlosing his or her capital. In the words of Muhammad Yunus "to make money is happiness but making other people happy is super happiness"

7. An opportunity for the lower class and unprofessional people is created by social businesses by making them to have access to professional experience, steady income, empowerment and the opportunity to empower others. This opportunity brought by social business is not just one that will change the lives of the poor but it is an opportunity that will make the poor to be relevant in the industry. Social business has made the lives of a lot of people better with happiness and self-sufficiency and meaningful life. 2

Social business gives the opportunity for investors to be selfless. According to professor Yunus in a poverty free nation articleoffers a reason to investors for selflessness. Professor Muhammad Yunus has put an emphasis in his article Vision 2050: A Poverty-Free World that "the mightiest mistake in describing capitalism is the process of misunderstanding or confusing the nature of humans. People that run several business are seen as beings that are 1dimensional with the sole purpose of maximizing their profit. There exists in this definition a wrong description of humans due to the fact that the definition doesn't contain the area of life itself but that humans can also be seen as multi-dimensional and not as machines that exist to just make money.It can't be denied that humans have their selfish aspect but in every human also lies a selfless part.With Social business, humans will have the opportunity to execute their 'self-sacrifice' whichcan hardly be found in the traditional profit making business. This is achieved by surprising or eliminating the personal gain motive and applying societal benefit motive.

CONCLUSION

A great no return investment opportunity is seen in Social Business. Satisfaction is the generated return that can be gotten from social business making the investor having a legacy of creating an unending circle of benefit for the poor. There is a great respect by people to the individuals who do impactful selfless acts for the benefit of the. There is always a special recognition given to the by the society which one cannot measure or compare with money. The measurement of success in a traditional profit business-making venture is by the amount of profit, which they earn in a given period.

ACKNOWLEDGEMENT

In order to prepare this research, we have relied on following up on our own work, by defining the role of social work in the development of business and educational projects under implementation. We noted that, by supporting the idea of social work, and introducing this idea within our educational institutions, which is international Brayan primary private school,of the system and institutions, all of which led to the work of

the search, and compare between social work and traditional work and the impact of each one in the institutions concerned and impact within the community quickly.

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Biotechnological Communication

Biosci. Biotech. Res. Comm. 12(2): 464-471 (2019)

BBRC Bioscience Biotechnology Research Communications

Bioethanol production from pulp of fruits

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ABSTRACT

Increase in the need of petroleum results a remarkable rise in its prices. Therefore the need arises to discover alternative cheaper sources for fulfillment of world population demand. Hence the main objective to satisfy this need leads to the development of easier techniques using cheaper and eco-friendly raw materials for the production of Bio-ethanol. One such technique is production of Bio-Ethanol, an alternative to the present petrol and diesel, from fruit wastes by using well known yeast *Saccharomyces cerevisiae RK1*. The use of mixture of three fruits namely Banana, Grapes and Mango was used as a possible substrate for production of cellulosic ethanol by modifying parameters such as aeration. Pretreatment, hydrolysis and fermentation were carried out during this study. After fermentation of mixed fruits pulp without sucrose and fruits pulp with sucrose produced 0.67% ethanol and 1.32% ethanol respectively. Thus, the main objective behind this study is to produce bio-ethanol using cheaper substrates. The present study indicates a promising future for generation of ethanol at commercial scale from cellulosic wastes. Phylogenetic tree shows similarity with yeast sp.

KEY WORDS: BIOETHANOL, ECO-FRIENDLY, FERMENTATION, FRUIT PULP, *SACCHAROMYCES CEREVISIAE*

INTRODUCTION

The fruits and vegetables are more prone to spoilage than cereals due to their nature and composition and this spoilage occurs at the time of harvestings, trasportation, storage, marketing and processing resulting in generation of wastes. Fruit wastes rich in reducing sugars are interesting feed stocks for production of first generation bio-ethanol (Joshi and Sandhu, 1996). Vegetable wastes are rich in cellulose, hemicellulose and lignin. So the production of second generation bio-ethanol from these could be an useful process (Zheng *et al.*, 2009; Del Campo *et al.*, 2006), earlier technologies have used reused fruits and vegetable waste to exploit the properties of food matrices to integrate human and animal diets, (Laufenberg et al., 2003).

Municipal solid waste has become a severe problem for disposal in developed and developing countries due

ARTICLE INFORMATION:

Corresponding Author: skrlipase@gmail.com
Received 23rd March, 2019
Accepted after revision 30th May, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/32

to the shrinking landfill capacity. Biofuel is one of the best alternative fuels in order to trounce the energy crisis and solve pollution problems (Zainab *et al.,* 2014). Since the need of bioethanol as a universal energy source has been increasing, the production of bioethanol must be increased using cheaper and eco-friendly raw materials. Bioethanol production from renewable ligno-cellulosic materials carries potential to reduce burgeoning world dependence on petroleum because of decreasing net emissions of carbon dioxide, the principal greenhouse gas, (James, 1997). According to Indian Agriculture Research Data Book 2004, the losses in fruits and vegetables are to the tunes of 30 percent. Taking estimated production of fruits and vegetables in India at 150 millions tones, the total waste generated comes to 50 million tons per annum. Efficient management of these wastes can help in preserving vital nutrients of our foods and feeds and bringing down the cost of production of processed foods, besides minimizing pollution hazards (Gautham and Gularia, 2007).

Due to energy crisis in 1970, it lead to the development of low - cost, sustainable and renewable energy resources such as ethanol.Ethanol has been described as one of the most exotic synthetic oxygen – containing organic chemicals because of its unique combination of properties as a solvent, a germicide, an antifreeze, a fuel, a depressant and especially of its versatility as a chemical intermediate for other organic chemicals. Investigations have carried out in Production of ethanol from food and agricultural waste now a day by bioprocessing in research. Fruits Wastes such as banana, orange, pineapple and pea peels were saccharification and fermentation by co-culture of *Aspergillus niger* and *Saccharomyces cerevisiae* (Girisha Malhotra, 2013). Pretreated Banana and Mango fruit wastes hydrolyzed and fermented for ethanol Production (Arumugam *et al.,* 2011).Other fruits wastes Grape, apple and banana, papaya or mixed fruit wastes using *Saccharomyces cerevisiae* (Kowshik, 2018).

The *Carica papaya* (pawpaw) agricultural waste, using dried active baker'syeast strain (*Sacchromyces cerevisiae*) also help in production of ethanol (Akin- Osannaiyee*t al.,* 2008),Ethanol obtain from Different Fruit Wastes Using *Saccharomyces cerevisiae* (Janani *et al.,* 2013) Hence bioethanol production could be the route to the effective utilization of the Municipal and agricultural waste (Shah *et al.,* 2017).

In Fermentation of fruits by the action of yeast (*Sacchromyces cerevisiae*) that provide zymase enzyme result in conversion of sugar to produce alcohol, carbon dioxide and other by- products. The percentage yield of the alcohol production influenced by Nitrogenous compounds which are generally essential for the growth and development of yeast (*Sacchromyces cerevisiae*) in the fermentation processes (Nzelibe *et al.*, 2001). The main aim of this research work is the biomasses used in bioethanol production are fruits pulp.The three biomasses with potential interest for bioethanol production have been used. The biomasses studied include grapes, banana and mango. The ripen fruit pulp raw materials fermented and enzymatic hydrolysis using yeast (*Sacchromyces cerevisiae*) have the ability to be converted Sugar into bioethanol. Basic objective of present studies:

1. Isolation of microorganisms from fruit and vegetable wastes and characterization for saccharifying activity.
2. Optimization of growth conditions for the fermentation process.
3. Selection of efficient bacterial and yeast strains for alcohol production.

MATERIALS AND METHODS

Isolation and Identification of Yeast

Yeast was isolated from fruits wastes by using GYE agar plate. Morphological character was studied by microscopy which shows oval and budding cell. Identification of yeast isolates was done by studying their morphological characteristics with the reference yeast, *Saccharomyces cerevisiae* cell identification was done by 18s rRNA sequencing method.

18s rRNA sequencing method

DNA Extraction

Lysis/homogenization: Cells grown in monolayer were lysed by suspending1-3 colonies aseptically and was mixed with 450 µl of "B Cube" lysis buffer in a 2 ml micro centrifuge tube and lyse the cells by repeated pipetting. 4µl of RNAse A and 250 µl of "B Cube" neutralization buffer were added. The content was vortex and incubated the tubes for 30 minutes at 65˚C in water bath. To minimize shearing of the DNA molecules, DNA solutions were mixed by inversion. Centrifuged the tubes for 20 minutes at 14,000rpm at 10˚C. Following centrifugation, the resulting viscous supernatant was transferred into a fresh 2 ml micro centrifuge tube without disturbing the pellet. 600 µl of "B Cube" binding buffer was added to the content and mixed thoroughly by pipetting and incubated the content at room temperature for 5 minutes. 600 µl of the contents was Transferred to a spin column placed in 2 ml collection tube. Centrifuged for 2 minutes at 14,000 rpm and discarded flow-through. The spin column and the collection tube were reassembled then transferred the remaining 600 µl of the lysate. Centrifuged for 2 minutes at 14,000rpm and discard flow-through. 500 µL "B Cube" washing buffer I was added to

the spin column. Centrifuged at 14,000 rpm for 2 mins and discarded flow-through the spin column was Reassembled and added 500 µl "B Cube" washing buffer II and Centrifugated at 14,000 rpm for 2 mins and discarded flow-through. The spin column was transferred to a sterile 1.5-ml micro centrifuge tube. 100 µl of "B Cube" Elution buffer was added at the middle of spin column. Care should be taken to avoid touch with the filter. The tubes were incubated for 5 minutes at room temperature and Centrifuged at 6000 rpm for 1 min. The above mentioned step 14 and 15 were repeated for complete elution. The buffer in the micro centrifuge tube contains the DNA. DNA concentrations were measured by running aliquots on 1% agarose gel. The DNA samples were stored at -20°C until further use.

PCR Protocol

Polymerase Chain Reaction (PCR) is a process that uses primers to amplify specific cloned or genomic DNA sequences with the help of a very unique enzyme. PCR uses the enzyme DNA polymerase that directs the synthesis of DNA from deoxynucleotide substrates on a single-stranded DNA template. DNA polymerase adds nucleotides to the 3` end of a custom-designed oligonucleotide when it is annealed to a longer template DNA. Thus, if a synthetic oligonucleotide is annealed to a single-stranded template that contains a region complementary to the oligonucleotide, DNA polymerase can use the oligonucleotide as a primer and elongate its 3` end to generate an extended region of double stranded DNA.

Composition of the Taq Master Mix :Taq DNA polymerase is supplied in 2X Taq buffer 0.4mM dNTPs, 3.2mM MgCl20 and 02% bromophenol blue

PRIMER DETAILS

Primer Name	Sequence Details	Number of Base
LR7	5' TAC TAC CAC CAA GAT CT 3'	17
LR0R	5' ACC CGC TGA ACT TAA GC 3'	17

Add 5 µL of isolated DNA in 25 µL of PCR reaction solution (1.5 µL of Forward Primer and Reverse Primer, 5 µL of deionized water, and 12 µL of Taq Master Mix). Perform PCR using the following thermal cycling conditions:

1. Denaturation

The DNA template is heated to 94°C. This breaks the weak hydrogen bonds that hold DNA strands together in a helix, allowing the strands to separate creating single stranded DNA.

2. Annealing

The mixture is cooled to anywhere from 51°C. This allows the primers to bind (anneal) to their complementary sequence in the template DNA.

3. Extension

The reaction is then heated to 72° C, the optimal temperature for DNA polymerase to act. DNA polymerase extends the primers, adding nucleotides onto the primer in a sequential manner, using the target DNA as a template.

Purification of PCR Production

Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). The PCR product was sequenced using the primers. Sequencing reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

Sequencing protocol

Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

BIOINFORMATICS PROTOCOL

The sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of query sequence with the closely related sequence of blast results wasperformed followed by multiple sequence alignment. The program MUSCLE 3.7 was used for multiple alignments of sequences.The resulting aligned sequences were cured using the program G blocks 0.91b. This G blocks eliminates poorly aligned positions and divergent regions (removes alignment noise).Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering.

BIOETHANOL PRODUCTION

Preparation of raw materials

The waste fruits of banana, mango and grapes fruit waste were taken from the local market and washed

properly to separate out the soil particles, spoilage particles and other adhering particles. 200 grams of each fruit were weighed and mixed in mixer with 200 ml of distilled water (1:1 ratio). 500 ml of substrate was taken in a 1000 ml flask.

Pretreatment of raw material

0.5 g NaCl and 0.25 KH_2PO_4 was added to substrates-fruits pulp and mixed well. The fruit pulp was separated into two flasks 200 ml and 100 ml and autoclaved for 20 minutes at 121^{0}C and 15lb pressure. In acid hdyrolysis, acid concentrations between 0 to 4% v/v were used, high concentrations yielded high solubilized material. Dilute acid pretreatment was chosen since it is the most preferred and widely used method (Balat *et* al., 2008). Phosphoric acid was used since after neutralization of hydrolysates with NaOH, a salt formed and can remain in the hydrolysates, as it is used by microorganisms. The amount of reducing sugars was estimated by dinitrosalicylic acid (DNSA) method (Miller, 1959).

Inoculation of yeast

A 24 hr grown inoculums of isolated yeast in GYE broth was added to each flask 20 ml was inoculated into the flasks of 200ml fruits pulp. Also, we have added sucrose to the flasks containing fruit pulp, 1% sucrose was added to 200ml flasks respectively.

Fermentation

Lab-scale batch fermentations were carried out on 250 ml of pretreatment mix fruit waste of liquefied substrate. Another pretreatment mix fruit waste of liquefied flask with sucrose. inoculums were inoculated with the yeast culture (1 × 106 cell/ml), in 1 liter flask, under static conditions, at 28°C. Samples were taken at 24, 72, 120, and 168 h to quantify ethanol production and sugar estimation respectively.

The maximum sugars are converted into bioethanol. The reducing sugar utilization during fermentation was analyzed by DNS method (Shah *et al.,* 2017) and the bioethanol production was analyzed by using Dichromate method (Byadgi *et al.,* 2015).

Recovery of the product

After the 36 hours fermentation processes a little sum each one sample was taken and centrifuged. The supernatant was gathered and the volume of the alcohol was dictated by the Dichromate method. At that point whatever is left of the sample was distilled by using batch distillation unit to collect the ethanol from distinctive fruit wastes (Sanchez, 2007). The distillation unit comprised of three parts: a reboiler, condenser funnel and a distillate flask. The vapors began to climb into the still head and passed through the condenser channel. The constant dissemination of cold water around the condenser funnel supported in cooling the alcohol rich vapors back to liquid state. The condensed liquid collected in the distillate and iodine test used to check the ethanol presence. The qualitative estimation of the presence of hydroxyl group was confirmed by FTIR. The quantitative estimation of bioethanol production was analyzed by using Dichromate method.

RESULTS AND DISCUSSION

Isolation of yeast

Yeast cell were isolated from fruit waste and identified by morphological and 18s RNA sequencing method *Saccharomyces cerevisiae* RK1.it was also reported by Nahvi et al. (2002) isolated yeasts from fruits source.

FRUIT PULP

Currently, researchers have great interests on the production of ethanol by using biomass. According to Janani *et al.,* mixture of Fruit pulp was chosen because it consists of useful sugars and monomers of sugars that could be fermented to produce ethanol and found suitable to be used as alternative energy source. The distilled liquid was found to be ethanol.

In the hydrolysis process the cellulose present in the substrate is converted into ethanol naturally or using the cellulose degrading bacteria after the pre-treatment process. Fermentation of sugars released during hydrolysis of substrates which was carried out by yeast at pH 6 and 28°C temperature, to convert into bioethanol. Fermentation was carried out with fermented samples being collected every twenty four hours for analysis of reducing sugar by DNS method for substrate utilization and is shown in the graph below. The bioethanol production was analyzed by using Dichromate method (Byadgi *et al.,* 2015). The sample was distilled in the distillation unit. The presence of hydroxyl group was confirmed by FTIR.

In FTIR result graphs band stretch 3331.34 and 3329.06 both indicate O-H function group present in fruit sample without sucrose and fruit sample with sucrose respectively.Gene Bank Accession number of *Saccharomyces cerevisiae RK1* (Gene bank Assesion No. MK225573).

In this study after fermentation Process by *Saccharomyces cerevisiae* mixed fruit pulps without sucrose produced 0.67gm % ethanol show in fig 1. Whereas with sugar 1.32gm% ethanol show in fig 4. Alcohol production was comparatively low concentration in work carried out by Gosavi et al., (2017). Gosavi also reported that fermented sugar present in fruit waste also affect

Table 2. Alcohol and Sugar estimation from fruits pulp Sucrose

Incubation Day	Alcohol in Gram%	sugar in Gram%
Day0	0	2.53
Day2	1.05	1.5
Day4	1.24	0.24
Day6	1.32	0.18
Day8	1.02	0.11

Table 1. Alcohol and Sugar estimation from fruits pulp without Sucrose

Incubation Day	Alcohol in Gram%	sugar in Gram%
Day0	0	1.7
Day2	0.63	0.7
Day4	0.67	0.6
Day6	0. 66	0.5
Day8	0. 51	0.4

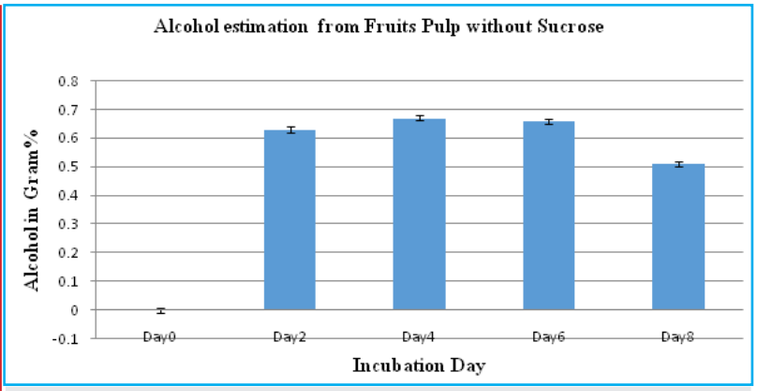

FIGURE 1. Alcohol estimation from Fruits Pulp without Sucrose

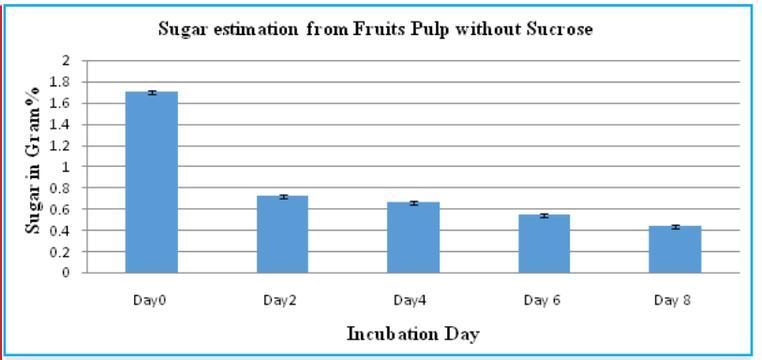

FIGURE 2. Sugar estimation from Fruits Pulp without Sucrose

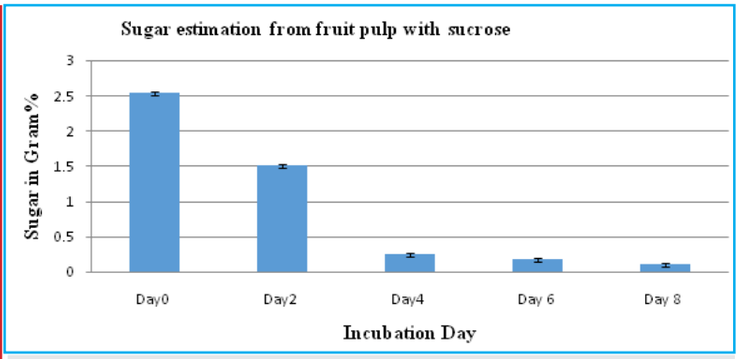

FIGURE 3. Sugar estimation from fruit pulp with sucrose

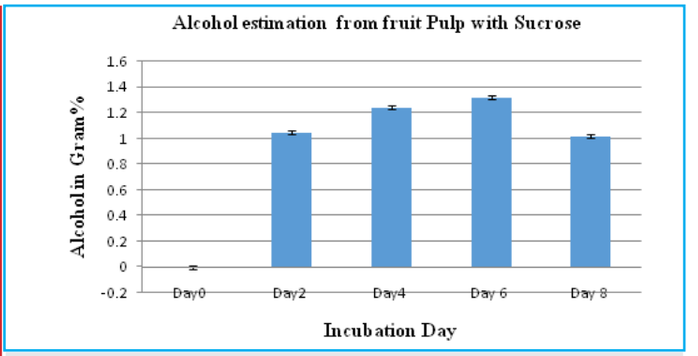

FIGURE 4. Alcohol estimation from fruit Pulp with Sucrose

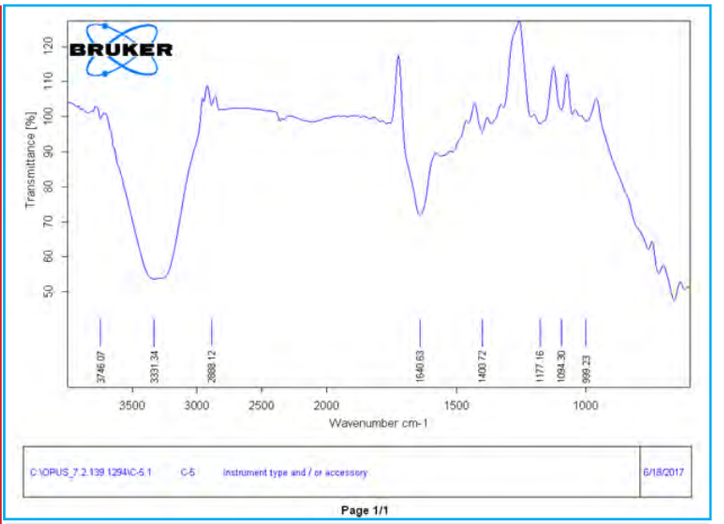

FIGURE 5. FTIR results of distilled alcohol from fruit sample without sucrose

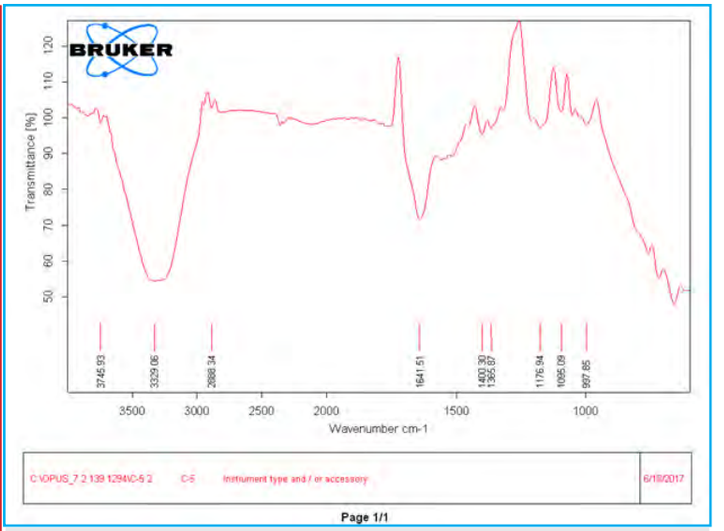

FIGURE 6. FTIR results distilled alcohol from fruit sample with sucrose

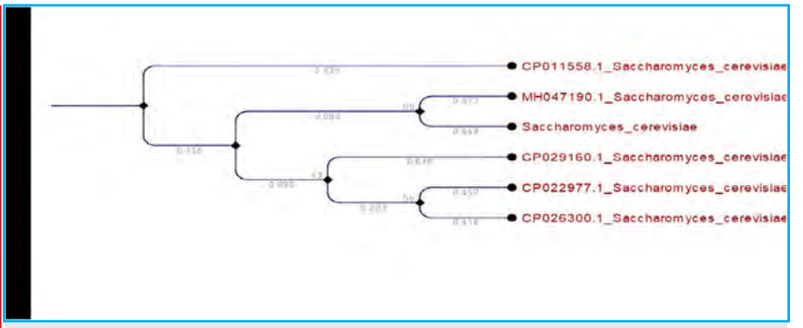

FIGURE 7. Phylogeny tree of yeast

alcohol production so it was observed clearly that fruits pulp containing sucrose increase alcohol production. Highest Alcohol production observed after 4 day (92 hrs) in both with and without Sucrose sample. Isolated *Saccharomyces cerevisiae* have high efficiency in alcohol production using less sugar as reported by Janani et al. Sugar level steadily decreases day by day in fermented broths. Experiments P-value of control is 0.000133 whereas P-value of ethanol produced without sucrose and with sucrose are 0.991334, 0.995558 respectively.

CONCLUSION

The present work deals with the studies on production of bioethanol from fruit pulp. Experiments were carried for Pretreatment of the substrate, Hydrolysis and Fermentation of the hydrolysate. The *Saccharomyces cerevisiae* RK1 was used to ferment the reducing sugar into ethanol. The presence of hydroxyl group was confirmed by FTIR. The ethanol yield as estimated using Dichromate method. Based on these results, it can be concluded that the production of bioethanol from fruit pulp was successful. We can predict that on re-distillation higher concentration of alcohol can be obtained, which can be used as biofuel. As this process is cost-effective and gives out no toxic substances, it could be produced at an industrial level.

ACKNOWLEDGEMENTS

The authors wish to express their thanks to the Department of Biotechnology, Pramukh Swami Science and H.D. Patel Arts College, Kadi.

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Computer Science Communication

Biosci. Biotech. Res. Comm. 12(2): 472-477 (2019)

BBRC Bioscience Biotechnology Research Communications

An approach to solve graph coloring problem using adjacency matrix

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ABSTRACT

Vertex coloring of a given a graph G= (V, E) consists of assigning a color to each vertex in such a manner that every adjacent vertices have assigned different colors. Graph coloring problem belongs to the class of combinatorial optimization problem and studied due to its lot of applications in the area of data science, networking, register allocation and many more. In the processes of allocation of the colors for vertices in the graph will done in such a manner that number of used color is minimum. Most of the existing algorithms available in the literature normally deals the problem by taking consideration above constraint at time of assigning the color to the vertices but sometimes it generates some more constraints during assignment of colors to vertices in the process and needed to be handled explicitly and thus it increases the running time of the algorithm. In this research paper we propose an algorithm based on adjacency matrix representation of graph along with matrix representation of the available colors that solve the problem without generating any additional constraints at intermediate stages in coloring process.

KEY WORDS: ADJACENCY MATRIX, COLOR MATRIX, EXPLICIT CONSTRAINTS, GRAPH COLORING

INTRODUCTION

Graph coloring is one of the most and frequent studied combinatorial optimization problems in graph theory. A graph G can be defined as pair (V, E) where V is the set of vertices and E is set of edges. The graph coloring problem can be defined as to assign the color to every vertex of the graph by keeping the constraints that no two adjacent vertex have same color and in this process of assigning the color total number of used colors should be minimum. The minimum number of colors that will used to color the vertices of the given graph is called chromatic number of the graph. Graph coloring problem is one of the NP-Hard combinatorial optimization problems which is studied for its complexity associated with computation and for its numerous applications in real world problems. These real world problems mapped into graph coloring problem and solved using a graph coloring algorithm. A few of its

ARTICLE INFORMATION:

Corresponding Author: ajayshuk@gmail.com
Received 6th April, 2019
Accepted after revision 5th June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/33

applications found in literature are scheduling in different area of applications (Gamache et al 2007, Zufferey et al 2008), time slots allocation in time table (Werra 1985, Burke et al 2007), allocation of register (Werra et al, 1999), assignment of frequency (Smith et al, 1998), application in communication network (Woo TK et al, 2002), Sudoku, pattern matching and applications in parallel computing, (Hao et al, 2017).

Mostly the algorithms that are used to solve the graph coloring problem can be categorized into two different categories named as approximate algorithms & exact algorithms (Méndez Díaz I. et al, 2008, Lucet C. et al, 2006, Méndez-Díaz I, et al 2006, Malaguti E, Monaci M, Toth P, 2011, Segundo PS, 2012). Approximate algorithms includes various techniques to solve the problem such as construction method for register allocation (Chaitin GJ, 2004), iterated local search (Caramia M, DellOlmo P, 2008), algorithms based on population (Porumbel DC, Hao JK, Kuntz P, 2009, Dowsland KA, Thompson JM, 2008), algorithms based on hybrid approach (Galiner P, Hertz A, Zufferey N, 2008) and other methods based on hybrid local search (Prestwich SD, 2008).

Algorithms available in literature for solving graph coloring problem are mostly approximate in nature due to its hardness and little are exact. For real world applications, the encoding into graph coloring problem is often large and may be not suitable for exact algorithms due to difficulty to solve the large instances after enumeration of search space of the problem.

The constraints in graph coloring problem can be expressed as no two adjacent vertices have same color which is quite simple but this constraint may infer few more constraints among non-neighboring vertices in the graph. The methods available in the literature to deal such problem involves the encoding of the problem using k number of colors into Boolean satisfiability problem and then algorithm search & explore the additional constraints generated among non-adjoining vertices by the use of conflict driven clause learning .

In this paper we propose a new algorithm to find the solution for graph coloring problem based on adjacency matrix representation of the given graph for k available colors. In the process of coloring of the vertices in the graph the proposed algorithms that uses only the related to adjacent vertices in the graph and does not generate any additional constraints during coloring process at intermediate stages and thus it avoids any additional constraints to be handled in the process.

LITERATURE REVIEW

In literature there are various techniques available to solve graph coloring problem. In an approach which is based on the conflict driven clause learning with backtracking (Zhaoyang Zhou et al 2014). The approach by the researchers addresses the solution of k-colorability problem with conflict driven clause learning. Researchers have formulated the solution of the problem without encoding the constraints between adjoining vertices in SAT form. Whenever a dead end has encountered algorithm called an implicit constraints generated at intermediate stage. All the constraints searched in this way was represented as CNF clause.

Algorithm1 cdclgcp(G,S,l)

```
//The input data for the procedure will Graph G, coloing solution set //S which is partial initially & recursive level l.
// in the graph Kv is set of vailble colors with each vertex v in G.
// The algorithm1 executed initially by calling cdclgcp(G,NULL,0).
// The output will be colour solution set S.
Start :
If( the graph G is NULLL graph )
return S
Call procedure unitpropagation (G,S,l)
If (kv is empty corresponding to a vertex in the graph or there is a NULL clause)
{
Find the reason R by analysing the conflict.
If (reason is empty)
return NULL
If ( R is non empty)
{
Compute
Implicit constraints =
Implicit constraints U ( R)
Call procedure backtracking for 2nd largest level(R)
}
Else
return NULL
}
From the graph G choose a vertex v using heuristics
do
{
Delete color C1 from the colorlist of available colors of adjacent vertices of v.
mark the each removal of color C1 as a reason in l+1 level.
S=cdclgcp((G\v,SU(marked reason), l+1)
If (S is not NULL)
return S
Add C1 to Kv of every adjacent vertex to v
}
while( each color C1 explored in colorlist Kv)
return NULL
End.
```

The unitpropagation procedure simply discover and generates other unit clauses, unit vertices using propagation. In case the occurrences of conflicts, the reason is analyzed using CNF representation and analyze conflict () procedure.

The algorithms proposed by the researchers is runs in two phases. In the first phase the algorithms search & constructs set of implicit constraints in the graph coloring process and uses it to crop the large search space generated. In the process researchers have applied DSATUR heuristic for the construction of implicit constraints. Next phase of the proposed algorithm constructs the coloring solution set using unit propagation method.

Another approach reported in literature where researchers have tried to give the solution of the graph coloring problem based on genetic algorithm & fuzzy logic (Beigh Tabiya Manzoor et al, 2016). Researchers have defined the following parameters for the optimization in their algorithm

a. Initial population (used random generation)
b. For selection (used alpha cut based selection)
c. For crossover (used 1- point crossover with probability .8)
d. In mutation phase they have used swapping with probability 0.01

On the basis of above parameters they have proposed the algorithm that runs in the following manner:

Algorithm 2

1. Random initialization of population
2. Evaluation of fitness value
3. Selection of population based parameter defined
4. Applying crossover on resulting population in previous step
5. Apply mutation & replacement operator on data set obtained in previous step.
6. If result obtained is required solution set then exit else goto step 2.

Researchers have proposed above algorithm to solve the problem using optimization techniques based on fuzzy logic & genetic algorithm and tried to give the optimal solution.

Few more techniques have reported in literature in which researchers have proposed the solution by transforming the vertex coloring problem into coupled oscillatory networks (Parihar Abhinav et al, 2018). Another approach is based on graph encoding in the form of adjacency list and researchers have given the solution of the problem with the help of color adjacency list representation of available colors (Shukla, Ajay Narayan et al, 2019). One application area of the problem has been reported in which researchers have tried to address the problem of Device-to-Device communication in wireless networks for improving spectrum utilization and proposed the solution for resource sharing based on graph coloring concept (Tinghan Yang et al, 2017). The other area of applications of the graph coloring problem that has recently reported (Xudong Zhu et al, 2017, Mohanad Mohammed Abdulkareem et al ,2017)where application problem has transformed in graph coloring problem to find the solution .

Due to versatile applications of graph coloring problem the methodology used for getting the solution of problem & its complexity is always of the interest of the researchers. A few different approaches to solve the problem based on the types of graphs they have considered for their research problem has discussed in literature (Jaffke L et al, 2017, V.V. Lozin et al, 2017, Petr A. et al, 2016, Arumugam, S et al, 2017).

The graph coloring problem has also addressed for its solution based on cellular learning automaton for getting efficient solution (Rezapoor Mirsaleh, M., Meybodi, 2016). Graph that is free from induced subgraph another approach may be applied to solve the problem by finding the path & cycles among the given number of vertices (Shenwei Huang, 2016). Another variation of coloring problem is in partition graph coloring problem where with help of optimization technique new results have evolved (Stefka Fidanova, Petrica Pop, 2016). Similarly graph coloring problem has also used to address pricing problem (Morrison David R et al, 2016) and in fuzzy coloring for fuzzy graphs (Samanta, S. et al, 2016) for its efficient solution.

MOTIVATION FOR OUR APPROACH

Mostly the solution proposed by researchers have based on explicit constraints of the graph coloring problem along with auxiliary procedure used for handling the other constraints generated during the coloring of vertices in the graph. Some researchers have used learnt clauses management to reduce the search space for coloring by applying certain heuristics. On the other hand some of them have used some other techniques based on genetic algorithm.

In my approach I have devise a new approach that uses only defined constraints for graph coloring problem in the literature and in the coloring process there will be no generation of any additional constraint that required to be handled explicitly. So this approach will reduce the running time for solving the problem.

The proposed approach is based on the representation of given graph in the form of adjacency matrix where the entry in the matrix corresponding to adjacent vertices is 1 otherwise it will 0. Suppose if the given graph is having n number of vertices then it requires to be stored in an nXn square matrix to represent the graph.

In addition to above if there are k number of available colors that will use to assign the colors among the vertices of the graph, we have created additional color matrix of the dimension nXk.

Finally a 1-D array of size n in the algorithm is used to store color that have assigned to every vertices of the graph.

Data Structure Used For The Problem:

As stated in previous section we present our proposed algorithm matrix representation of the graph, set of available colors and a one dimensional array for storing final color information corresponding to every vertex in the graph.

Suppose given a 3-colorable graph as shown in the figure1 and its adjacency matrix has shown in Table1.

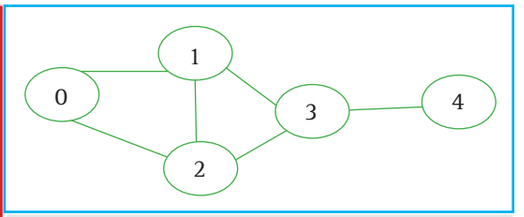

FIGURE 1. Colorable graph

Table 1. Adjacency matrix representation of figure 1

	0	1	2	3	4
0	0	1	1	0	0
1	1	0	0	1	0
2	1	0	0	1	0
3	0	1	1	0	1
4	0	0	0	1	0

We have additionally used a color matrix and the matrix representation of available colors for graph can be described as follows:

Table 2. initial color matrix representation for the graph

	0	1	2
0	1	1	1
1	1	1	1
2	1	1	1
3	1	1	1
4	1	1	1

In the above Table 2 the first row of the matrix represents the number of available colors where each color is given a discrete value say for example o represents Red, 1 represent Green and 2 represents Blue color. The first column in figure 3 represents the vertices in the given graph. The values in the color matrix is initially filled with 1 that indicates any color may be assigned to any vertex initially at the starting of the algorithm and in later stage color matrix will updated as 0 or 1 . The updation of values in the color matrix will done in the manner that if cm[i][j] is 1 then the jth color may be assigned to vertex i in the graph otherwise it is 0 the jth color cannot assign to vertex i.

Final colors assigned to the vertices of the will stored in the form discrete values into one dimensional array color[].

PROPOSED ALGORITHMS

In the proposed algorithms we are using adjacency matrix for graph, color matrix, number of available colors and finally assigned color array as global.

Algorithm 3 assigncolor (int i)

```
{
int i1=i;
for j=0 to k-1
{
if(cm[i1][j]==1)
{
color[i]=j;
ic=j; // here ic is global
cm[i][j]=0;
break;
}
}
}
```

In the above algorithm we are assigning the color to the vertex i that can be obtained from the color matrix after testing the feasibility of assignment of the color in from the execution of for loop.

Algorithm 4 updatecolor(int j)

```
{
for l=0 to k-1
{
if(cm[j][l]==1&&ic==l)
cm[j][l]=0;
}
}
```

Above algorithm4 runs to update the values in the color matrix corresponding to those vertices which have previously assigned some color & these values becomes 0 in the color matrix cm[][].

Algorithm 5 GCP Color( )

```
{
for i=0 to n-1
{
assigncolor(i);
```

```
for j=i+1 to n-1
if(a[i][j]==1)
updatecolor(j);
}
```

Initially algorithm 5 will call algorithm3 to assign the color to vertices of the graph .Once color has assigned to a particular vertex in the graph , calls algorithm 4 to update the color matrix.

WORKING OF PROPOSED ALGORITHM & ITS ANALYSIS:

Before starting of the algorithm we are assuming that the given graph has stored in the form of adjacency matrix a[][] where entry if a[i][j] is 1 then vertex j is adjacent vertex to i otherwise if it is 0 j is a non-adjacent vertex to i. Similarly color matrix cm[][] is initially assigned as 1 for its every value and it has been updated in algorithm4 .

We have executed above proposed algorithm on several graphs and found it will always terminated by assigning the colors to the vertices of the work in the form of exact algorithm that is it will uses the minimum colors to color the graph.

Suppose the algorithm runs on the given graph in figure 1 after the assigning the colors to the vertices say 0 the content of color matrix (Table 3) and color assignment array (Table 4) will as follows:

Table 3. content of color matrix after assigning vertex 0 as Red.

	0	1	2
0	0	1	1
1	0	1	1
2	0	1	1
3	1	1	1
4	1	1	1

Table 4. color array after assigning color Red (0) to vertex 0 in the graph

0				

Analysis of proposed algorithm:

The formal analysis of the proposed algorithms is simple. We have implemented the proposed algorithm on windows based Turbo C although it can be implemented on any platform without affecting its complexity. Suppose the number of vertices in the given graph is n and total number of available colors is k. The formal analysis of algorithms proposed may be done in the following manner:

- The procedure assigncolor( ) will called n times which in turns executes maximum k times for every calling so the total number of execution time will be(n*k)times.
- The procedure updatecolor( ) will called n-1 times for its every calls by GCPColor and in this case the total number of calls of procedure updatecolor( ) will be (n*(n-1)) times.
- The instructions in procedure updatecolor( ) itself executed maximum k times in every call.
- So the total number of times instructions executed in procedure updatecolor( ) will be (n*(n-1)*k)

Finally the total number of instructions executed in the proposed algorithm will be (n*k)+(n*n-1*k).It is therefore complexity of the proposed algorithm will be O(n*(n-1)*k).

CONCLUSION & FUTURE SCOPE:

In the proposed research work we tried to give the solution for graph colouring problem with the help of traditional data structure available in the literature by adopting a completely new approach. Although the solution for the problem available in the literature in the form of either approximate or exact algorithm but every approach have its own flaw. In some cases algorithms will work for dense graph but fails to work satisfactory in case sparse graph. In our case the proposed approach will work with same capability in any kind of graph without affecting its complexity.

Since the graph coloring problem is a NP class problem, so there will be always possibility of improving the running time of algorithm and this may be improved by either changing the data structure that reduces the complexity for the searching the adjacent vertices for updating the color list corresponding to each vertex in the graph or by use of certain heuristics that reduces the search space updating the color matrix.

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Microbiological Communication

Biosci. Biotech. Res. Comm. 12(2): 478-484 (2019)

BBRC Bioscience Biotechnology Research Communications

Comparative effect of bio fertilizers under drip fertigation system on nutrient uptake and yield performance in the green gram, *Vigna radiata*

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ABSTRACT

A field experiment was carried out on the effect of bio fertilizers under drip fertigation system on nutrient uptake and yield performance in Green gram *Vigna radiata*. It was conducted at water technology centre fields, college farm, College of Agriculture, PJTSAU, Rajendranagar, Hyderabad, Telangana during *kharif* 2016 – 2017 with one of the objectives of nutrient uptake and yield performance of Green gram, variety (MGG – 295) under drip fertigation system. The experiment was laid out in randomized block design with three replications along with 10 treatments. The liquid bio inoculants viz., *Rhizobium* and PSB were used under drip fertigation system. The results of the field experiment indicated that the treatment T6 with combination of 100 % RDF along with LBBF drip fertigation recorded significantly highest N, P and K uptake were 62.23, 26.60 and 51.33 kg ha-1 respectively and significantly highest seed yield (1019.50) kg ha-1 recorded with treatment (T6) when compared to all the treatments. Further, the percent of seed yield increased over control by 23.93 %.

KEY WORDS: BIO FERTILIZERS, BIOFERTGATION, MICROBIAL INOCULANTS SEED YIELD, NUTRIENT UPTAKE

INTRODUCTION

The high inputs of chemical fertilizers have not only caused environmental problems but also become a cause of concern for human health. Bio fertilizers have been identified to address the issue of excessive use of chemical fertilizers as they are one of the sustainable source of supplementation to chemical fertilizers for Agriculture. The term Bio fertilizers generally are defined as preparation containing live or latent cells of efficient

ARTICLE INFORMATION:

Corresponding Author: shravani4335@gmail.com
Received 18th April, 2019
Accepted after revision 19th June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/34

strains of Nitrogen fixing, Phosphorus solubilizing or cellulolytic microorganisms used for application to seed or soil. Intensified use of bio fertilizers emerging as an environmentally-friendly alternative soil fertility management practice with potential to increase and cheaply sustain crop yields compared with continuous application of inorganic fertilizers alone (Sharma *et al.,* 2013). It is also play an important role for supplementing the essential plant nutrients for sustainable agriculture. Bio fertilizers are low cost and economically viable technology which improves plant growth and development. These are reduce the environmental pollution caused by chemical fertilizers, protect plants against many soil-borne pathogens and help the plant to grow under stress conditions (Brahmaprakash and Sahu, 2012).

Liquid bio fertilizers of good quality hold great promise in agriculture because of benefits over the conventional carrier based bio fertilizers such as *Rhizobium* and PSB inoculants are often exposed to un favorable stresses when inoculated seed were planted in the soil. These stresses include such things as low or high pH, high temperature, low moisture and high salt concentrations. Therefore, the *Rhizobia* and PSB inoculants should be tolerant to these stresses (Liu *et al.,* 2009), have protecting materials in the inoculants formulation or have adaptive mechanisms that increase their tolerance. Because of these advantages over the conventional carrier based bio fertilizers, the liquid bio fertilizers has great importance in yield increased aspects in agriculture.

Green gram [*Vigna radiata* (L.) Wilczek] also known as mung bean is a self-pollinated leguminous crop which is grown during Kharif (July- October) as well as summer (March- June) seasons in arid and semi arid regions of India. It is primarily a rainy season crop but with the development of early maturing varieties, it has also proved to be an ideal crop for spring and summer season. It is tolerant to drought and can be grown successfully on drained loamy to sandy loam soil in areas of erratic rainfall. It is a native of Central Asia. It is a short duration crop, fits well in various multiple and intercropping systems. India is a leading green gram cultivator, with up to 55% of the total world acreage and 45% of total production (Singh *et al*., 2013; Rishi *et al.,* 2009). It is one of the most important pulse crops for protein supplement. The major advantage of liquid bio fertilizers having long shelf life, (Bhavya *et al.,* 2017).

Efficient management of water is of outmost importance for sustaining and enhancing Agricultural production (Palanisami *et al*., 2012). Drip fertigation allows precise timing and uniform distribution of fertilizer nutrients, and is an efficient and agronomically sound method of providing soluble plant nutrients directly to the active plant root zone. Biofertigation is the efficient and precise use of beneficial microorganisms through a microirrigation system. Biofertigation offers vast scope for minimizing the use of chemical fertilizers. fertigation is definitely advantageous over the surface irrigation with basal application of fertilizers with optimum moisture supply and timely nutrient application (Jeyajothi and Pazhanivelan, 2017). There are more chances for increasing the yield, quality, fertilizer-use efficiency, water-use efficiency, and economic output. With drip fertigation and in combination with organics, there is possibility for organic farming to be intensified in the future.

MATERIAL AND METHODS

The field experiment was conducted at water technology centre fields, college farm, College of Agriculture, PJTSAU, Rajendranagar, during *kharif* 2016 – 2017. The location is geographically situated at 17° N Latitude and 78° E longitude at an altitude of 542.3 m above MSL. The soil of an experimental site was sandy loam in texture, moderate in organic carbon, low in nitrogen and medium in available phosphorus and high in potash and slightly alkaline (pH 7.6) in chemical reaction. The seed of green gram (Cv. MGG 295) was obtained from Agriculture Research Station, Madira, Khammam. Seed treatment with microbial inoculants (carrier*):* After quality testing of the bioformulation, the good quality bio inoculants were used for the field experimentation to study the performance on yield of green gram crop. Twenty grams of jaggery was dissolved in 200 ml of water. Jaggery solution was prepared as per the volume of seed. The *Rhizobium &* PSB cultures were thoroughly mixed for slurry preparation. Seeds were treated with this mixture carefully, so that seed coat was not injured and a uniform coating was made. Treated seeds were dried under shade on gunny bags and then used for sowing. Seed treatment with microbial inoculants (liquid): Inoculums of PSM & *Rhizobium* were prepared by dissolving 10 g of jaggery in one litre of boiled water and subsequently cooled and then added to the broth culture in required quantity, so as to obtain at least 1.0 - 1.5 x 10^8 cells per ml. Soil application of liquid culture based bio fertilizers: PSB and *Rhizobium* culture was applied in the soil @ 1 l ha^{-1} in 10 days interval for the treatment $T_{8.}$

The experiment was laid out in a Randomized Block Design with 10 treatment combinations. The treatments are T_1: 100 % RDF, T_2 : 100 % RDF + CBBF Seed treatment, T_3: 100 % RDF + CBBF Soil treatment, T_4: 100 % RDF + LBBF Seed treatment, T_5: 100 % RDF + LBBF Soil treatment, T6: 100 % RDF + LBBF Drip fertigation, T_7: 100 % RDF + LCBF Seed treatment, T_8: 100 % RDF + LCBF Soil treatment, T_9: 100 % RDF + LCBF Drip fertigation, T_{10} Control: Only bio fertilizers. Sowing was done on 15th July, 2016 by hand dibbling two to three seeds

at each hill at a recommended spacing of 30 cm × 10 cm. Recommended dose of fertilizer for green gram is 20: 50: 00 N P K kg ha^{-1}. Fertilizer *viz.,* nitrogen, phosphorus were applied in respective plots as per the recommendation by using the urea and SSP.

The fertilizer solution was prepared by dissolving the required quantity of fertilizer with water in 1:5 ratio and liquid bio fertilizers *Rhizobium* (10 ml) + PSB (10ml) injected into the irrigation system through venturi assembly. Fertigation interval was scheduled once in 7 days interval. The recommended doses (20: 50: 00 NPK kg ha^{-1}) of inorganic fertilizers *i.e.,* urea (46 % N) and single super phosphate (16 % P_2O_5) were applied as basal to the surface irrigated treatments except fertigation treatment (T_6 & T_9) combinations. Data were collected for green gram nutrient uptake and seed yield and haulm yield at harvest stage. The nutrient content uptake values obtained as percentage in the analysis was multiplied by the respective dry matter content for computing N, P and K uptake expressed in kg ha^{-1}.

$$\text{Nutrient uptake (kg ha}^{-1}\text{)} = \frac{\text{Percentage of nutrient content} \times \text{Total dry matter production (kg ha}^{-1}\text{)}}{100}$$

RESULTS AND DISCUSSION

The significantly highest uptake of N registered in treatment T_6 with combination of 100 % RDF and LBBF drip fertigation was 62.23 54.37 kg ha^{-1}. On the other hand the lowest uptake 41.30 kg ha^{-1} of N was noticed in T_{10} (Control). When compared to control (Table 1 & Fig: 1) in all other treatments, combination N uptake was significantly higher. The significantly the highest uptake (26.60) kg ha^{-1} of phosphorus noticed in treatment T_6 with combined application of 100 % RDF and LBBF as drip fertigation (Table 1 & Fig: 1). When compared to control in all other treatments, combination N uptake was significantly higher. Whereas, the lowest 16.50 kg ha^{-1} uptake of P was observed with T_{10} (control).The data regarding to uptake of K was significantly influenced by the drip fertigation with liquid based bio fertilizers *Rhizobium* and PSB along with mineral fertilizers significantly increased the 51.33 kg ha^{-1} of K uptake by the greengram crop when compared to all the treatment combinations. The lowest uptake of K 43.33 kg ha^{-1} was noticed (Table 1 & Fig: 1) in T_{10} (control).

Total highest N P K uptake was recorded with the treatment drip fertigation with liquid based biofertilizer might be due to increased nutrient contents in root zones with the application of *Rhizobium* and PSB. PSB facilitated P availability to plant by solubilizing insoluble P by production of organic acids and resulted in better P uptake by greengram with application of biofertilizers. Enhanced the uptake of potassium may be due to the synergistic effect between N and P. Availability of various nutrients in the soil for plant uptake depends on the soil solution phase, which is mainly determined by the soil moisture availability. The higher available soil moisture, provided by continuous water supply under drip irrigation, had led to higher availability of nutrients in the soil and thereby increased the nutrient uptake by the crop. It is also be responsible for expansion of root surface area and enhanced plant-microbe interaction resulting in more nutrient uptake was reported by (Yuming *et al.*, 2003).

It is clearly indicated (Table 2 & Fig: 2) that the number of pods plant-1 was significantly influenced due

Table 1. Influence of different formulations of biofertilizers application on nutrient uptake of greengram at harvesting stage.

Treatments	N (kg ha^{-1})	P kg ha^{-1})	K (kg ha^{-1})
T_1 : 100 % RDF	45.80	18.17	41.77
T_2 : 100 % RDF + CBBF Seed treatment	56.27	23.77	47.87
T_3 : 100 % RDF + CBBF Soil treatment	53.60	20.77	45.60
T_4 : 100 % RDF + LBBF Seed treatment	58.10	24.10	49.77
T_5 : 100 % RDF + LBBF Soil treatment	52.23	21.03	46.07
T_6 : 100 % RDF + LBBF Drip fertigation	62.23	26.60	51.33
T_7 : 100 % RDF + LCBF Seed treatment	53.27	21.23	47.87
T_8 : 100 % RDF + LCBF Soil treatment	50.60	19.80	45.43
T_9 : 100 % RDF + LCBF Drip fertigation	54.37	23.53	46.80
T_{10} Control : Only biofertilizers	41.30	16.50	43.33
SE(m)	2.91	1.39	1.48
CD(P=0.05)	8.71	4.17	4.43
CV	9.54	11.21	5.50

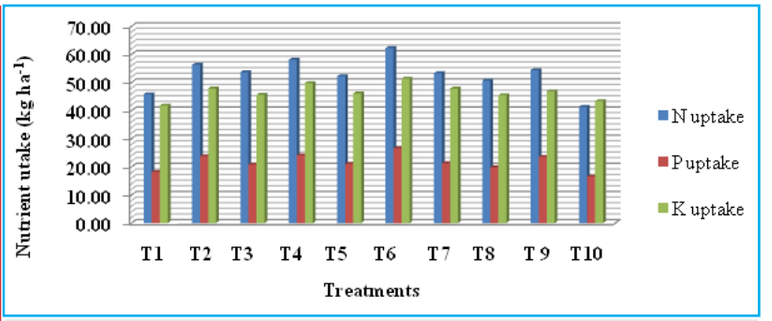

FIGURE 1. Influence ofdifferent formulations of biofertilizers application on nutrient uptake of greengram at harvesting stage

Table 2. Influence of different formulations of biofertilizers application on yield attributing characters of greengram at harvest stage

Treatments	No.of pods plant^{-1}	No. of Seeds pod^{-1}	Test weight of seeds (g)
T_1 : 100 % RDF	11.67	10.33	29.40
T_2 : 100 % RDF + CBBF Seed treatment	16.67	11.00	37.30
T_3 : 100 % RDF + CBBF Soil treatment	14.33	11.00	36.20
T_4 : 100 % RDF + LBBF Seed treatment	17.33	12.00	37.80
T_5 : 100 % RDF + LBBF Soil treatment	14.67	12.00	36.30
T_6 : 100 % RDF + LBBF Drip fertigation	19.67	13.00	38.77
T_7 : 100 % RDF + LCBF Seed treatment	14.67	11.00	36.50
T_8 : 100 % RDF + LCBF Soil treatment	13.33	10.33	31.50
T_9 : 100 % RDF + LCBF Drip fertigation	16.00	11.67	37.00
T_{10} Control : Only biofertilizers	10.00	8.00	22.30
SE(m)	1.12	0.67	0.87
CD(P=0.05)	3.37	2.02	2.61
CV	13.14	10.62	4.41

to drip fertigation with liquid based biofertilizers. The treatment T_6 with 100 % RDF and LBBF drip fertigation was recorded significantly higher (20) number of pods plant-1 when compared to the other treatments. Whereas, the lower number of pods plant-1 were recorded with treatment T_{10} (Control) and T_1 (100 % RDF) were 10.00 and 11.67 pods plant-1 respectively.

The significantly maximum (Table 2 & Fig:3) number of seeds (13.00) pod^{-1} was recorded in drip fertigation plot treated with liquid based biofertilizers i.e T_6 treat-

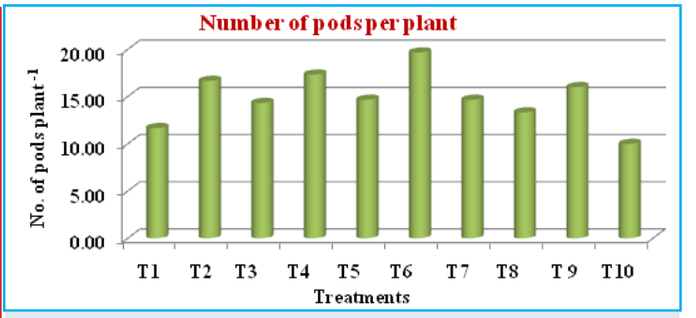

FIGURE 2. Influence of different formulations of biofertilizers application onnumber of pods plant-1of greengram at harvest stage.

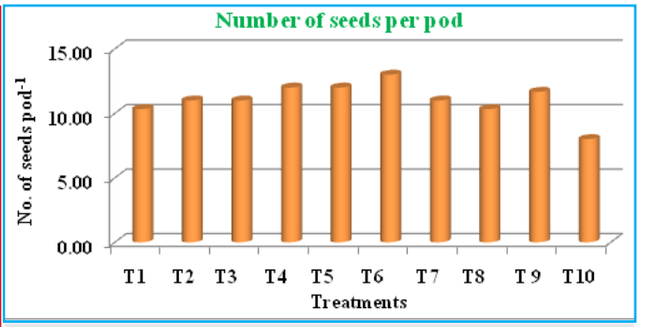

FIGURE 3. Influence of different formulations of biofertilizers application onnumber of seeds pod-1of greengram at harvest stage.

Table 3. Influence of different formulations of biofertilizers application on seed yield and haulm yield of green gram

Treatments	Seed yield (kg ha-1)	Percent increase over RDF (%)	Haulm yield (kg ha^{-1})	Per cent increase over RDF (%)
T_1 : 100 % RDF	0821.67	-	1980.23	-
T_2 : 100 % RDF + CBBF Seed treatment	0945.70	15.09	2450.20	23.73
T_3 : 100 % RDF + CBBF Soil treatment	0875.50	06.55	2310.20	16.66
T_4 : 100 % RDF + LBBF Seed treatment	0960.77	16.83	2506.70	26.32
T_5 : 100 % RDF + LBBF Soil treatment	0880.23	07.08	2350.07	18.48
T_6 : 100 % RDF + LBBF Drip fertigation	1019.50	23.93	2543.50	28.16
T_7 : 100 % RDF + LCBF Seed treatment	0890.20	08.29	2380.57	20.17
T_8 : 100 % RDF + LCBF Soil treatment	0850.23	03.45	2256.97	13.83
T_9 : 100 % RDF + LCBF Drip fertigation	0930.80	13.20	2420.23	22.00
T_{10} Control : Only biofertilizers	0721.77	-	1750.23	-
SE(m)	26.32	-	72.65	-
CD(P=0.05)	78.82	-	217.55	-
CV	5.12	-	5.48	-

ment with combination of 100 % RDF and LBBF drip fertigation. The lowest number of (8.00) seeds pod^{-1} was noticed in T_{10} (control).

The significantly (Table 2 & Fig: 4) maximum (38.77 g) test weight of seeds was registered in treatment T_6 with combination of 100 % RDF combined with LBBF Drip fer-

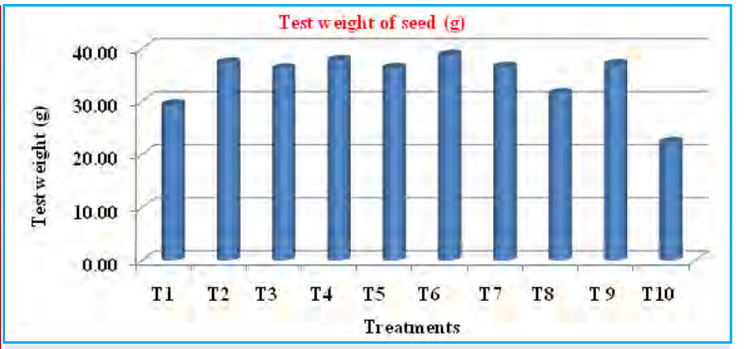

FIGURE 4. Influence of different formulations of biofertilizers application ontest weight (g) of greengram at harvest stage.

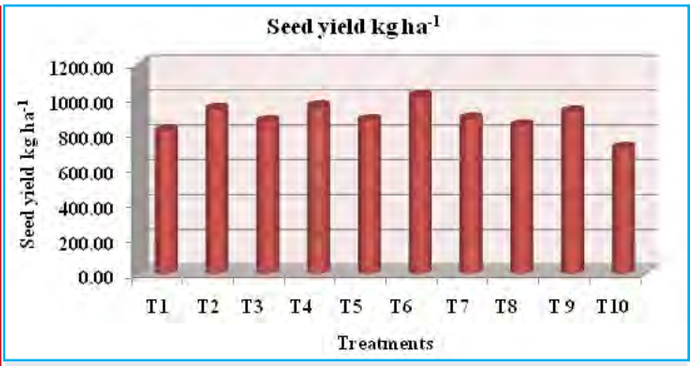

FIGURE 5. Influence of different formulations of biofertilizers application on seed yield of greengram

tigation. The lowest (22.30 g) test weight was observed in T_{10} (control). When compared to control in all other treatments test weight of seeds were observed higher.

The seed yield of green gram significantly varied (Table 3 & Fig: 5) due to application of liquid biofertilizers through drip fertigation. It was indicated that the treatment T_6 with combination of 100 % RDF along with LBBF drip fertigation recorded significantly higher (1019.50) kg ha^{-1} seed yield when compared to all the treatments. The lowest seed yield (721.77) kg ha^{-1} was recorded by treatment T_{10} (Control). Further, the percent of seed yield increased over T_1 (RDF) by T_6 was 23.93%.

Data regarding (Table 3 & Fig: 6) haulm yield of green gram was significantly influenced by drip fertigation with liquid based biofertilizer. Haulm yield is directly related with increase in vegetative growth of the plant. It was observed that the treatment T_6 with combined application of 100 % RDF along with LBBF as drip fertigation recorded significantly higher (2543.50) kg ha^{-1} haulm yield when compared to remaining treatments. Among all the treatments, the lowest (1750.23) kg ha^{-1} haulm yield was recorded in treatment T_{10} (Control). Further, it was observed that the percent increased in haulm yield of greengram over T_1 (RDF) by T_6 was (28.16 %).

The highest yield and yield attributing characters *viz.,* number of pods $plant^{-1}$, number of seeds pod^{-1}, test weight (g), Seed yield (kg ha^{-1}) and haulm yield (kg ha^{-1}) was significantly increased due to combined application of liquid biofertilizers and mineral fertilizers with dripfertigation. It might be due to biofertigation can precisely deliver the bio inoculants in the root zone (Gomathy *et al.,* 2008). It is an added advantage whereas microbial inoculants are supplied through biofertigation could be a potential organic input for precision farming, which can be easily delivered through fertigation system for effective colonization of root zone of crop plants. Effective microorganisms were applied in the field along with inorganic materials and it had more water use efficiency and fertilizer use efficiency.

The fertigation with liquid based biofertilizers and 100 % RDF resulted in higher availability of all three major nutrients (N, P and K) in the soil solution, which led to higher uptake and better translocation of assimilates from source to sink, thus in turn increased the yield. Similar linear response was obtained in long duration pigeonpea under drip fertigation by Vimalendren and Latha (2014). Hence, the Precision farming is one among the integrated management approaches of agriculture,

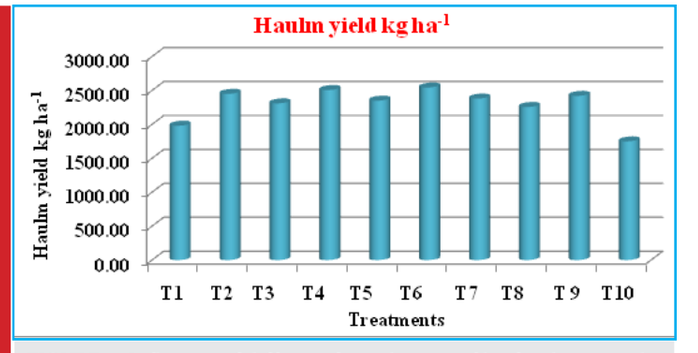

FIGURE 6. Influence of different formulations of biofertilizers application on haulm yield of greengram

which include fertigation and combined practice of organic and inorganic farming to get highest yield and to minimize the cost of farming (Bharathi *et al.,* 2017). Similar results found in (Jeyajothi and Pazhanivelan, 2017) increased drymatter production, nutrient uptake, seed yield per ha^{-1} and stalk yield per ha^{-1} obtained from 125 percent RDF through WSF under drip fertigation. Hence, the perusal of the yield and nutrient upatke data showed the favourable effect of drip fertigation on yield of Greengram. The yield per hectare was significantly improved by the application of major nutrients through fertigation and liquid bofertilizers as biofertigation, which boosted the overall vegetative growth and biological efficiency of the plant. The findings of the foregoing experiments have clearly established that fertigation is definitely advantageous over the surface irrigation with basal application of fertilizers.

CONCLUSION

Precision farming is one among the integrated management approaches of agriculture, which include fertigation and combined practice of organic and inorganic farming to get highest yield and to minimize the cost of farming. Fertigation system of precision farming is considered as effective delivery of nutrients exactly at the root zone of crop, which minimize the loss as well as reduce the environmental hazards caused by the chemicals. This technology ensures the fertilizer use effectively to a greater extent. Biofertigation can precisely deliver the bio inoculants in the root zone. It is an added advantage whereas microbial inoculants are supplied through biofertigation as it has more water use efficiency and fertilizer use efficiency, quality etc. Effective microorganisms can also applied in the field along with inorganic materials. The results clearly confirmed that bio fertigation could be an effective system which can increase the ultimate output of yields.

ACKNOWLEDGEMENT:

Authors acknowledge the help and cooperation of Dr. V. Ramulu, Principal Scientist (Agro), Water Technology Centre, College of Agriculture, PJTSAU, Rajendranagar, Hyderabad, for providing facilities to successfully carry out the research work.

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Technological Communication

Biosci. Biotech. Res. Comm. 12(2): 485-490 (2019)

BBRC Bioscience Biotechnology Research Communications

The issues of energy efficiency in cloud computing based data centers

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ABSTRACT

Cloud computing is a revolution nowadays, which changes the ways in which the IT-based services are being delivered to end users. It is growing or we can also say grown-up technology that offers many benefits whether in terms of economy or in terms of cost-effective resource utilization. Cloud services are being offered via data centres situated at various locations and these data centres make use of virtualization that leads to various benefits to end users. All the Giants in the field of cloud computing like Google, Amazon, and Microsoftrely totally on the data centres to fulfil the dynamic demand raised by the services. However, all the benefits of cloud-based services lead to various energy-related issues. Energy efficiency is very important from the future perspective of information communication technology (ICT). So nowadays lots of efforts are being made towards minimizing the power consumption of data centres. In thepresent paper, all the issues related to energy efficiency discussed and investigated. The main aim of the current paper is to study the various energy efficient techniques for the data centres and compare these approaches, to do so data has been used from various research articles, papers, and internet.

KEY WORDS: CDC, DVFS, ENERGY SAVING, ENERGY CONSUMPTION, POWER SAVING, QOS

INTRODUCTION

Cloud computing can be considered as a model that deliver services and resources dynamically over the internet as per the requirement of end users. It provides the option to the end user to transfer their work and services into the cloud and use these services around the globe whenever required with the help of internet connectivity. By this, way end user able to use the quality of service in a reliable and efficient manner (Tianfield et al., 2018)

With the invention of cloud-basedservices, remote collaboration becomes very easy and a large number of users can work in a collaborative manner (Damien

ARTICLE INFORMATION:

Corresponding Author: anujbit@gmail.com
Received 1st April, 2019
Accepted after revision 1st June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/35

et al, 2011). The end user can use applications as well as storage through cloud-based services, as the cloud-based services delivered as platform-based services, Infrastructure based service and software-based services. These models known as service delivery models. All these delivery models deployed as a public cloud, private cloud or as a hybrid cloud (YI et al, 2018).

Cloud computing makes use of virtualization for using the software and hardware in an efficient way. The idea behind it is to deliver the services to the end users based on their demand and the user just need to pay according to the usage. Resources are available in the large amount and all these resources are available to use by the users (Yadav et al, 2019). These are some basic characteristics of cloud-based services. The evolution of cloud computing revolutionizes the owner based approach to the subscription-based approach, by delivering the services on demand and in scalable fashion (Kotas et al, 2018).

Nowadays there are many cloud service providers, which provides cloud resources. These resources may be hardware based or software based resources, and these resources are used by the variety of users on pay as you use basis (Yadav et al 2019).

All the giants of cloud-based service providers (Amazon, Google, Microsoft, Sun, IBM etc) expanding their work by building their data centers across the globe(Mell et al, 2011). All data centers are used to host the cloud-based applications, which can be categorized according to the requirements; some of them can be viewed as business applications, gaming services, scientific data processing, and multimedia information delivery (Qureshi et al, 2018).

In addition, to run these data centers enormous amount of energy or power is needed (Clark et al, 2005). The power needed for network devices, monitors, cooling fans, monitors, air conditioners etc. This power demand is increasing day by day as in 2012 power consumption of data centers across the globe is 65% greater than its previous year. Moreover, nowadays it's more than 100% (Andy et al, 2008).

In spite of the fact that cloud-based services gives financial benefits as discussed above, there are some concerns as well. These concerns are due to large power consumption and emission of carbon, and that has become a serious concern. Data centers used to store a large amount of data in the cloud and to do so heavy amount of energy is used in form of heat. Moreover, nowadays a serious concern around the globe is global warming, so we can say cloud-based services may be view as one of the source that leads to global warming because there are many gases (carbon dioxide, carbon monoxide etc)released while using cloud-computing services (Markandey et al, 2018).

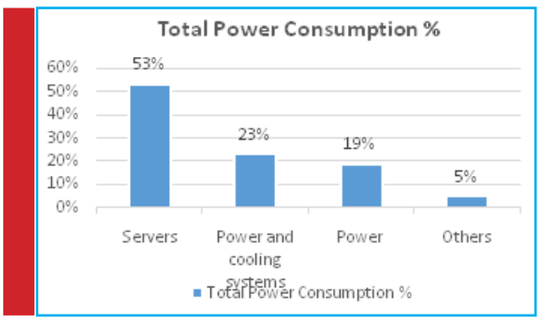
consumption of 1.2 MV, which used for around 1200 houses power consumption (Toress et al, 2018). If energy and power consumption of data center reduced, it will not only benefit the global warming vice but also as economy wise and that would be a great contribution to environmental sustainability. As per the estimation of Amazon.com and their data centers, the total cost of operations of servers is around 53% of total budget and cost related to energy comes out to be 42% of total cost as shown in Table 1.1 and chart. This energy consumption cost includes power required to working of systems as well as total cooling infrastructure (Hamilton et al, 2009).

As energy plays an important role in global warming-related issues, if energy consumption gets reduced by data centers it will be best for the climate-related issues. Therefore, energy minimization will lead to improvement in productivity and reliability of the system. And energy minimization not only reduces the cost but it will also be useful in protecting the environment. So the reduction of energy consumption of the cloud data center and cloud computing systems is a challenge and it is growing rapidly with time. To counter this situation

Table 1.1. Energy distribution in the data center

Factor	Total Power Consumption %
Servers	53%
Power and cooling systems	23%
Power	19%
Others	5%

So energy consumption is one of the major areas of concern in service distribution of cloud computing. The energy and power requirement of data centres is very high, as data centres use this for the computation and cooling. Due to this energy, cost becomes very high and there is large emission of carbon. The energy consumption of data centers situated worldwide calculated as 1.4 % of total EEC (electrical energy consumption) and it is growing every year at a rate of 12%.A datacentre situated in Barcelona pays around £1 million for the power



effective measure must be taken so that environment-related issues could be addressed well within time. In this paper energy, related issues discussed so that data center based cloud computing results become environment-friendly. Remaining paper is organized as in section 2 differences between cloud infrastructure and a data center pointed out; in section, 3 energy consumption issues of data centers are discussed. In section 4 points regarding sustainable energy pointed out, section 5 constitute the information about the factors that lead towards energy consumption, section 6 contains the approaches used by various researchers for energy efficiency in data centers. Finally, the paper concludes in section 7 with the conclusion.

Data CentreVs Cloud Infrastructure

The data center is a facility that has a collection of servers, routers, switches, and other computing-related devices. In addition to this data center also have different components that are required to run a data center and these components comprise of backup systems, power supply systems, security-related devices etc. data center is a solution where there is a large collection of software and hardware resources. These resources are placed at a certain location and are intact of each other. In today's IT enterprise world, term data center ignites the business applications (Cisco Web, 2018).

Another side cloud is a virtual established infrastructure, which is available and provides services locally or remotely with the help of network connections. Within the range of cloud, computing environment users can access the variety of resources with ease. The difference between two summarized in table 2.1 given below

Table 2.1. Data Centre Vs Cloud Infrastructure

On Premise Data Center	Cloud based Infrastrucrre
Cost is high and less scalability	Not costly , used needs to pay according to usage
Less flexible	Largely flexible and customizable
Not remote access of data	Data can be access from any location
Implementation takes lots of time	Can be implemented in less time
No automatic updates	Updates on regular basis
Need specialized members for maintenance	Manage and maintain by the provider

Therefore, if we look towards the differences, we can say the data center is a broader term when we compare it with the cloud (Quora Web, 2018). However, both of these are closely associated with one another.

Energy consumption issues in Data Centres and Cloud Infrastructures

As we already discussed that a data center is a broader termthan cloud computing that has a large collection of servers, power-related devices for an example, NSA data center situated in the USA consume more than 70 MW electricity. If we discuss in detail we can say, nowadays with the increased demand for cloud-based applications by the organizations, many data centers come into existence. In general, a data center with an area of 50000 square feet needs 5 MW electricity. This much electricity is enough for 5000 households per year. All the cooling equipment's required for the datacentre needs to run round the clock (Slashdot web, 2018).

Therefore, energy and cost minimization is the new requirement for the researchers to develop new technology or algorithms (Ghamkhari et al, 2013).

Data centers and the Challenge in Sustainable Energy

As discussed in the previous section that cloud computing provides demand based solution to end user for their problems and to ensure the reliability and availability of these solutions Cloud Data Centre (CDC) should be functional 24/7. All the activities performed during the various operations generate a large amount of data and that data is stored and processed at the data centers (Dou et al, 2017).

However, the methods of processing, storing and creating the data lead towards energy costs, carbon emissions and issues that disturbs the environment. As data centres consumes numerous amount of power, we are facing the problems and challenges of the sustainable energy economy. With the increase in the demand or cloud-based services, the demand of data centers also got increased. In addition, the demand is increasing day by day in many folds. If demands kept increasing like this, data centers are expected to require 8000 TeraWatt hours (TWh) of energy by 2030 as shown in figure 4.1 (Buyya et al, 2018).

At present, all the data center service providers are looking towards the ways by which carbon footprints of

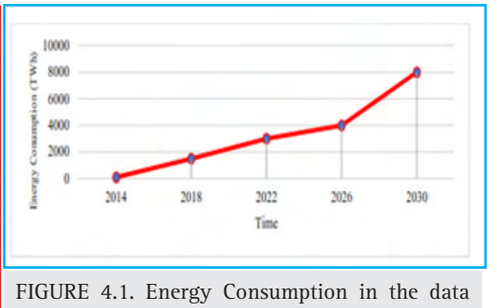


FIGURE 4.1. Energy Consumption in the data center

their infrastructure get reduced. All the giants of cloud data center service providers are looking towards using renewable energy sources such that their data centers provide the services with minimal emission of carbon footprints and greenhouse gas emissions (Li et al, 2018).

Considering all this in consideration maintaining a mechanism to provide cooling is required. However, by developing such solutions cost will also increase. One solution to solve thisIssue is to use the waste heat energy and make free the cooling units. To do so waste heat locations needs to be find out. For enabling the sustainability in cloud, data centres positions is very critical. Data centres needs to be based on:

- Approachability of available green resources
- Chances for waste heat recovery and
- Positioning of free cooling systems

Thus, all these issues are required to be address for enablement of energy-efficient cloud-based systems.

Energy consumption factors in Data Centre

If we know exactly how the servers of data centres consume the energy than we can take the corrective measures to control it. Servers consume lots of energy in the cloud computing environment and this consumption is dynamic which keeps changing according to server utilization. The consumption also depends upon the type of application or computation is running on the server, like data retrieval applications take less time as compared to data processing applications. All the electrical equipment's, networking equipment's also contributed to the energy consumption.

In addition to all these server idle time, energy consumption also contributes to energy consumption but it is very less.

Power supply for UPSs supplied through power conditioning systems. All the UPSs require continuous charging so that that power can supplied to required equip-

Table 6.1. Energy saving approaches in cloud computing

Authors	Technique Used	Aim	Approach Applied
(Stillwell et al, 2009)	Virtual Machine consolidation and resource throttling	Lower energy under some performance constraints	Use of Heuristic algorithms
(Kusic et al, 2009)	Virtual Machine consolidation and Server power switching	Low power under some performance constraints	Use of Resource provisioning framework
(Kim et al, 2009)	Leveraging heterogeneity, DVFS	Minimum energy under performance constraints	Lowest-DVS, Advanced-DVS, Adaptive-DVS
(Li et al 2013)	load balancing of actual resources present across virtual Machines and load migration across virtual machines	Balanced resource utilization and power saving	An algorithm on Cloud Sim toolkit
(Ghribi et al, 2013)	Resource Migration and virtual machine scheduling	Energy saving based upon the load on the system	The algorithm on Java simulator
(People et al, 2013)	Workload Scheduling	Power saving and better server utilization	Java-based tool
(Murtazaev et al, 2011)	Applies Virtual consolidation method	By lowering the number of active server 's energy consumption also reduced.	New simulation tool
(Lago et al, 2011)	Virtual Machine Scheduling and Migration	Virtual Machines in non federated homogeneous and heterogeneous data centers, also, improve power consumption in loads	The algorithm on Cloud Sim toolkit
(Belonglazov et al, 2010)	Effective dynamic relocation of virtual machines, DVFS	Minimize power consumption, satisfy performance requirements	Cloud Sim toolkit
(Buyya et al, 2010)	Resource allocation and scheduling adaptive utilization	Qos, Minimize energy consumption, Green resource allocator	Cloud Sim toolkit
(Buyya et al 2018)	Thermal-aware Scheduling, Capacity Planning, Renewable Energy, Waste Heat Utilization, energy-aware resource management technique	a decrease in carbon footprints of cloud data centers, energy efficiency of power infrastructure and cooling devices by integrating them	Conceptual model

ment's. All these UPSsdisseminate electricity at very high voltage to the power distribution units. Another major source of energy usage in the data center is cooling systems. All the cooling systems used to maintain the necessary temperature and humidity. Cooling in the data center is started with CRAH (Computer room air handler).CRAH is used to transfer heat generated by servers to an icy water cooling loop. Moreover, this process requires a large amount of energy. So the efforts must be made towards minimizing all these factors (Fan et al, 2007) (Uchechukwu et al, 2012)

Therefore, if we want to categories the power consumption, it is broadly of 2 types, one is power consumption during idle time and other is power consumption during the processing.

$$Pt=Pi + Pp$$

Where Pt=Total power consumption,
Pi=Power consumed in idle time and
Pp=Power consumed during processing

Energy Saving approaches in cloud computing

The performance and energy usage of systems depends upon various parameters. Some basic approaches that used to save energy could be the turn on and off the server as per the requirements, putting servers in sleep mode during idle time, Use of DVFS (Dynamic Voltage/Frequency Scaling) and intelligent use of virtual machines or Docker containers for better resource utilization. Many researchers putting efforts for reduction of energy consumption in cloud-based data centers. Some of the approaches are as follows in table 6.1

CONCLUSION

Nowadays cloud computing is a driving force which defines the new way in the IT industry and almost every organization is moving towards it due to its benefits. With the increase in demand cloud systems posses large IT resources, and to run all these resources lots of power and energy is required.

Due to this, it becomes an issue for ecological and economic reasons. In the current paper,the need for energy conservation in cloud computing is investigated and we have identified the issues regarding the cloud computing environment that are related to the data centers. The solution regarding the reduction of energy and power for cloud computing also investigated in the paper. It has been observed that there are some components in the architecture of cloud computing that leads to energy saving. When we talk about the hardware, CPU can lead towards more energy saving than other components like hardware (server and nodes) and memory. In addition to these several solutions regarding the problem also studied, that provides better management of power consumption in cloud computing. All the approaches generally consider few factors like Server scheduling, QoS (Quality of service), network topology, workload scheduling, virtual machine migration, waste heat utilization, load balancing etc. As a future work, all the solutions provided by the researchers can be compared and a new algorithm can be developed that provide the maximum energy efficiency and minimization in CO_2 emission, without reduction the QoS in cloud computing.

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Medical
Communication

Biosci. Biotech. Res. Comm. 12(2): 491-495 (2019)

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Effectiveness of balance training program in improving the functional mobility and risk of fall in obese and osteoporotic patients

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ABSTRACT

Obesity is considered as a rising problem in India and around the world generally seen in individual having sedentary life style and at older ages and risk of Falls especially in osteoporotic population. In order to increase the quality of life in elderly & reduce the incidence of fall it becomes a health priority in clinical intervention. The patients were divided sequentially into two groups: the group who were to performed Borg balance training session Exercise Group (group A) consisting of 22 patients and other Control group (group B) consisting of 20 patients were oriented for risk of fall through a seminar and calcium substitutes were advised for osteoporosis. With improvement seen in the functional mobility and functional balance there was also an significant difference seen with reduction in the frequency of fall in between groups supervised exercise followed by home based exercise protocol and regular telephonic follow-up proved to improve on functional mobility, functional balance and reduce frequency of fall.

INTRODUCTION

Obesity is considered as a rising problem in India and around the world generally seen in individual having sedentary life style and at older ages. Increase in body weight and sedentary lifestyle often lead to loss of calcium deposits from bones thus causing osteopenia and osteoporosis (Benedetti et al., 2018, Marcucci et al., 2015) and increasing age and obesity becomes and contributory factor. The total population in India is above 1.3 billion, and approximately 10% of population are elderly, estimated around 50 million of Indian population are

ARTICLE INFORMATION:

Corresponding Author: dr.poojachaudhuri@gmail.com
Received 3rd May, 2019
Accepted after revision 4th June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/36

osteoporotic or having T-score less than -2.5. Recent studies also show that younger adults may also have osteoporosis (Denova-Gutiérrez et al., 2018, Ambrose et al., 2019), even though male population having common cause of mortality and morbidity the studies done are limited. In elderly obese and osteoporotic population often leads to prevalence of non-communicable diseases, further creating several socioeconomic issues acts as a burden to public health. Falls depend upon two factors i.e. intrinsic and extrinsic factors which comprises of poor balance, urinary incontinence, depression etc. and certain environmental factors respectively. Recent studies shows population with deficit balance is one of the biggest causes for fall in with a high correlation amongst it, (Ozcan et al 2005, Multani et al., 2010, Prato et al., 2017, Malhotra et al., 2018). In order to increase the quality of life in elderly & reduce the incidence of fall, it becomes a health priority in clinical intervention.

Besides this many studies recommend that regular exercises decreases the risk of fracture by increasing the bone density and mass , improving on muscle strength , maintaining good posture and therefore lessening the risk of fall (Senderovich et al., 2017). Whereas emphasis can also be made on maintaining and enhancing balance to prevent falls. In a sedentary lifestyle usually the lack of exercise and awareness about good balance is limited, (Chahal et al., 2018). Various studies also show that only exercise may or may not fulfil the role in reducing the incidence of fall and same is not significantly established in population suffering from osteoporosis. The aim of the study is to establish the effectiveness of balance training program in incidence, frequency of fall and functional mobility and in elderly osteoporotic female.

MATERIAL AND METHODS

42 female subjects were selected from R.L.J.T. Hospital, Churela. The patients with osteoporosis falling under bone mineral densitometry i.e. T score less than -2.5 according to WHO guidelines were included in the study. The patient's bone mineral densitometry tests were done for heel region. The subjects suffering from any neurological disorder, visual and auditory disorder and those women having secondary cause for osteoporosis, Patients using assistive aids and having knee osteoarthritis and other contraindication for which they cannot be included for duration of study were excluded.

The patients were divided sequentially into two groups: the group who were to performed Borg balance training session Exercise Group (group A) consisting of 22 patients and other Control group (group B) consisting of 20 patients were oriented for risk of fall through a seminar and calcium substitutes were advised for osteoporosis. All subjects who were included, procedure were explained about the research and a written consent was occupied.

Demographic data: Personal and clinical details were collected through medical sheet, during assessment more focus was on recent history of fall and frequency of fall in previous years, any use of drugs related increasing risk of falls.

Functional mobility: To evaluate the functional mobility timed "up and go test" was used, the activities performed by the individual were getting up from the chair, walking for 3 meter and returning back to chair and sitting down again. The stipulated time for completion of the activities without a balance deficit is 0 seconds

Functional balance: The Berg balance scale was used, the scale consists of 14 items that are similar the activities of daily living. Total maximum score achieved is 56, reading were taken from 0-4 point. Scoring attained less than 45 were considered as diminished balance .The instrument used was a ruler, stop watch, a chair and step stool. Time to complete the activities maximum of 15 minutes per individual was given (Lima. et al., 2018).

Frequency of fall: The total number of fall in last 1 year was noted during the preliminary assessment, Patients were asked to document the number fall during the duration of the study. The difference between the two was calculated for both the groups. Procedure: The exercise protocol consists of 80 minutes of exercises performed in group once a week in supervision of a qualified physiotherapist. As per the patient age mild to moderate exercise were taught to them also there were ask to maintain a daily diary to mark the number of sessions the exercise were performed supervised and non-supervised for at least 12 weeks.

Patient preparation: Before training exercises patient were instructed to perform few stretching exercises and warm up exercises for at least 10-15 minutes consists of light stretching exercise included stretching of upper & lower extremities followed by walking combined with light movement exercises upper extremities.

Training protocol: 30-40 minute of balance exercises both in dynamic and static position were performed, included walking on toes followed by heels, tandem walking, side walking, walking with rising alternate hand and leg, static exercises included standing on a line and one leg standing (Howe et al,. 2011, Multani 2011). Patients were instructed increase the duration of hold in the static exercises Non-supervised exercises :Patients were also instructed to repeat same exercises taught for minimum 30 minutes of exercises at home in front of mirror as a feedback for 3-4 times in a week, all were instructed to note the number of days the exercise were performed.

Data analysis:42 female subjects participated in the study with 22 subjects in group A and 20 subjects in

group B (5 out of total subjects enrolled for the study discontinued because of personal reasons and family engagements. the data was analysed using Chi-square test and Mann-Whitney, using SPSS software 2018 version. Significant level for P values at <0.05 was considered.

RESULTS AND DISCUSSION

Comparing the demographic details and history of disease parameters and previous treatment taken by the patients there was no significant difference found in the in between group A and group B as explained in table.1, similarly when the base line data was taken for exercises and control group there was no significant difference for Functional mobility (TUGT), Functional balance (BBS) and Frequency of fall in between both groups explained in table 2. The subjects selected in both the group adhered to the protocol and only one individual dropped out from the study.

Table 1. Illustrates about the demographic details of both groups noted at the onset of the study

Variables	Exercise Group Mean ± SD	Control Group Mean ± SD	P-Value
Age (years)	60.23 ± 3.94	61.3 ± 4.25	0.412**
H/o Fracture	0.380 ± 0.49	0.35 ± 0.48	0.842**
Drug History	0.57 ± 0.50	0.7 ± 0.470	0.406**
Hypnotics Drugs	0.71 ± 0.46	0.55 ± 0.510	0.287**
T- Score (Heel)	-2.75 ± 0.16	-2.705 ± 0.17	0.373**

Data expressed in means ± SD
* Significant
**Non-Significant

Table 2. Illustrates the Reading recording at the onset of the study for Timed "Up & Go" Test (TUGT), Berg Balance Scale (BBS) and frequency of fall in exercises and control group.

Outcome	Exercise Group n=21	Control Group n=20	P-value
Functional mobility (TUGT)	48.19±3.23	47.83±4.33	0.764**
Functional balance (BBS)	14.22±5.30	14.45±5.66	0.894**
Frequency of fall	1.12±1.40	0.77±1.44	0.435**

Data expressed in means ± SD
*Significant
**Non- Significant

Table 3. Variance between the initial and final reading for Timed "Up & Go" Test (TUGT), Berg Balance Scale (BBS) and frequency of fall in exercises and control group.

Outcome	Exercise group n=21	Control Group n=20	P-value
Functional mobility (TUGT)	-2.67±3.12	+3.26±2.33	0.0001*
Functional balance (BBS)	4.45±6.07	-1.11±3.98	0.001*
Frequency of fall	-0.89 ±2.16	+1.02±0.82	0.001*

Data expressed in means ± SD
*Significant
**Non- Significant

When comparing the base line data and the final data for exercise and control group, where the functional mobility was measured (TUGT) there was an high significant difference seen for interventional group vs control group (-2.67±3.12 vs +3.26±2.33 $P < 0.0001$), likewise when comparing the exercise group and control group for Functional balance (BBS) there was a significant difference seen in between group (4.45±6.07vs -1.11±3.98, $P < 0.001$) proving improvement in the ability to distribute body weight without a fall.

With improvement seen in the functional mobility and functional balance there was also an significant difference seen with reduction in the frequency of fall in between groups (-0.89 ±2.16 vs +1.02±0.82, $P < 0.001$) resulting that patient when performed exercises for the given duration improved on the checked parameters.

In the past decades studies have been performed for balance and exercises training in osteoporotic patients. The current study was performed for osteoporotic patients for 6 month duration, improving upon the functional balance and functional mobility and reducing the frequency of fall of individual after intervention of exercises. The increases in the functional mobility was seen in patient undergone exercise protocol and was evaluated with time up and go test, comparable results were seen in studies performed by Asmidawati et al., (2014), where home exercise program was performed by patient and resulted in improvement in turning and mobility performance in elderly, with the study the author demonstrated that patient who participated in study especially intervention group showed better result as compared to control group. Balance training program includes mainly aerobic, strengthening and flexibility exercises (Seco et al., 2013), it is difficult to isolate the effectiveness of different types of exercises performed together balance training (Orr et al., 2008), but is has been seen with exercises performed regularly produces

positive result and benefit in term reducing the risk of fall, (Kuptniratsaikul et al., 2011).

The present findings recommend that balance training and exercises produces more evident results than that of strengthening exercises the outcome was reduction in timing of TUGT in subjects performing exercises. The results are significant as related studies shows increase in the risk of fall with conceded mobility (Davis et al, 2010). The mobility and balance is one of the significant constituent of routine activities (Rosenberg et al., 2011). With regular training and improved balance plays an important role in reduction of risk of fall, few studies reveals relationship of exercise training in prevention of fall at older adults, but some studies claims regular activities reduction in frequency of fall (Ambrose et al., 2019), similar studies suggest the characteristic of exercises and intensity of exercise performed on regular interval yields significant results (Gschwind Y.J et al., 2013) Regular exercises and observance towards daily routine can improve the fundamental parameters of balance and mobility, (Osho O et al., 2018).

Adherence of the exercise can also be improved in a home based with certain instruction through a manual or a diary, telephonic reminder & one to one session with physiotherapist to learn and perform correct form of exercises. The environment plays an important role for developing interest towards the exercises also , physical and psychological support from family have proved beneficial results, the results from the present study reveal the benefit of exercises and its relationship with functional mobility, functional balance and frequency of fall in the interventional group.

CONCLUSION

The adapted system of exercises for elderly obese and osteoporotic patients under supervision of physiotherapist which were followed by home based exercise protocol and regular telephonic follow-up proved to improve on functional mobility, functional balance and reduce frequency of fall.

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Microbiological
Communication

Biosci. Biotech. Res. Comm. 12(2): 496-503 (2019)

BBRC
Bioscience Biotechnology
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Antimicrobial and antioxidantactivity of the golden shower, *Cassia fistula*

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ABSTRACT

Cassia fistula, commonly known as golden shower, purging cassia or Indianlaburnum is a flowering plant in the family Fabaceae. The species is native to the Indian subcontinent and adjacent regions of Southeast Asia. Sputum were collected aseptically from patients and cultured on the appropriate bacteriological media. Bacterial isolates were identified by biochemical tests and antimicrobial susceptibility performed by standard methods. Out of 25 specimens, 12 species of various bacteria isolated. The prevalence of bacteria spp. isolated were as follows *Staphylococcus sp*, *Klebsiellasp*, *Pseudomonas sp*, *Escherichia coli*, *Proteus sp*. The susceptibility patterns varied from one bacterial isolates to the other depending on the drug. The susceptibility test against microbial isolates of 15 commercially available antibiotics were used. The Aqueous, Acetone leaf extracts of *Cassia fistula* was subjected for screening of in vitro antibacterial activity against selected major human pathogenic bacterial strains like *Staphylococcus sp*, *Klebsiella sp*, *Pseudomonas sp*, *Escherichia coli*, *Proteus sp* by agar well diffusion method. Ciprofloxacin antibiotic was used as positive control. The Acetone extraction shows more effective in 19 mm in 100µl concentration against *Escherichia coli*. Phytochemical analysis of Aqueous,Acetone solvent extracts of *Cassia fistula* were carried out and results confirmed that alkaloid, flavonoid, phenol, tannin, anthoquinone, saponin, carbohydrate, steroid, terpenoids, triterpenoids, phytosterols are present. Quantitative phyotochemical analysis of *Cassia fistula* leaves revealed the presence of Alkaloids (13%), Flavanoid(17%), Moisture (15%) , Ash (5%), protein and carbohydrate as 11500mg/ml and 1160mg/ml respectively. Antioxidant activity of Acetone, aqueous extracts were studied, such as the ferric reducing antioxidant power (FRAP), Hydrogen peroxide Scavenging activity, Fe2+ chelating ability was performed. The chemical constituents of organic plant extracts were purified by column chromatographyand were separated by thin layer chromatography (TLC).

KEY WORDS: *CASSIA FISTULA*, ANTIBIOTIC SUSCEPTIBILITY TESTING, AGAR WELL DIFFUSION METHOD, PHYTOCHEMICAL ANALYSIS, ANTIOXIDANT ACTIVITY

ARTICLE INFORMATION:

Corresponding Author: pavaimicro@gmail.com
Received 19th April, 2019
Accepted after revision 30th June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/37

INTRODUCTION

A global problem accounting for over 50 million deaths of each year and occurs in both community and health care settings (Zafar *et al.,* 2008). Respiratory infections are the commonest community-acquired infections of humans **(**Bannister *et al.,* 2006). Respiratory tract infection (RTI) is considered as one of the major public health problems and a leading cause of morbidity and mortality in many developing countries (Sharma *et al*., 2005, Jacobs *et al*., 2009 and Bipin Prajapati *et al*., 2011). An antibacterial is an agent that inhibits bacterial growth or kills bacteria. The term is often used synonymously with the term antibiotics; today, however, with increased knowledge of the causative agents of various infectious diseases, an antibiotic has come to denote a broader range of antimicrobial compounds, including anti-fungal and other compounds. With advances in medicinal chemistry, most of today's anti-bacteria chemically are semisynthetic modifications of various natural compounds. These include, for example, the beta-lactam antibacterials, which include the penicillins (produced by fungi in the genus *Penicillium*), the cephalosporins, and the carbapenems. Compounds that are still isolated from living organisms are the aminoglycosides, whereas other antibacterials—for example, the sulfonamides, the quinolones, and the oxazolidinones—are produced solely by chemical synthesis. In accordance with this, many antibacterial compounds are classified on the basis of chemical/biosynthetic origin into natural, semisynthetic, and synthetic, (Bindhu and Sabeena, 2019).

Many factors play in the emergence of resistance, (WHO, 2012) from poor utilization of antimicrobial agents, to the transmission of resistant bacteria from patient to patient and from healthcare workers to patients and vise versa, to a lack of guidelines for a appropriate and judicious use of antimicrobial agents, to lack of easy-to-use auditing tools for restriction (Aly and Balkhy, 2012). In recent years, the infectious diseases remain the leading cause of death worldwide infections due to antibiotic resistant ability of some microorganisms. However, synthetic antimicrobial agents provide broad spectrum characteristics, but often associated with the adverse effects on the host, including immune suppression, hypersensitivity and severe allergic responses. This situation reinforced the scientist communities looking for eco-friendly alternatives so that novel bioactive therapeutic agents can be made. Herbal medicine has a long history in the treatment of several kinds of disease (Holm *et al*., 1998). Their use for the treatment of disease has been practiced by man for many years and is still being widely practiced even today (Kokwaro, 1993).

Medicinal plants beer potent antimicrobial potential, many of them uses in traditional system of medicine, which are readily available in rural areas as relatively cheaper than modern medicine. Some investigators reported the medicinal values of this plant such as hypoglycemic properties of leaf, antibacterial and antifertility effects of seed extract. The present work was objected to carryout scientific study on antibacterial activity of the leaf extract of *Cassia fistula* against nine bacterial species in comparison with the contemporary commonly used antibacterial drug Cephradine. Medicinal plants always played an important role in the health development of mankind. In developing countries, 80% of populations are totally dependent on plants for their primary health care. The global emergence of multidrug resistant bacterial strains is increasing, limiting the effectiveness of current drugs and treatment failure of infections.A noval approach to the prevention of antibiotic resistance of pathogenic species is the use of new compounds from plant origin (Bindhu and Sabeena, 2019).

MATERIALS AND METHODS

Cassia fistula (leaves) were collected in Tirupur district, TamilNadu, India. The freshly collected leaves were washed with water and immediately sprayed with ethanol and dried under shade at room temperature .The dried leaves were powdered in a blender. The powdered plant leaf material was stored in sterile containers for further use.For aqueous extraction10 gram of dried powder of *Cassia fistula* was suspended in 100ml cold distilled water and hot water and mixture was soaked for 24 hours. The suspended solid was filtered through whatman No.1 filter paper and kept in water bath at 80˚C for 2hours. The dried crude extracts were stored at 4˚C for further use, (Negi & Dave 2010, Kulkarni *et al*., 2015). For solvent extraction5 gram of dried powder of C*assia fistula* suspended in 100ml of solvent Acetone and the mixture was soaked for 24 hours. The suspended solid was filtered through Whatman No.1 filter paper and kept in water bath at 80˚C for 2hours. The dried crude extracts were stored at 4˚C for further use. The 35 Pathogenic sputum samples were collected from various Hospitals in Tirupur. Sputum samples were maintained at 4°C. The collected sputum samples were inoculated on the selective media such as EMB, MacConkey, MSA and Cetrimide agar plates were incubated for 24 hours at 37°C. The growth of bacterial colonies was observed and results were recorded. The collected pathogens were identified on the basis of Gram's reaction and biochemical characteristics (Macfaddin, 1980) and results were identified with the help of Bergey's manual of systemic Bacteriology.

Kirby-Bauer disc diffusion method was adopted for susceptibility testing. Penicillin, Azhihaemycin, Cefoxitin, Chloramphenicol, Clindamycin, Erythromycin, Oxa-

cillin, Teicoplarin, Ciprofloxacin, Of loxatin, Linezolid, Tetracycline, Vancomycin, Rifampicin, Ceftazimide, Ceftriaxone, Cerpodoxime, Cefazolin, cefoperazone, Cephalexin, Cefpriome, Piperacillin, Gentamycin, Cotrimoxazole, Amikacin, Polymixin was used. The antibiotic disc was placed on the surface of the Mueller hinton agar medium by using a sterile forceps. The plates were incubated at 37°C for 24 hrs. The zones of inhibition were measured and tabulated (Bauer *et al*., 1966). The plant extract was tested for antibacterial activity by standard agar well-diffusion method. The sputum isolates were swabbed uniformly using sterile cotton swab. The wells of 6mm diameter were made on nutrient agar using gel puncture and then 100µl of plant extracts were loaded into the well and allowed to diffuse at room temperature for 2hrs. The plates were incubated at 37°C for 24hrs. Diameter of the inhibition zones were measured and tabulated (Perez, 1990).

For Qualitative Phytochemical Analysis the plant extracts and acetone, aqueous solutions were assessed for the existence of the phytochemical analysis by using the following standard methods. Wagner's test was performed to determine alkaloids. 0.5g of extracts was diluted with 10ml of acid alcohol and warmed then filtered. 2ml of dilute ammonia and 5ml of chloroform was added, shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 10ml of Acetic acid and 1ml of Wagner'sreagent was added to the resultant extract. Formation of cream or reddish brown precipitate indicated the presence of alkaloids, (Harborne, 1973). For Flavonoids determination 1 ml of extract, 5 drops of 5% sodium hydroxide was added. An increase in the intensity of yellow colored solution is seen which becomes colorless on the addition of few drops of 2M Hydrochloric acid. To determine the Phenols by using Ferric Chloride Test, few drops of 10% aqueous ferric chloride solution was added to 2 ml of the diluted leaf extract and thoroughly mixed. The presence of phenols indicated the formation of blue or green or violet colour. Ferric Chloride test was used to find tannins, 2ml of extract, few drops of 5% ferric chloride solution were added. The blue color indicated the presence of hydrolysabletannis, the green color indicated the presence of condensed tannins. For saponins test, 5 ml of extract was taken and shaken well for five minutes., after which table foam was formed.

The presence of carbohydrates in solvent extracts was determined by different methods such as, Fehling's test, Benedict's test, Molisch's test and iodine test. For-Benedict's test the 5 ml of Benedict's qualitative reagent added 0.5ml of the leaf extract and mixed well. Boiled it for 5 minutes in a boiling water bath. Appearance of yellow, green, red or reddish brown colour precipitate showed that presence of reducing sugar. For Glycosides Test 2ml of the diluted leaf extract added 1 ml of aqueous sodium hydroxide solution. Formation of yellow colour indicated the presence of glycosides .2 ml of the leaf extract (prepared in chloroform) added 2 ml of concentrated sulphuric acid drop by drop along the sides of the test tube. Red colour formed in the chloroform layer, indicated the presence of steroids .5 ml of each plant extract was mixed with 2ml of chloroform and 3ml of concentrated sulpuric acid (98%). Formation of a reddish brown coloured ring between the interfaces indicated the presence of terpenoids.

Few drops of Ninydrin reagent and 1ml of extract were added. Appearance of blue colour indicates the presence of protein. Approximately 2mg of dry extract was shaken with 1ml of chloroform and few drops of concentrated sulfuric acid were added along the sides of the test tube. Formation of red brown color at the interface indicates the presence of triterpenoids .2 ml of aqueous extract was added to 2 ml of 2N HCl & NH_3. The appearance of pink red turns into blue violet indicates presence of Anthocyanin. The extract was (2mg) dissolved in 2ml of acetic anhydride and heated to boiling, cooled and 1 ml of concentrated sulfuric acid was added along the sides of the test tube. A brown ring was formed at the junction and the upper layer turned to dark green color indicates the presence of phytosterols. Hydroxyanthraquinone test was performed to determine the anthraquinone glycosides. To 1ml of extract, few drops of 10% potassium hydroxide solution were added, the appearance of red color confirmed the test.

For quantitative estimation - Alkaloids5 g of the sample was weighted into a 250ml beaker and 200ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4 hrs. This was filtered and the extract was concentration a water bath to one quarter of the original volume concentrated ammonium hydroxide was added drop wise to the extract until the preparation was complete. The whole solution was allowed to settle and the precipitated was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloids which was dried and weighed (Harborne, 1973). For flavonoid analysis 10 g of the plant sample was extracted repeatedly with 100ml of 80% aqueous methanol at room temperature. The whole solution was filtered through Whatman filter paper No.42 (125mm). The filtrate was later transferred into a crucible and evaporated into dryness over a water bath and weighed to a constant weight (Bohrm and Abyazan, 1994). The total ash content of a substance is the percentage of inorganic residue remaining after the organic matter has been ignited .2gm of the pulverized samples was placed in a crucible and ignited in a muffle furnace at 555°c for 6 hours. It was then cooled in desiccators and weighted at room temperature to get the weight of

the ash. For the estimation of moisture 2g of sample was placed in a dried pre-weighed petri dish and placed in an oven to dry at 105°C for two hours. The dried samples were weighed and the experiments were repeated until constant weight was obtained. (A. O. A. C1990). The protein estimation was done by Lowry *et al* method as per standard protocol (Lowry *et al.,* 1951).

For the estimation of carbohydrates 1.0 g of sample was taken into boiling tubes and hydrolyzed by keeping them in boiling water bath for 30minutes to 1 hr with 5ml of 2.5 N HCL. It was cooled to room temperature and neutralized with solid sodium carbonate until the effervescence ceases and made up the volume to 100ml and centrifuged. 0.5ml and 1ml of the supernatant were collected and used for further analysis. The volume was make upto 1.0ml in all the tubes including the sample tubes by the addition of distilled water .4ml of 0.2% Anthrone reagent was added to each test tube followed by heated in a boiling water bath for 10minutes. Test tubes were cooled at room temperature. Dark green color was appeared on heating the samples. The optical density (OD) value of the colored solution was then measured through 630nm wavelength in a colorimeter against blank. The amount of carbohydrate present in the sample was calculated, using the method of Hedge and Hofreiter, (1962).

Antioxidant activity of the extract was done in reducing assay, chelating assay andscavenging of hydrogen peroxide assay. About 1ml of extract/fractions (50-300µg/ml) was prepared in distilled water and mixed with 2.5ml of phosphate buffer (0.2M, pH6.6) and 2.5ml of 1% potassium ferricyanide. The mixture was incubated at 50oC for 20min. 2.5ml of 10% trichloroacetic acid was then added to the mixture and centrifuged at 3000rpm for 10 min.1ml of aliquot of supernatant was mixed with 2.5ml of distilled water and 0.5ml FeCl3 (0.1%) and absorbance was measured at 700nm. Increase in absorbance was interpreted as increased reducing activity. 1ml of extract with different concentration was mixed with 3.5ml methanol and then the mixture was mixed with ferrous chloride (2mM, 0.1ml) and ferrozine (1mM, 0.2ml) for 10 min at room temperature. The absorbance was measured at 562 nm against a blank in which the extract was not added. The % age inhibition was calculated as: % Inhibition = B_0-B1/B_0*100

Where, B_0 is the absorbance of control, B1 is the absorbance of reaction mixture (Harpreet Walia *et al*., 2011). To the Different concentration of the plant extract ranging from (20-100 µg/ml) and (200-1000µg/ml) the hydrogen peroxide prepared with phosphate buffer (PH 7.4) was added in the volume of 0.6ml. Then the mixture was kept at room temperature for 10 minutes and the absorbance was measured at 230 nm using UV-visible spectrophotometer .% of inhibition = Control OD-sample OD / Control OD*100 (Gill *et al*., 2010). For column chromatography air dried powder *Cassia fistula* was soaked in methanol for overnight. 0.5 g of crude extract was used for column (6mm×2mm) chromatography. Silica gel (mesh 60-120) was used as column packing material. The column was eluted up to 5 fractions. Collected fractions were applied on TLC using Acetone; Methanol; Acetic acid (75:08:50µl). The spotswere detected and the presence of certain compounds such as organic compound, carbohydrate, carboxylic acid and phenol was identified due to colour change by spraying reagents.

RESULTS AND DISCUSSION

A total of 35 sputum samples were collected from various hospitals in and around Tirupur. The 10 samples showed no growth and 25 sample shows of respiratory tract infections. From this 25 samples 12 isolates were selected and identified as *Staphylococcus* sp*, E.coli, Pseudomonas* sp*, Klebsiells* sp*,* and *Proteus* sp*,* based on morphological and biochemical characterization. It was shown in Table no. 1. From this 12 bacterial isolates, 8 isolates were gram negative and remaining 4 were gram positive. Based on the gram's reaction commercial antibiotics were chosen. For gram negative isolates were subjected to the Kirby-bauer disc diffusion method was adopted for susceptibility and zone of inhibition were measured and shown in Table no: 2 In this study, five *Staphylococcus* sp., one *Proteus* sp., two *Klebsiella* sp., two *Escherichia coli*, three *Pseudomonas* sp., strains were isolated and identified from different types of sputum samples by Biochemical characterization. In the present study, the antibacterial activity of commercial antibiotics against four *Staphylococcus* sp., was done by disc diffusion method. The Cefotaxime shows Maximum zone of inhibition of range 25mm and Rifampicin shows that Minimum zone of inhibition of range 7mm.Manikandan and Asmath (2013) had reported the susceptibility profile of *Staphylococcus aureus* with the maximum inhibition of 97% for Amikacin and minimum inhibition of 21.3% for Ampicillin.

In the present study, the antibacterial activity of commercial antibiotics was done by disc diffusion method. The antibiotics Piperacillin, Ciprofloxacin shows Maximum zone of inhibition of range 28mm and Polymixinb shows that Minimum zone of inhibition of range 13mm against *Proteus* sp. The polymixin shows Maximum zone of inhibition of range 14mm and ciprofloxacin shows that Minimum zone of inhibition of range 13mm against *Escherichia coli*. The polymixin shows Minimum zone of inhibition of range 13mm against *Klebsiella* sp., other antibiotics shows Resistant. The cefoperazone shows Maximum zone of inhibition of range 14mm and

Table 1. Morpholological and Biochemical characterization of sputum isolates

S. no	Bio-chemical test	Iso 1	Iso 2	Iso 3	Iso 4	Iso 5	Iso 6	Iso 7	Iso 8	Iso 9	Iso 10	Iso 11	Iso 12
1	Gram staining	+ve cocci	+ve cocci	+ve cocci	+ve cocci	-ve Rod	-ve rod	-ve rod	-ve rod	-ve Rod	-ve rod	-ve rod	-ve rod
2	Mannitol salt agar	G	G	G	G	-	-	-	-	-	-	-	-
3	Macconkey agar	P	P	P	P	NLF	-	-	MLF	MLF	-	-	-
4	Eosin methylene blue agar	-	-	-	-	-	MS	MS	-	-	-	-	-
5	Cetrimide agar	-	-	-	-	-	-	-	-	-	BG	BG	BG
6	Indole	-	-	-	-	-	+	+	-	-	-	-	-
7	Methyl red	+	+	+	+	+	+	+	-	-	-	-	-
8	Voges proskauer	+	+	+	+	-	-	-	+	+	-	-	-
9	Citrate	-	-	-	-	+	-	-	+	+	+	+	+
10	Catalase	+	+	+	+	+	+	+	+	+	+	+	+
11	Oxidase	-	-	-	-	-	-	-	-	-	+	+	+
12	Result	*Staph 1*	*Staph 2*	*Staph 3*	*Staph 4*	*Pro 1*	*E 1*	*E2*	*K1*	*K2*	*Ps1*	*Ps2*	*Ps3*

Note:'+' indicates positive , '-' indicates negative , NLF-Non lactose fermenting, MLF-Mucoid lactose fermenting,G-Golden color colonies, P-Pink color colonies, BG-Blue green color colonies,Staph-Staphylococcus sp,Pro-Proteus sp, E-E.coli,K-Klebsiella sp, Ps-Pseudomonas sp

Table 2. Antibiotic sensitivity of isolates for commercial antibiotics

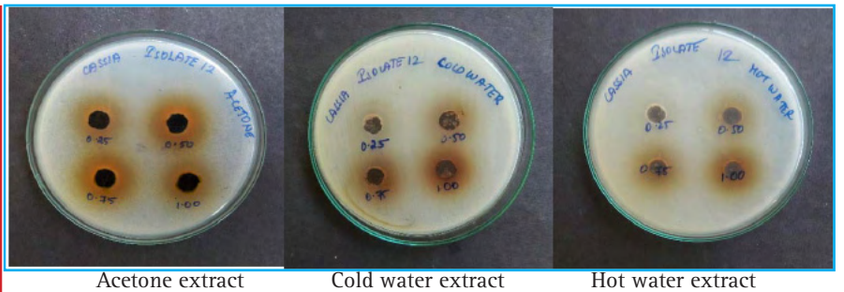
S. No	Isolates	Resistance	Sensitivity
For gram negative isolates			
1	Pro 1	9	6
2	E1	13	2
3	E2	12	3
4	K1	14	1
5	K2	14	1
6	Ps1	13	2
7	Ps2	13	2
8	Ps3	13	2
For gram positive isolates			
9	Staph 1	13	2
10	Staph 2	13	2
11	Staph 3	13	2
12	Staph 4	13	2

Note: Staph-Staphylococcus sp., Pro-Proteus sp., E-E.coli., K-Klebsiella sp., Ps-Pseudomonas sp.,

Cefotaxime and amikacin shows that Minimum zone of inhibition of range 12mm against *Pseudomonas* sp.,

Manikandan and Asmath (2013) had reported the susceptibility profile of *E. coli*with the maximum inhibition of 86% for Amikacin and minimum inhibition of 14% for Sparfloxacin. The susceptibility profile of*Pseudomonas aeruginosa* with the maximum inhibition of 87% for Amikacin and minimum inhibition of 01% for Ampicillin. Solvent extraction of *Cassia fistula* leaf powder was extracted using cold water, hot water and acetone. An attempt has been made to assess the antibacterial properties of Cassia fistula for 12 sputum isolates were shown in **Table no: 3 & Figure no: 1**

Phytochemical analysis of three solvent extracts of *Cassia fistula* were carried out and results confirmed that alkaloid, flavonoid, saponin, carbohydrate, steroid, terpenoids, triterpenoids, anthraquinones, phytosterols, phenol are present.Kulkarni *et al.*, (2015) reported the phytochemicals analysis of three solvents (Aqueous, Methanol, Petroleum ether) showed the presence of 11



Acetone extract Cold water extract Hot water extract

FIGURE 1.

Table 3. Antibacterial activity of *C. fistula* extracts for Sputum isolates

S. No	Isolates	Extracts	Zone of inhibition in mm			
			25%	50%	75%	100%
1	Pro 1	C	0	0	15	16
		H	0	12	14	14
		A	13	15	15	18
2	E1	C	0	13	14	14
		H	0	13	13	15
		A	15	17	17	19
3	E2	C	0	0	13	13
		H	0	12	13	15
		A	12	13	15	16
4	K1	C	0	0	13	13
		H	0	11	12	15
		A	14	15	17	17
5	K2	C	0	12	13	15
		H	0	12	13	13
		A	13	14	17	18
6	Ps1	C	0	12	12	16
		H	0	13	13	15
		A	12	15	16	18
7	Ps2	C	0	0	13	16
		H	0	0	13	14
		A	12	13	15	17
8	Ps3	C	13	15	16	17
		H	0	0	14	16
		A	14	15	18	18
9	Staph 1	C	12	12	15	17
		H	12	13	15	15
		A	12	14	15	17
10	Staph 2	C	11	13	15	15
		H	0	10	14	16
		A	13	15	17	18
11	Staph 3	C	0	0	11	13
		H	10	10	12	14
		A	12	13	16	17
12	Staph 4	C	0	0	13	16
		H	0	0	10	13
		A	13	14	16	16

Note: Staph-Staphylococcus sp,Pro-Proteus sp, E-E.coli,K-Klebsiella sp, Ps-Pseudomonas sp

important phytoconstituents viz., alkaloid, flavonoid, saponin, carbohydrate, anthraquinones, phenol compounds, glycosides, fats, gums and mucilages, proteins and amino acid.Antioxidant increasing absorbance indicates an increase in reductive ability. The *Cassia fistula* leaf extract of acetone, aqeous extract was used

Table 4. Phytochemical analysis of *Cassia fistula* for bio active compounds

S. No.	Parameters	Different Extraction		
		cold water	Hot water	Acetone
1.	Alkaloid	+	+	-
2.	Flavonoid	+	+	+
3.	Phenol	-	-	+
4.	Tannin	-	-	-
5.	Saponin	+	+	-
6.	Carbohydrate	+	+	+
7.	Glycoside	-	-	-
8.	Steroid	+	+	+
9.	Terpenoids	+	+	+
10.	Proteins	-	-	-
11.	Triterpenoids	+	+	+
12.	Anthocyanin	-	-	-
13.	Anthoquinone	+	+	-
14.	Phytosterols	+	+	+

'+' indicates Presence, '–' indicates Absence

for reducing power assay in different concentration of 20,40,60,80,100 µl in absorbance of 700nm and the result was showed in **Table no: 5**

The compounds were separated by column chromatography. The fractions of F1, F2, F3, F4 were collected and thin layer chromatography was done . In this study the Acetone extract of *C. fistula* leaves was subjected to purification by column chromatography with the combinations of solvents i.e. Acetone + Hexane (10+90%), Acetone + Ethyl acetate (60+40%), Acetone + Methanol (55:45%), Acetone + Hexane (50:50%), Acetone + Hexane (50:50%) (0.146, 0.33, 0.568, and 0.7355 respectively) and the column was eluted in different fractions.

In this study TLC of selected fractions reported the presence of certain compounds by spraying different reagents such as p-Anisaldehyde gives a range of colors indicates Carbohydrate, Bromocresol Green stain yellow-green on a blue background indicates the presence of Carboxylic acid, Iodine which reveals the appearance of dark spot indicates organic compounds.TLC results of different organic solvents (Methanol, Chloroform, Toluene, Petroleum ether, Ethyl acetate) when used singly the elution resulted in No separation, spots are not clear, Blur spot, Blur spot and No separation respectively were reported by Bhawna Sunil Negi & Bharti P. Dave (2010).

CONCLUSION

From this present study we conclude that *Cassia fistula* leaf extracts have significant antimicrobial and anti-

Tabl 5. Antioxidant activity of *Cassia fistula* leaf extracts

S. No	Concentration	Extracts	Reducing assay (Absorbance at 700 nm)	Scavenging assay (% of inhibition)	Chelating assay (% of inhibition)
1	20µl	Acetone	0.95	11	15
		Cold water	1.04	17	15
		Hot water	0.82	10	20
2	40 µl	Acetone	1.07	28	28
		Cold water	1.06	22	25
		Hot water	0.88	22	38
3	60 µl	Acetone	1.08	43	42
		Cold water	1.12	31	36
		Hot water	1.02	39	51
4	80 µl	Acetone	1.36	56	56
		Cold water	1.30	48	48
		Hot water	1.12	48	65
5	100 µl	Acetone	1.44	64	69
		Cold water	1.38	55	61
		Hot water	1.20	62	77

oxidant properties. Sputum isolates had a high level of resistance to examine antibiotics. Acetone extract of *Cassia fistula* leaves showed 18-19mm zone of inhibition, surprisingly cold water extract also showed 14-17mm zone of inhibition and hot water shown 14-16mm of inhibition. Health sector should educate the public to use natural remedies instead of antibiotics.

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Biotechnological Communication

Biosci. Biotech. Res. Comm. 12(2): 504-511 (2019)

BBRC Bioscience Biotechnology Research Communications

Role of Protochlorophyllide oxidoreductase C in protection of plants from singlet oxygen-induced oxidative stress

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ABSTRACT

Exposure of plants to extreme environmental conditions greatly reduces their growth by increasing the concentration of reactive oxygen species (ROS). The elevation in the ROS accumulation directly impacts the photosynthetic machinery of the plant. Among the ROS, singlet oxygen ($^1O_2$) cannot be detoxified by any antioxidative enzyme-mediated reactions. Therefore, $^1O_2$ is the major cause of damage to plants during day time. Spraying plants with a chlorophyll precursor 5- aminolevulinic acid (ALA) increases the accumulation of Chl biosynthetic intermediate protochlorophyllide (Pchlide) which acts as a photosensitizer. Upon light exposure of ALA-treated plants, overaccumulated Pchlide in chloroplasts gets excited and transfer their absorbed energy to oxygen to generate $^1O_2$ via type-II photosensitization reaction. The $^1O_2$ immediately damages thylakoid membranes and induces wilting in the whole plant. The *por*C-2 mutant that lacks the photoenzyme protochlorophyllide oxidoreductase C (PORC) fail to photo-transform overaccumulated Pchlide to Chlide upon light exposure therefore, leading to the excess generation of $^1O_2$. This leads to severe damage to photosynthetic apparatus that destroys chlorophylls and the photosynthetic reactions as indicated from Chl a fluorescence imaging, reduced *Fv/Fm* and *Fv/Fo* ratio, the quantum yield of PSII and electron transport rate. Conversely, plants overexpressing protochlorophyllide oxidoreductase C (PORCx) are capable of efficiently photo-converting photodynamic Pchlide to Chlide that reduces the $^1O_2$ production in ALA-treated and light-exposed plants. Reduced $^1O_2$ produced in PORCx plants causes less damage to the photosynthetic machinery and plants do not bleach. Results demonstrate the pivotal role of protochlorophyllide oxidoreductase C in the protection of plants from $^1O_2$-induced oxidative stress during day time.

KEY WORDS: *ARABIDOPSIS THALIANA* · IMAGING PAM · OXIDATIVE STRESS · PROTOCHLOROPHYLLIDE OXIDOREDUCTASE C · SINGLET OXYGEN

ARTICLE INFORMATION:

Corresponding Author: baishnabtripathy@yahoo.com
Received 15th April, 2019
Accepted after revision 20th June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/38

INTRODUCTION

In a cell, metabolic reactions are tightly regulated to minimize endogenous ROS production. Several antioxidant enzymes and antioxidant molecules work in coordination to regulate the redox status in plants. However, this balance gets disrupted when plants are exposed to extreme environmental conditions (Dat *et al*., 2000). Excess light exposure over reduces the photochemical electron transport chain, thereby reducing the overall photosynthetic efficiency of the plant (Nishiyama *et al*., 2001). Apart from chlorophyll molecules present in the antenna and reaction center complex, the membrane-bound chlorophyll (Chl) biosynthetic intermediates can also absorb excess excitation energy and gets excited. Since they are not directly connected to the reaction centers, the energy absorbed by the intermediates cannot be channeled to the photochemical reaction (Tripathy, Mohapatra and Gupta, 2007). Instead, they return to their ground state by transferring their energy to molecular oxygen to give rise to $^1O_2$ via type II photosensitization reaction (Foote, 1991; Montoya *et al*., 2005). Accumulation of $^1O_2$ results into peroxidation of membrane lipids (Triantaphylides *et al*., 2008), the irreversible oxidation of D1 proteins (Krieger-Liszkay, Fufezan and Trebst, 2008) and gravely damages PS I and PS II (Tripathy and Pattanayak, 2010). Experiments conducted on *chlorina1* mutant (lack chlorophyll b) shows severe phenotypic damage when exposed to light (Ramel *et al*., 2013). In the absence of chlorophyll b, the photosystem II chlorophyll protein antenna complex becomes non-functional and produces $^1O_2$ at the natural site of their production due to naked PS II centers. HPLC analysis of enzymatic and nonenzymatic mediated lipid peroxidation products such as octadecatrienoic hydroxyl acids (HOTEs) increased when exposed to high light for 2 days (Ramel *et al*., 2013; Yang *et al*., 2019).

Carotenoids are the natural quencher of singlet oxygen, which are present in close abundance in the antenna molecule to dissipate the excess energy absorbed as heat (Baroli *et al*., 2000). However, in the reaction center, the distance between the reaction center and beta carotene molecule is too large to allow energy transfer, therefore results in the formation of $^1O_2$. (Laloi and Havaux, 2015). The Chl biosynthetic intermediates are bound to the plastidic membrane (Mohapatra and Tripathy, 2007). The carotenoids present in the thylakoid and envelope membranes are not in close proximity of Chl biosynthetic intermediates. Therefore, carotenoids fail to quench the $^1O_2$ from the excited states of Chl biosynthetic tetrapyrroles and allow the production of intermediate derived $^1O_2$ in response to high light stress. Instead, $^1O_2$ oxidized carotenoid metabolites actively participate in downstream regulation of gene expression in response to high light stress, (D'Alessasndro and Havaux 2019).

Application of tetrapyrrole precursor, ALA induce accumulation of chlorophyll biosynthetic intermediate, Pchlide (Tripathy and Chakraborty, 1991; Chakraborty and Tripathy, 1992; Fujii *et al*., 2017). Its conversion into nonphotodynamic intermediate is catalyzed by a light-dependent enzyme, Protochlorophyllide oxidoreductase (POR) which requires light and NADPH to reduce Pchlide to Chlide at the C17 and C18 on the D ring (Armstrong *et al*., 1995; Oosawa *et al*., 2000; Pattanayak and Tripathy, 2002). The evolution of POR enzyme occurred very early among oxygenic photosynthetic organisms (Vedalankar and Tripathy 2019). External application of ALA induces production of Pchlide which cannot be converted into Chlide due to the limited supply of POR in WT plants. In rice plants, two isoforms of POR are present, in which POR A is expressed during early development whereas, PORB is present throughout maturity (Kwon *et al*., 2017).

In the model plant *Arabidopsis thaliana*, multiple isoforms of POR enzyme exist, namely POR A, POR B, and POR C (Armstrong *et al*., 1995; Reinbothe *et al*., 1996; Oosawa *et al*., 2000; Su, Armstrong and Apel, 2001; Pattanayak and Tripathy, 2002). Among them, POR C is highly expressed in response to light and is chiefly present in the photosynthesizing tissues (Oosawa *et al*., 2000; Masuda *et al*., 2003). Therefore, overexpression of POR C rapidly converts the accumulated Pchlide into Chlide and thereby reducing the possibility of Pchlide derived $^1O_2$ oxygen production (Pattanayak and Tripathy, 2011). In contrast, POR C knock-down mutants (*porC-2*) (Masuda *et al*., 2003) have a limited supply of PORC. We have taken the mutant to modulate Pchlide derived $^1O_2$ generation in plants to ascertain the role of PORC during oxidative stress. In the present study, WT, PORC overexpressors (PORCx) (Pattanayak and Tripathy, 2011) and *porC-2* mutants were used to modulate the Pchlide sensitized $^1O_2$ production to study its impact on the photosynthetic efficiency of plants. We have demonstrated the pivotal role of protochlorophyllide oxidoreductase C in the protection of plants from $^1O_2$-induced oxidative stress in light.

MATERIALS AND METHODS

Plant material and growth conditions: Arabidopsis seeds were soaked in double distilled water for 48h at 4°C for seed stratification. Seeds were sterilized with 4% sodium hypochlorite solution and washed 5 times with autoclaved double distilled water. The sterilized seeds were plated on half-strength Murashige and Skoog media (Sigma). After 12 days of plating, the seedlings were transferred to autoclaved agropete and vermiculite

potting mixture (6:1) and grown at 100 µmol photons m^{-2} s^{-1} at 21°C under 14 h light/ 10 h dark photoperiod.

ALA treatment:1 mM aqueous ALA solution was used to spray on three-week-old pot grown Arabidopsis plants. Later, plants were covered with aluminum foil and kept in the dark for 12 hours to accumulate Pchlide. Control plants were sprayed with distilled water.

Light treatment: After 12 h of dark incubation, ALA-treated and untreated plants were exposed to low light (75 µmol photons m^{-2} s^{-1}) for different time intervals.

Imaging PAM: After completion of light treatment, plants were again incubated in the dark for 20 min to open all the reaction centers. Images were captured from IMAGING PAM MAXI chlorophyll fluorometer (Walz, Germany) and the fluorescence parameters were calculated from ImagingWin software (Walz, Germany). Chlorophyll fluorescence parameters such as the maximum quantum yield of PS II (*Fv/Fm*) was determined by the equation *Fv/Fm= (Fm-Fo)/Fm* where *Fo* is the dark fluorescence yield, *Fm* is the maximum fluorescence yield and *(Fm-Fo)* is the variable fluorescence (*Fv*). Maximum efficiency of the water diffusion reaction is analyzed from the ratio of variable to minimum fluorescence yield (*Fv/Fo*). Other parameters such as Electron transport rate of photosystem II was calculated from the equation ETR (II) = ø PS II x PAR x 0.5 x 0.84 where ø PS II is effective PSII quantum yield (calculated by $(F'_m - F_t)/ F'_m = \Delta F/ F'_m$ where F'_m is referred as the maximum fluorescence yield when the samples are illuminated, and F_t is the fluorescence yield at any given time (t). PAR abbreviates for photosynthetically active radiation, 0.5 is the factor of the ratio of PS II and PS I (1:1), 0.84 is the value that correlates with the percentage of incident photons are absorbed by the leaf to drive photosynthesis. The non-photochemical quenching of the maximum fluorescence was calculated by *(Fm- F'm)/ F'm*. Quantum yield of nonregulated energy dissipation Y(NO) is calculated by the equation 1/(NPQ+1+qL (*Fm/Fo*-1)), where *qL* represents the fraction of reaction centers that are open according to the lake model (Baker, 2008).

RESULTS AND DISCUSSION

Overexpression of PORC protect plants from 5-Aminolevulinic acid (ALA) induced oxidative stress: WT, PORCx, and *porC-2* plants were grown under the low light regime (100 µmol photons m^{-2} s^{-1}). After three weeks, plants were sprayed with 1 mM aqueous ALA solution and covered with aluminum foil and kept in the dark for 12 h to allow phototransformation and were kept under low light (75 µmol photons m^{-2} s^{-1}) for 1 or 2 h. Upon exposure to light, due to the limited presence of POR enzyme, plants failed to photo-transform overaccumulated Pchlide to Chlide which resulted in the build-up of excess Pchlide pool that acted as a photosensitizer. The excess Pchlide absorbed a supra-optimal amount of light that could not be transferred to the photosynthetic reaction centers to synthesize reducing equivalents. Instead, the absorbed light energy was transferred to molecular oxygen to generate $^1O_2$ via type II photosensitization reaction of tetrapyrroles (Tripathy and Chakraborty, 1991; Chakraborty and Tripathy, 1992).

The severity of $^1O_2$-induced damage to plants was monitored by IMAGING PAM that measured Chl a fluorescence. Figure 1 shows false color images of chlorophyll fluorescence at a time t (*Ft*), from WT, PORC overexpressor, and its knock-down mutant *porC-2* plants treated without and with 1 mM ALA. In ALA-treated *porC-2* mutants, the fluorescence was seen from only a few partially green patches of bleached leaves.

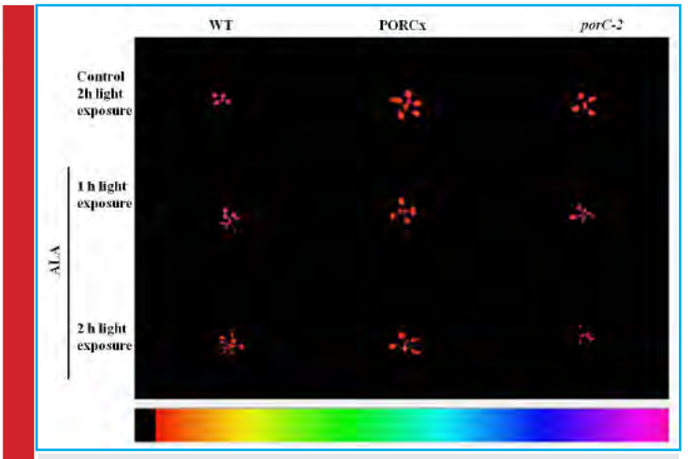

FIGURE 1. Overexpression of PORC protect plants from 5-Aminolevulinic acid (ALA) induced oxidative stress.

The *porC-2* mutant plants due to lack of PORC enzyme were the most affected as they failed to photo-transform overaccumulated Pchlide to Chlide. Under identical conditions, the overexpressor plants had a lot of fluorescence emanating from greener leaves whose images were recorded by the fluorometer.

WT, PORC overexpressor and its knock-down mutant (*porC-2*) plants of three week stage was sprayed with 1mM ALA or distilled water and kept in the dark for 12 h to accumulate photosensitizer Pchlide. Chlorophyll a fluorescence images at time t (Ft) was imaged by using Imaging-PAM (Walz, Germany) after 1 h and 2 h of light exposure. The color code beneath the image depict values from 0 to 1.

Singlet oxygen accumulation decreases chlorophyll fluorescence yield: External application of tetrapyrrole precursor ALA to three-week-old WT, PORCx and *porC-2* plants induced oxidative stress when ALA-treated dark incubated plants were exposed to low light (75 µmol photons m^{-2} s^{-1}), i.e., lower than the intensity at which they were grown (100 µmol photons m^{-2} s^{-1}). To understand if ALA-induced oxidative stress influence the maximum PS II quantum yield in dark incubated plants, the *Fv/Fm* ratio was analyzed in both control and ALA-treated samples after 1 h and 2 h of light exposure (Fig.2a). The *Fv/Fm* ratio of dark incubated ALA-treated WT and *porC-2* plants was reduced by 8% and 14% after 1 h of light treatment. Under identical conditions, the *Fv/Fm* ratio of PORCx plants was unaffected. After 2 h of light treatment, the *Fv/Fm* ratio declined by 14% in WT, 20% in *porC-2* and to a small extent (5%) in PORC overexpressors.

To ascertain the intensity of damage caused by $^1O_2$ to the oxygen evolution complex, the *Fv/Fo* ratio in ALA-treated dark incubated plants was monitored upon light exposure (Fig. 2b). The *Fv/Fo* ratio denotes the activity of water splitting complex of PSII. Due to the limited availability of PORC enzyme in *porC-2* and WT plants, the accumulation of Pchlide caused a burst in $^1O_2$ production that significantly damages the oxygen-evolving complex. The maximum decrease in *Fv/Fo* ratio was observed in *porC-2* plants followed by WT whereas PORCx plants had smaller impairment of photosynthetic oxygen evolution machinery. The *Fv/Fo* ratio declined by 23% and 18% after 2 h of light exposure in *porC-2* and WT plants respectively. However, under identical conditions, minimal damage was observed with respect to the *Fv/Fo* ratio of PORCx plants.

To understand the effect of accumulated singlet oxygen due to ALA treatment on the quantum yield of PS II, the chlorophyll a fluorescence parameters were analyzed in ALA or distilled water treated dark incubated WT, PORCx and *porC-2* mutant plants after light exposure. (a) the ratio of variable to maximum fluorescence

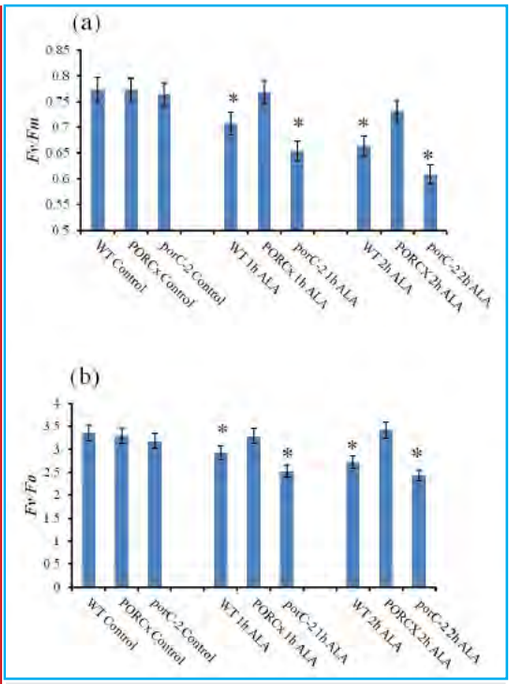

FIGURE 2. Pchlide derived singlet oxygen decreases chlorophyll fluorescence yield.

(*Fv/Fm*) represents the maximum photochemical efficiency of PS II (b) *Fv/Fo* denotes the relative activity of water splitting complex on the donor side of PS II. Each data point is the average of 5 replicates and error bars represent ±SE. Asterisks indicate significant differences determined by t test (*P<0.05).

ALA-induced oxidative stress decreased the quantum yield of PS II photochemistry and electron transport rates: The impact of $^1O_2$-induced oxidative stress on the overall photochemical quantum yield of PS II (ø PSII) was determined at 530 µmol photons m^{-2} s^{-1} light intensity using the equation described in materials and methods. In ALA-treated dark incubated *porC-2* plants, the effective quantum yield of PS II decreased by 50.3% within an hour of light exposure that later declines to 72.6 % after 2 h of light treatment (Fig.3a). In the WT plants, the effective quantum yield decreases from 35% and 63% due to 1h and 2h of light treatment respectively. However, in the PORC overexpressors, the decline in ø PSII was 17 % after 2 h of light exposure. The decrease in the electron transport rate (ETR) of PS II measured at 530 µmol photons m^{-2} s^{-1} light intensity was determined in control, and ALA-treated plants. ALA sprayed *porC-2*, and WT plants, exposed to light 75 µmol photons m^{-2} s^{-1} for 2 h had 50% and 35 % reduction in ETR respectively (Fig. 3b). Under identical conditions, the ETR of PORCx plants was declined by 17.41% after 2 h of light exposure.

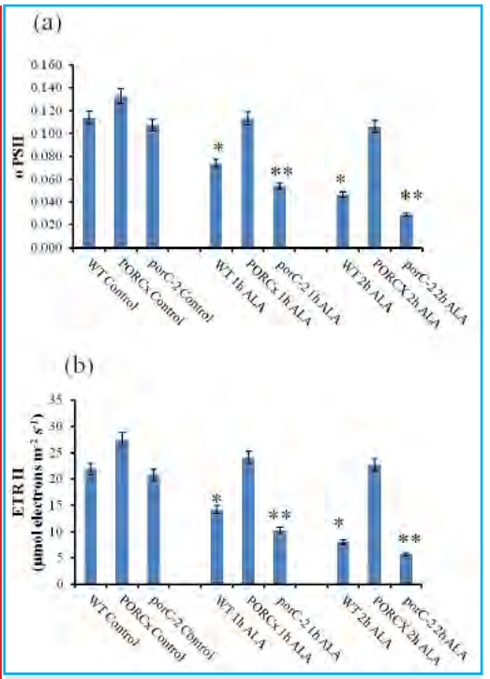

FIGURE 3. Exogenous application of ALA decreases quantum yield and electron transport rates of *porC*-2 mutant.

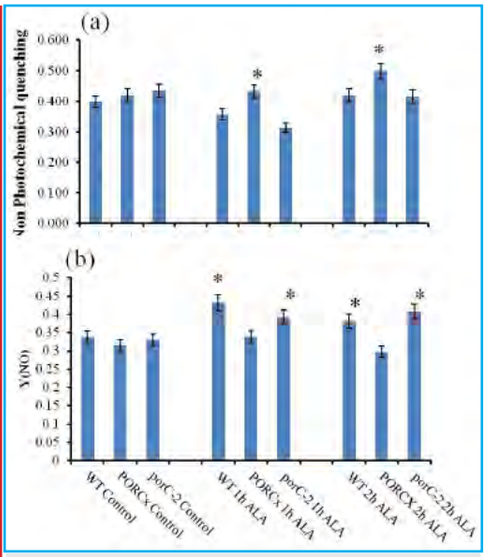

FIGURE 4. Activation of heat dissipation machinery in PORCx in response to ALA-induced oxidative stress.

Pulse amplitude modulated chlorophyll a fluorescence parameters were analyzed from ALA or distilled water treated samples. (a) Quantum yield of photosystem II (b) Electron transport rate of photosystem II was calculated using Imaging Win Software. Each data point is the average of 5 replicates and error bars represent ±SE. Asterisks indicate significant differences determined by t test (*P<0.05, **P<0.01). Activation of heat dissipation machinery in PORCx in response to 102-induced oxidative stress: NPQ is an indicator of activation of heat dissipation machinery in response to stress. Increased NPQ in PORC overexpressor indicates the activation of gradient-dependent heat dissipation machinery (Fig. 4a). Whereas, in *porC-2* and WT, the decrease in NPQ indicate the absence of activation of the protective mechanism. In the control conditions, the Y(NO), that denotes quantum yield of energy dissipation in PS II, were similar in all the three types of plants (Fig. 4b). After ALA treatment, the increase in Y(NO) in *porC-2* and WT indicates that both the processes of photochemical energy conversion and protective regulatory mechanisms are unable to cope up even with the low light (75 µmol photons $m^{-2} s^{-1}$). Under identical conditions in PORCx plants, the decrease in the Y(NO) indicates that due to excess of PORC enzyme, the Y(NO) remained unaffected after 2 h of light exposure.

Pulse amplitude modulated Chlorophyll a fluorescence parameters in ALA and distilled water treated samples were determined by Imaging PAM. (a) Non photochemical quenching (NPQ) (b) Quantum yield of non regulated energy dissipation in PS II. Each data point is the average of 5 replicates and error bars represent ±SE. Asterisks indicate significant differences determined by t test (*P<0.05). In a plant cell, $^1O_2$ is produced as a byproduct of cellular metabolic function. The limited amount of $^1O_2$ can be easily quenched by carotenoids (Ramel *et al.*, 2012). However, when plants are exposed to photooxidative conditions, the balance between $^1O_2$ productions and quenching gets perturbed, resulting in an elevation in $^1O_2$ level. The accumulation of $^1O_2$ not only oxidizes several biomolecules but severely impact the metabolic processes (Tripathy and Pattanayak, 2010). In plants, chloroplasts are the major source of $^1O_2$ generation as the tetrapyrroles that act as photosensitizers are exclusively located in the organelle (Chakraborty and Tripathy, 1992; Triantaphylides *et al.*, 2008; Ambastha *et al.*, 2017).

ALA is the precursor of tetrapyrroles such as chlorophyll, haem, and phycobilins. In the dark, the accumulation of Pchlide modulates ALA synthesis by a feedback mechanism. Regulation of ALA synthesis is essential to prevent the accumulation of photodynamic chlorophyll metabolic intermediates. This enables plants to evade photooxidative stress (Rebeiz *et al.*, 1988; Tripathy and Chakraborty, 1991; Chakraborty and Tripathy, 1992). Unlike other isoforms of POR, which are prominently present in the etiolated seedlings, PORC expres-

sion is present throughout the leaf development and it increases in response to high light. In PORCx plants, the rapid conversion of Pchlide to Chlide takes place due to maximum availability of PORC enzyme (Pattanayak and Tripathy, 2002, Pattanayak and Tripathy, 2011). Chlide produced as a result of phototransformation is immediately converted into chlorophyll that associates itself to the chlorophyll binding proteins present in the thylakoid membranes and transfers the absorbed energy to the reaction centers to drive photosynthetic reactions.

To understand the effect of Pchlide derived $^1O_2$ on the photosynthetic machinery of plants, their chlorophyll fluorescence parameters were studied by IMAGING-PAM (Kandoi *et al.*, 2016). The energy absorbed by Chl can be utilized through either one of the three processes. Most primarily, the energy absorbed by the Chl molecule is directed to initiate the photochemical reactions. Secondly, the absorbed energy is dissipated in the form of heat, and third, the excited Chl molecules return to the ground state by fluorescence. These are three competing processes. Analysis of modulated chlorophyll fluorescence in response to different light pulses provide valuable information regarding the photosystem II activity in a quick and non-invasive manner. The ratio of variable to maximal fluorescence (*Fv/Fm*) indicates the health status of dark-adapted plants. Under control conditions, a dark-adapted, healthy and non-stressed plant emits maximum fluorescence (*Fm*) after illuminated with a short pulse of saturating light Therefore the ratio of variable to maximum fluorescence was closer to 0.78 in non-stressed leaves, which is an effective parameter to study the maximal quantum efficiency of PS II (Björkman and Demmig, 1987). Similarly, under stress, a decline in the light-adapted maximal fluorescence (F'_m) indicates the release of the absorbed energy as heat due to activation of nonphotochemical quenching (NPQ). Likewise, other parameters of chlorophyll a fluorescence can be interpreted to obtain information regarding the quantum yield and PSII-dependent electron transport rate.

In this study, we have observed that plants overexpressing PORC enzyme were capable of tolerating ALA-induced oxidative stress. The *porC-2* mutant has T-DNA inserted in the 4th exon of PORC gene (Masuda *et al.*, 2003). The unavailability of PORC enzyme reduces efficient phototransformation of Pchlide to Chlide. The nontransformed Pchlide upon receiving light gets excited like a Chl molecule and returns to the ground state by generating singlet oxygen via type-II photosensitization process of Pchlide (Tripathy and Chakraborty, 1991; Chakraborty and Tripathy, 1992). Further, spraying *porC-2* plants with ALA increases the accumulation of Pchlide and Pchlide derived $^1O_2$. Therefore, maximum damage to the photosynthetic machinery was observed in the *porC-2* mutant.Enhanced level of $^1O_2$ can reduce photosynthetic efficiency by oxidizing structural and functional proteins associated with the PS II. During electron transport across the PS II, D1/D2 heterodimer constitutes the core of reaction center. D1 protein participates in the charge separation and electron transfer events during the electron transport. Production of $^1O_2$ in the plastidic membrane due to the accumulation of membrane-bound Chl biosynthetic intermediates (Pchlide) can easily target D1 protein. When the oxidative damage caused by $^1O_2$ increase beyond the D1 repair machinery, impairment in the reduction of P680 directly influences the electron transport rate and quantum yield of PS II (Edelman and Mattoo, 2008).

The increased production of $^1O_2$ in *porC-2* mutant bleached substantial amounts of Chls leaving a few green patches in the leaves. The fluorescence was emitted only from a few green patches that were not severely bleached. The WT plants using its endogenous POR could photo transform some of the Pchlide to Chlide, resulting in lower Pchlide accumulation than *porC-2* mutant. Therefore, WT had lesser bleaching of leaves due to reduced generation of $^1O_2$ than *porC-2* mutants, and consequently, the fluorescence was emitted from a higher number of green leaves. The fluorescence imaging clearly reveals that the PORCx plants were protected from photooxidative damage having fluorescence emission from a large number of leaves due to the minimal generation of $^1O_2$ in light.

The Fv/Fm ratio, a measure of the quantum efficiency of PSII, in dark-adapted leaves, declined in WT and *por*C-2 plants. The *PORC*x plants always had higher *Fv/Fm* ratio than that of the WT and *por*C-2 plants. In the same vein, the *Fv/Fo* that measures the oxygen-evolving activity in the oxidizing side of PSII implies the activity of water splitting complex, which is very sensitive to redox changes, was lower in light exposed *por*C-2 plants. It was due to $^1O_2$-induced impairment of oxygen-evolving complex associated with PSII. Similarly, the quantum yield of PSII and the estimated PSII-dependent ETR were higher in PORCx plants due to reduced oxidative damage to the oxygen-evolving complex. Conversely, the heat dissipation of absorbed photons measured as NPQ and quantum yield of non regulated energy dissipation Y (NO) (Genty, Briantais and Baker, 1989) was higher porC-2 mutants exposed to light. Present results demonstrate the important role of protochlorophyllide oxidoreductase C in the protection of plants from 1O2-induced oxidative damage in light.

ACKNOWLEDGEMENT

This work was supported by BSR Faculty Fellowship from the University Grants Commission to BCT.

Conflict of interest :The authors declare that they have no conflict of interest

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Microbiological
Communication

Biosci. Biotech. Res. Comm. 12(2): 512-522 (2019)

BBRC
Bioscience Biotechnology
Research Communications

Influence of grinding time on the bioleaching of copper from copper slag

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ABSTRACT

The present study aims to compare copper recovery from pulverized ground copper slag obtained from pyrometallurgical processing of copper. The method employed for copper leaching/recovery is a microbial catalyzed process with a solid% of 10% (w/v). Grinding/pulverization of the copper slag was carried out for a variable time period of 30, 60, 90 mins and the copper content in all the ground slag was 3.88% (w/w). Reason for varying grinding time was to allow liberation copper from the complex mineral matrix to enhance Cu leaching. 2.5 Liters batch bioreactor was used with one- Liter working volume for bioleaching of copper slag using the mixed culture of chemolithotrophic acidophilic iron and sulfur-oxidizing microorganisms. The Cu recovery from bioleaching of copper slag with a variable grinding time of 30, 60 and 90 mins was 77.8, 84.7 and 86.6% respectively. The recovery% obtained was highest with 90 mins but even 60 mins grinding time also resulted in an appreciable percentage of recovery.

KEY WORDS: COPPER SLAG, REDOX POTENTIAL, SULPHATE, ACIDOPHILLIC, IRON OXIDISING MICROORGANISM, SULPHUR OXIDISING MICROORGANISM, COPPER, OXIDATION

ARTICLE INFORMATION:

Corresponding Author: csgahan_mbio@curaj.ac.in
Received 19th April, 2019
Accepted after revision 25th June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/39

INTRODUCTION

Metallurgical and mineral processing industries are generating a bulk amount of wastes like slimes, slag, sludge, etc., posing several environmental issues creating a greater challenge for the waste disposal. Slags generated from copper forming process via pyro-metallurgical processes is one the important challenges faced by many industrial houses involved in copper production. Copper slag has been one of the potential industrial wastes generated during the smelting and converting steps in copper matte production from copper ores. The estimated global production of copper metal has reached 19.7 million tons by 2017 and for every ton of copper metal produced, around 2.2 tons of slag is generated annually (Gorai et al., 2003; Jarošíková et al., 2017; Mikoda et al., 2019). Copper slag consists of a matrix of iron silicates, aluminum oxide and included droplets of matte, metal, and blister Copper (Cu). Almost all copper slag contains 0.6 to 3% of Cu (Carranza et al., 2009; Roy et al., 2016). Conventionally, copper slag is either dumped directly or used in the preparation of value-added products like cement, glass, tiles, abrasive, cutting tools, etc.(Gorai et al., 2003; Jarošíková et al., 2017; Murari et al., 2015). Owing to the hazardous nature of copper slag due to its heavy metals content, dumping them directly may pose major threat to the environment. However, in either of the scenario the valuable copper present in copper slag goes to the land with a loss of revenue(Agnello et al., 2018; Kaksonen et al., 2016; Potysz et al., 2018a, 2018b). It is expected that by 2025, the global demand for copper is expected to rise to 35 million tons (Market Survey Series MS-33, 2011). Therefore, the need of the time has driven the industries and research organizations to develop alternative methods of copper recovery considering copper slagas a potential secondary source of copper (Mikoda et al., 2019; Piatak, 2018). Slags are found to be highly reactive in the acidic medium; the solubilization of several oxidic phases of copper slag has been found to increase in the acid solution (Kaksonen et al., 2017, 2016; Roy et al., 2016; Urosevic et al., 2015). The use of ferric sulfate (11.5 g Fe^{3+}/l) and a combination of ferric sulfate-sulfuric acid as a lixiviant have led to 90% copper recovery at 2% pulp density at 60 °C (Carranza et al., 2009; Kaksonen et al., 2016; Muravyov et al., 2012). Several studies employing bioleaching as a potential environment-friendly, energy extensive and economical alternative approach for the recovery of metal values from solid wastes have been reported(Ding et al., 2019; Dong et al., 2013; Fu et al., 2008). The oxidative dissolution of metal (from its sulfide or oxide) into the solution takes place by the action of biooxidized ferric ion and biogenic protons (Zhang et al., 2018). The efficacy of bioleaching over chemical leaching has been mentioned in a study, where biogenic acid production by acidophilic *Acidithiobacillus ferrivorans* and *Alicyclobacillus* spp. has led to 80% Cu dissolution. It is worth mentioning here that the study was conducted on a shake flask, where elemental sulfur (S^0) was fed to the bacteria for H_2SO_4 production (Kaksonen et al., 2016). A recent article reported good recovery of metals from slag employing bioleaching using a sulfur-oxidizing microorganism (*Acidithiobacillus thiooxidans*) for 28 dayswith 1% pulp density and 10g/l elemental sulphur (Mikoda et al., 2019). A research study on the physical beneficiation of copper slag by flotation technique followed by bioleaching by a mixed culture of iron-oxidizing and sulfur-oxidizing microorganisms (*At. ferrooxidans and At. thiooxidans*) in shake flask could dissolve 93% of Cu into the solution at pH 1.5 within 12 days(Panda et al., 2015). It is noteworthy thatin many bioleaching studies on copper slag, a grinding step prior to leaching has been followed, due to their refractory nature. The reason for grinding is that the occluded phases of Cu in the slag matrix can be easily liberated by mild grinding (Carranza et al., 2009; Panda et al., 2015). Therefore, in the present study, the copper slag was ground for three different time intervals like 30 minutes, 60 minutes and 90 minutes prior to the bioleaching steps. Three bioleaching experiments were conducted on a bench scale 2.5 L bioreactor with a working volume of 1 L. The three different copper slag samples obtained from different grinding times were used as the feed material. The effect of grinding time of copper slag on the bioleaching mechanism and bio-catalyzed copper recovery by a mixed culture of iron oxidizing and sulfur-oxidizing microorganisms in an iron and sulfur-free 0K medium was investigated. All the three bioleaching experiments were compared for their Cu recovery and bioleaching kinetics and together with their order of reaction.

MATERIAL AND METHODS

Copper Slag

The copper slag used as a feed material in the bioleaching experiments was obtained from M/s Hindalco Industries Limited, India. The slag sample as received was coarse-grained and was subjected to grinding into fine particle sizes at Mineral Processing Department of CSIR-Institute of Minerals and Materials Technology (IMMT), Bhubaneswar, Odisha, India.A size fraction analysis was carried out on the ground copper slag samples followed by a copper content analysis. It was observed that about 80% of the as received copper slag sample was finer than 1800 microns. As the coarse size copper slag were not suitable for bioleaching experiments, a wet grinding

was carried out on a standard Ball Mill (12" x 12") under specified conditions for different time intervals of 30, 60 and 90 mins respectively. All ground products were analyzed for their size fractions (Table 1). It was observed that, with the increasing grinding time from 15 to 120 minutes the d80 of the product decreased from the size of 360 microns to 46 microns respectively. Among all the samples only three samples were selected for the study such as 30, 60 and 90 minutes with a particle size of 210, 83 and 68 microns respectively. These three different ground copper slags with varying grinding time were used for the bioleaching tests to evaluate the copper recovery from the copper slag.

Table 1. Size analysis of the different time ground products

Size in microns	Grinding Time cum Wt % Finer		
	30 min	60 min	90 min
500	100.0	-	-
212	84.1	-	-
150	61.6	100.0	-
100	44.2	92.4	100.0
75	31.2	68.2	87.9
63	23.9	59.1	71.2
45	20.3	52.3	71.2
38	13.8	38.6	52.3
0	0.0	0.0	0.0
d80, microns	210	83	68

Microorganisms

The microbial culture for bioleaching was obtained from Lulea University of Technology, Lulea, Sweden. Q-PCR analysis conducted by Bioclear B.V., Netherlands, revealed that it was a mixed culture of chemolithotrophic, mesophilic acidophilic Fe & S oxidizers. The microbial culture was dominated by *Acidithiobacillus ferroxidans (Fe and S), Leptospirillum ferriphilum (Fe oxidizer) followed by Acidithiobacillus caldus (S-oxidizer), and with approximately the same amount of Acidithiobacillus thiooxidans (S-oxidizer), Sulphobacillus sp. (Fe-oxidizer) and Ferroplasma (Archaeal species, Fe-oxidizer*). The microbial culture was grown and sub-cultured regularly on modified 9K medium supplemented with ferrous sulfate 22 g/l (4.5g/l of Fe2+) and 2mM of potassium tetrathionate as S source. The culture was grown at optimum temperature and pH conditions i.e., temperature 30°C (Franzmann et al., 2005), pH 1.50±0.05 in a 2 L bioreactor with an impeller speed of 220 rpm. The growth medium pH was maintained at 1.5 to provide a conducive environment for bacterial growth (Plumb et al., 2008; Tan et al., 1998) and prevent iron precipitation and jarosite formation (Fu et al., 2008; Kinnunen and Puhakka, 2005; Leahy and Schwarz, 2009). The bioleaching experiments were set up when the viable microbial cells reached their late log phase i.e. oxidized all the ferrous ions available in the growth medium (redox ~700 mV) and a good viable cell count was achieved.

Analytical techniques

Regular pH measurements were carried out by a bench top Rivera Eutech pH meter to ensure the optimized pH conditions for microbial growth and biooxidation. Three-point calibration of the pH meter with standard buffers of pH 1.68, 4.01 and 7.0 was done on a daily basis to ensure a slope percentage of 95- 100%. The measurement of redox potential (Rivera Eutech ORP meter) with a platinum electrode against Ag/ AgCl reference electrode was done at regular time intervals to check ferrous- ferric redox activity. Microbial growth was analyzed by the viable cell counting using a bright field microscope (Rescholar) with the 10x eyepiece and 100x objectives on an improved Neubauer hemocytometer. The estimation of ferrous ions was done by titrating the daily eluted bioleaching solution (with 1, 10 phenanthroline indicator) against cerium sulfate (Sundkvist et al., 2008). The Fe (Total) concentration of the bioleaching solution was measured by vis- spectrophotometer at 510 nm using ammonium acetate as buffer and 1,10-phenanthroline as indicator and the solution was saturated with hydroxylamine hydrochloride whereas the Fe (III) concentration of the bioleaching solution was calculated by subtracting concentration of Fe (II) from the Fe (Total).The sulfate ion concentration was measured by a turbidimetric method involving the formation of barium sulfate colloidal precipitates on the addition of barium chloride to the diluted samples; absorbance was taken at 420 nm as described in American Public Health Association, 1975 (APHA) (Kolmert et al., 2000). Cu content in bioleaching solutions was measured on regular time intervals by Atomic absorption Spectroscopy(Thermo Scientific-iCE 3000 Series).

Bioleaching Studies

Batch bioleaching experiments with iron and sulfur-oxidizing microorganisms (20% v/v) were conducted in 2 L baffled stirred tank reactor at a working volume of 1 L under controlled pH 1.5 and temperature 30°C±2. The pulp density of the feed materials viz. 30, 60 and 90 minutes grounded copper slag was 10% (w/v). On the basis of the time interval of grinding of copper slag the

bioleaching experiments were named as CSGT0.5h, CSGT 1h, and CSGT 1.5h for 30, 60 and 90 minutes grinding. The mineral salt growth medium (80% v/v) used for the experiments was iron free 9K mineral salt medium (Silverman and Lundgren, 1959). The actively grown culture of Fe & S oxidizers had 2.64 x 10^8 microbial cells/ml and redox potential ~700 mV. Homogeneous mixing of the pulp was obtained by an overhead stirrer with an impeller speed of 220 rpm. A hotplate was placed beneath the reactor for maintaining the temperature of the system at 30°C. The increase in pH was maintained at 1.5 by adding 5M H_2SO_4.The water loss due to evaporation during the study was compensated by the regular addition of deionized water.

RESULTS AND DISCUSSION

Characterization of Copper Slag

A representative sample of the copper slag after fine grinding was digested withaqua regia. The liquid obtained after digestion was analyzed by atomic absorption spectrophotometry for copper content analysis. The Cu concentration was measured to be 3.88%.XRD (Rigaku) was done for the mineralogical study of the bioleached residue by measuring the diffraction patterns at angles between 5° to 90° and step size 0.02 angle/sec using Cu-Kα (λ=1.540598) as an X-Ray source. The peaks of the phases of fayalite (Fe_2SiO_4), Iron Oxide (Fe_2O_3), Silicon Oxide (SiO_2), copper oxide and Copper (Cu^0) were prominent in the X-Ray Diffractogram (Figure 3).

Effect of Copper Slag grinding time on batch bioprocess dynamics

The increase and decrease in the pH during bioleaching operations can be controlled by several factors. The sudden increase in pH just after feed addition is due to the presence of acid consuming species in the feed material. The decrease in pH is may be due to biogenic H^+ ions produced by sulfur oxidation mechanism (equation 1) or reduction of ferric to ferrous ion after attacking the feed surface (equation 2) or jarosite precipitation. The increase in pH during the course of bioleaching may be due to ferrous biooxidation which is a proton consuming process (equation 3) or due to proton attack on the metal oxide surface and its subsequent dissolution (equation 4).

$$S^0 + 1.5O_2 + H_2O \rightarrow 2H^+ + SO_4^{2-} \quad (1)$$

$$MeS + 2Fe^{3+} + 1.5O_2 + H_2O \rightarrow Me^{2+} + 2Fe^{2+} + SO_4^{2-} + 2H^+ \quad (2)$$

$$Fe^{2+} + 1/4O_2 + H^+ \rightarrow Fe^{3+} + \tfrac{1}{2} H_2O \quad (3)$$

$$MeO + H_2SO_4 \rightarrow MeSO_4 + H_2O \quad (4)$$

The initial pH in all the experiments was high on the first day was due to the presence of acid consuming gangue minerals like silica and iron oxide (Mikoda et al., 2019; Potysz et al., 2016). The rise in pH on the

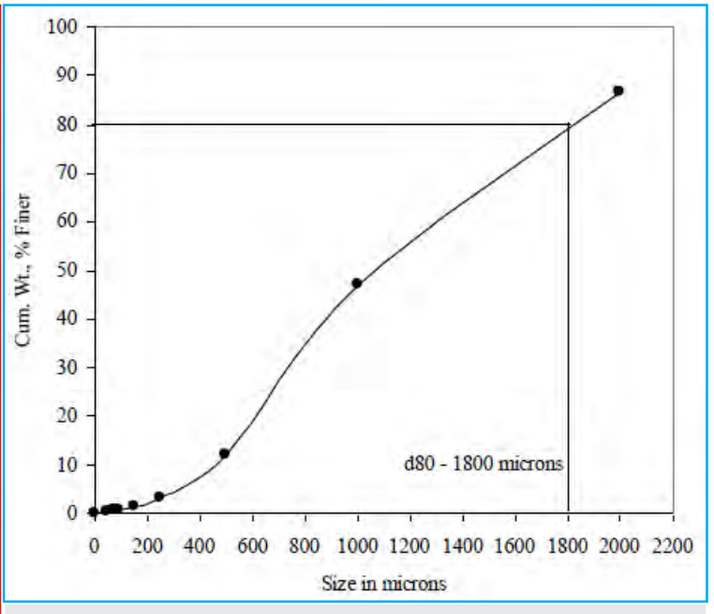

FIGURE 1. Size analysis of the as received copper slag sample

Table 2. Acid consumption in all the bioleaching experiments

Copper Slag	Acid Consumption, kg Conc. H_2SO_4/ ton spent catalyst
CSGT 0.5h	787.517
CSGT 1h	830.806
CSGT 1.5h	812.344

initial day was highest in the CSGT 1.5h experiment as the acid consuming phases are more exposed in it due to the longest grinding and finest particle size among (Figure 4). To avoid iron precipitation and cell stress/ death, the sudden increment in pH was maintained back to 1.5 by the addition of 5M H_2SO_4. The amount of acid consumption decreased gradually after the first day with the increase in Cu dissolution signifying the role of bacteria in the leaching process. The total amount of acid consumption was in the order- CSGT 1h > CSGT 1.5h> CSGT 0.5h (Table 2).

The viable planktonic cell count was also high in all the experiments (Figure 5). The growth of microbial cells in CSGT 1.5h remained in increasing order throughout the bioleaching process, whereas in CSGT 0.5h and CSGT 1h the cell count decreased after the 6th dayand remained similar after 3rd day respectively.The sulfate profile in all experiments was almost constant throughout which depicts no jarosite formation (Figure 6). The ferrous ion profile also suggests the presence of a good amount ofiron for bacterial activity (Figure 7). The redox potential graph explains the ferrous/ferric redox conversions effectively (Figure 8). The high ferrous ion concentration in the bioleaching experiments was due to the dissolution of fayalite (Equation 5-8). The ferrous ion produced served as an electron donor or the energy source for the microorganisms and the biooxidized ferric ion may have attacked the target species (Equation 9).

$$Fe_2SiO_4 \rightarrow SiO4^{4-} + 2Fe^{2+} \quad (5)$$

$$SiO4^{4-} + 4H^{+} \rightarrow H_4SiO_4 \quad (6)$$

$$H_4SiO_4 \rightarrow H_2SiO_3 + H_2O \quad (7)$$

$$H_2SiO_3 \rightarrow SiO_2 + H_2O \quad (8)$$

$$Cu^0 + 2Fe^{3+} \rightarrow Cu^{2+} + 2Fe^{2+} \quad (9)$$

In CSGT 0.5h, the ferrous ion concentration rose till 2nd day and then started decreasing tending to zero on the final day. As the bacterial cell count was fairly high in the experiment, good ferrous biooxidation was taking place. The ferric ion was getting accumulated in the system due to unavailability of the target metal (Cu) phases. This was confirmed by the redox profile of CSGT

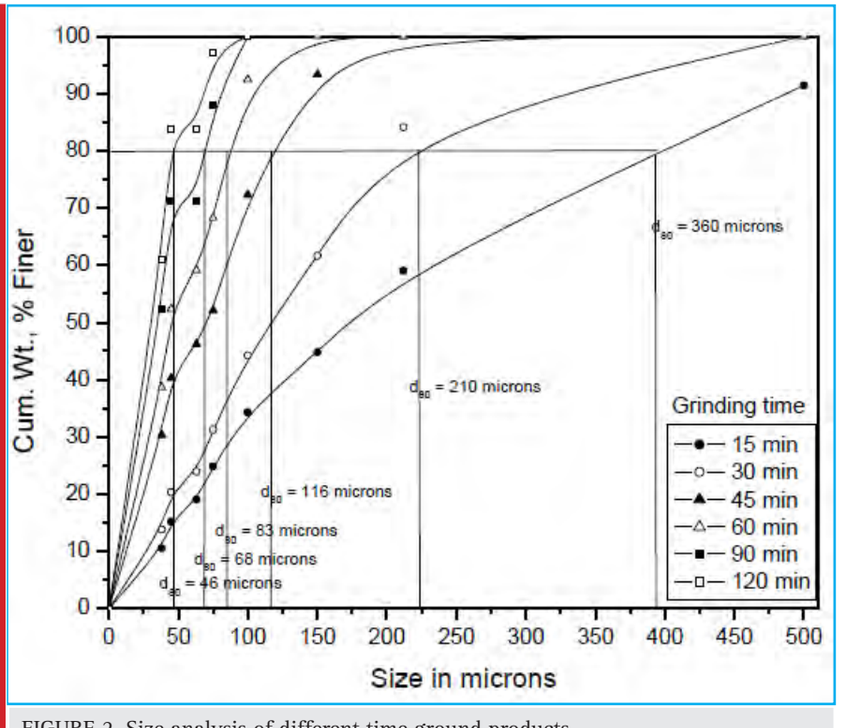

FIGURE 2. Size analysis of different time ground products

0.5h in which redox started to increase from 2nd day and reached up to ~700 mV on the 6th day after which the Cu dissolution was also stopped due to the hindrance in lixiviant penetration through the passivation layer of Fe species. Only 77.8% of Cu can be dissolved into the solution. In CSGT 1h and CSGT 1.5h, ferric ion was always present in the bioleaching medium for the reaction and the redox was also increasing. Cu dissolution was also high in CSGT 1h and CSGT 1.5h due to more availability of the phases of Cu.

Copper bioleaching kinetics and reaction order studies

The shrinking core and shrinking particle models were applied to study the heterogeneous reaction kinetics of Cu dissolution. To evaluate the rate determining step in the leaching mechanism, the steps involved in the solid-liquid reaction are considered (Levenspiel, 1999). The Cu dissolution may have the following step –First, diffusion of the lixiviant from the bulk solution to the solid; second, penetration and diffusion through product layer; third, a chemical reaction between lixiviant and solid; fourth, Product diffusion through product layer and the final step fifth is product diffusion into bulk solution. The first step cannot be the rate-determining step as leaching was carried out under mixing condition. The leaching reaction may also not be limited by the fourth and fifth steps since product diffusion is expected to be a fast process.Therefore, the rate-limiting step can be determined by evaluating the second and third steps. In shrinking particle model, the second step i.e., resistance in leaching through product layer diffusion is omitted as there is no product layer formation. The quantity of reacting lixiviant is proportional to the unreacted surface of the solid core, but the chemical reaction at the solid surface is slower than diffusion. Thus, the third step is evaluated for the rate-determining step using equation 10-

$$\left(\frac{kC}{r_o\rho}\right)t = 1-(1-\alpha)^{\frac{1}{3}} \qquad (10)$$

Here, α= fraction of Cu leached into the solution, t=time, k= rate constant,r_o=original radius of the solid particle, C= concentration of the leach solution, ρ= Density.
Assuming C, ρ, r_o to be constant, rate constant (k) can be calculated by plotting right-hand side of equation 10 with time (t) (Figure 9). The leach rate in chemically controlled leaching is inversely proportional to the radius of the particle.In the shrinking core model, the reaction is controlled by the formation of the product diffusion layer. The chemical reaction at the solid surface is faster than the diffusion. Thus, the second step is evaluated for the rate-determining step using equation 11-

$$\left(\frac{2MDC}{r_o^2\rho\beta}\right)t = 1-\left(\frac{2}{3}\right)\alpha-(1-\alpha)^{\frac{2}{3}} \qquad (11)$$

Here, α= fraction of Cu leached into the solution, t=time, r_o=original radius of the solid particle, M=molecular weight, D= Diffusion constant, C= concentration of the leach solution, ρ= Density, β= stoichiometric factor.

Similarly, assuming M, C, ρ, β to be constant, Diffusion constant (D) can be determined by plotting right-hand side of equation 11 against time (t) (Figure 10). The leach rate in diffusion controlled leaching is inversely proportional to the square of the radius.

It was observed that all three experiments were controlled by the formation of a product diffusion layer. The diffusion-controlled kinetics model gave a better fit with co-relation coefficients R^2= 0.93, 0.89 and 0.72 for CSGT 0.5h, CSGT 1h and CSGT 1.5h experiments respectively. The diffusion constants for CSGT 0.5h, CSGT 1h and CSGT 1.5h were 0.0103, 0.0164 and 0.0107 respectively. To obtain further clarity on the diffusion-controlled leaching mechanism, the mass transfer rate constant and rate determining step was also evaluated by the following mass transfer rate equation-

$$Q = k_m\, t^{0.5} \qquad (12)$$

Here, Q = amount of Cu, $t^{0.5}$ = time, days and km= mass transfer rate constant.

Q was plotted as a function of time ($t^{0.5}$) and the slope of the graph was k_m. The collective graph for all the experiments can be divided into two regimes namely fast and slow,which depicts two different diffusion controlled leaching modes on the basis of the Cu leaching rates (Figure 11). The initial region denotes surface diffusion controlled and final region denotes intra-particle or diffusion through product layer or combination of both. It can be concluded that the slow/final region denoting intra-particle or diffusion through product layer or combination of both is the rate determining step as the mass transfer constant (k_m values) for it was less in all the experiments.

As the Cu is embedded or occluded in the slag matrix, the Fe_2O_3 present in the feedmay have made a passivation layer around the Cu phases and hindered its leaching efficiency. It has also been reported that iron present in the slag gets oxidized to ferric as Fe_2O_3 or ferric hydroxides. The accumulation of these precipitates on the mineral surface forms a passivation layer which negatively affects the leaching kinetics (Roy et al., 2016). High redox potential and ferric ion concentration in all bioleaching medium support the above assumption.

The more is the grinding of the slag, more is the liberation of inbound copper, lesser is the particle size, lesser is the passivation by the diffusion layer and more is the leaching rates.The Cu leaching rates were also assessed in the experiments (Figure 12). The leaching rates of the experiments were in the order- CSGT 0.5h< CSGT 1h< CSGT 1.5h with the R^2 values of 0.833, 0.868 and 0.954

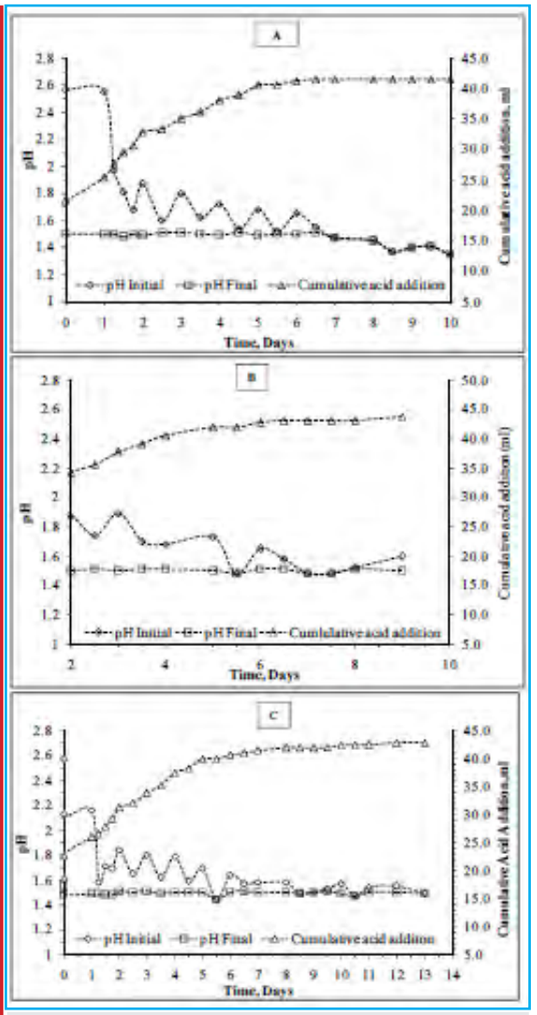

FIGURE 4. pH trend during bioleaching of different time grounded Copper Slag, A- CSGT 0.5h, B- CSGT 1h and C- CSGT 1.5h

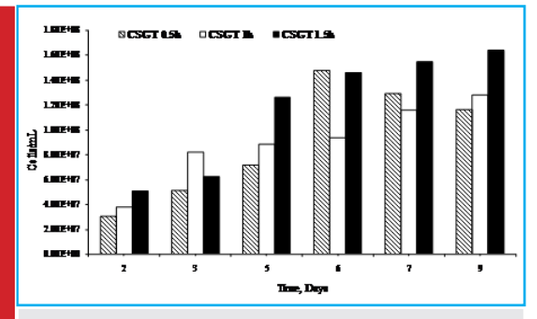

FIGURE 5. Viable planktonic cell count profile of all the bioleaching experiment

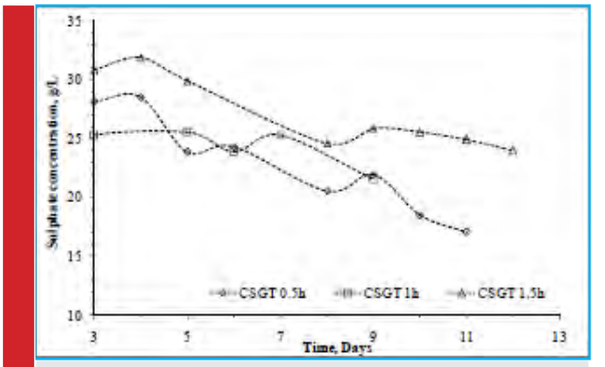

FIGURE 6. Sulphate concentration profile of all the bioleaching experiment

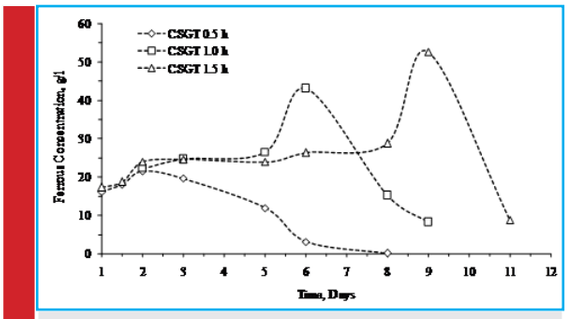

FIGURE 7. Ferrous ion concentration profile of all the bioleaching experiment

respectively. Therefore, the experiment CSGT 1.5h had the highest mass transfer rate and Cu leaching rate (rate constant=0.390) as the Cu phases are more exposed in it due to its longest grinding time and smallest particle size.

The Cu leaching reaction was evaluated for 1st order or 2nd order reaction kinetics. For 1st order model, 'lnC'

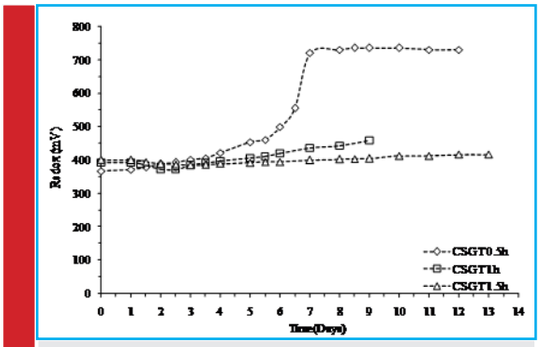

FIGURE 8. Redox Potential profiles of all the bioleaching experiment

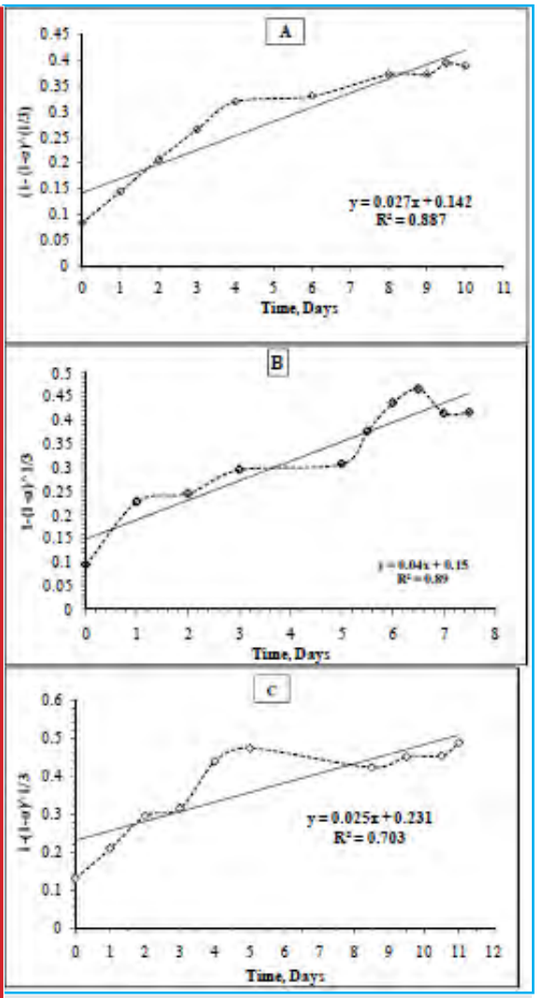

FIGURE 9. Chemically controlled leaching kinetics study of the bioleaching experiments, A-CSGT 0.5h, B-CSGT 1h and C-CSGT 1.5h

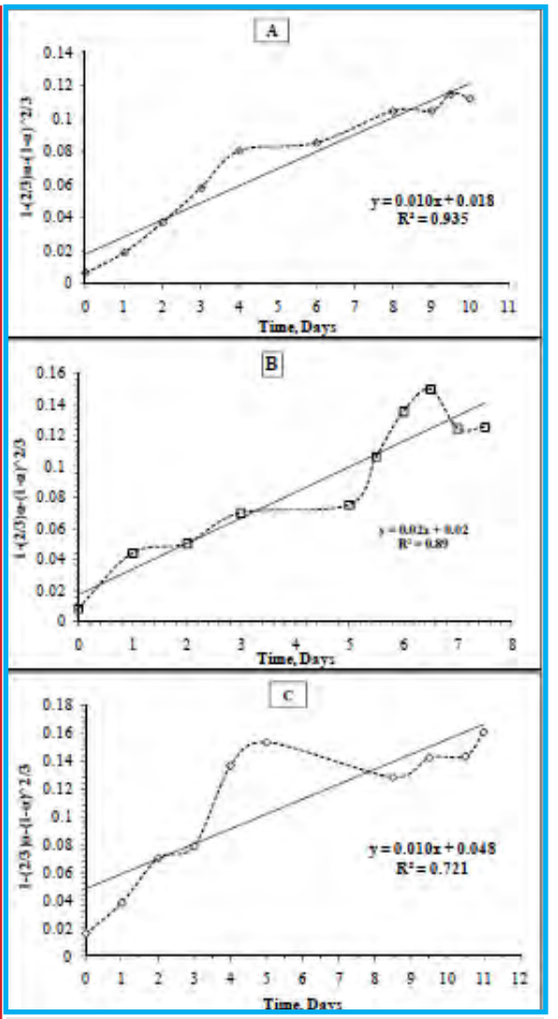

FIGURE 10. Diffusion controlled leaching kinetics study of the bioleaching experiments, A- CSGT 0.5h, B- CSGT 1h and C- CSGT 1.5h

was plotted as a function of 't' and for 2nd order model, '1/C' was plotted as a function of 't', where C is the metal ion concentration and t is the time (Figure 13, 14). The slope of each model gives the reaction rate 'k'. The correlation coefficient for the 1st order reaction model was found to be higher in all experiments CSGT 0.5h, CSGT 1h, and CSGT 1.5h. Therefore, Cu leaching kinetics is following 1st order reaction with a reaction rate of 0.090, 0.114 and 0.056 in the order of experiments.

Copper leaching yields

The copper leaching yields in all the experiments were calculated from the amount of copper measured in the feed. The Cu recovery in CSGT 0.5, CSGT 1h and CSGT

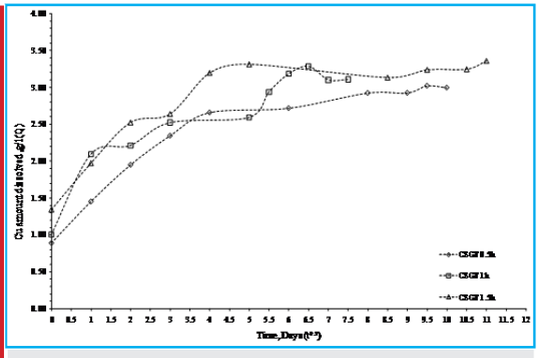

FIGURE 11. Amount of copper dissolved as a function of time

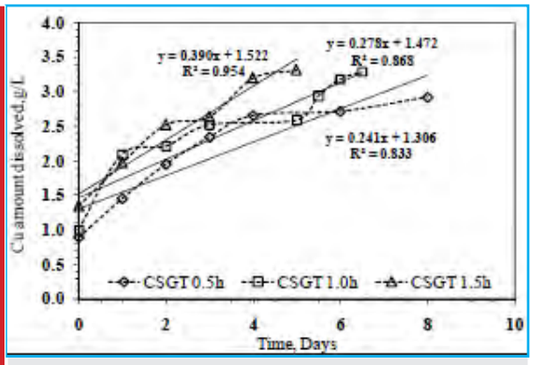

FIGURE 12. Plot of amount of Cu dissolved as a function of time for leaching rate assessment

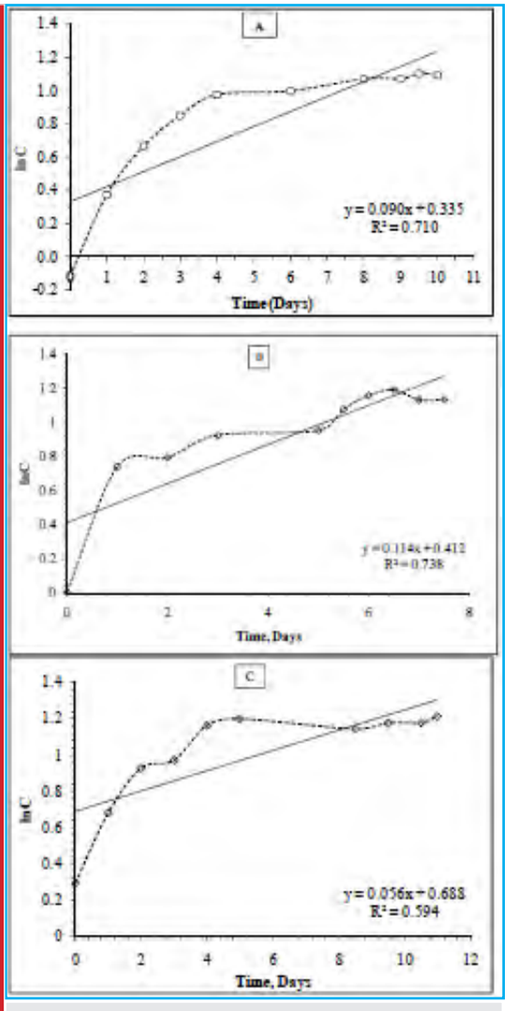

FIGURE 13. 1st order reaction study of Copper bioleaching in all the experiments, A- CSGT 0.5h, B- CSGT 1h and C- CSGT 1.5h

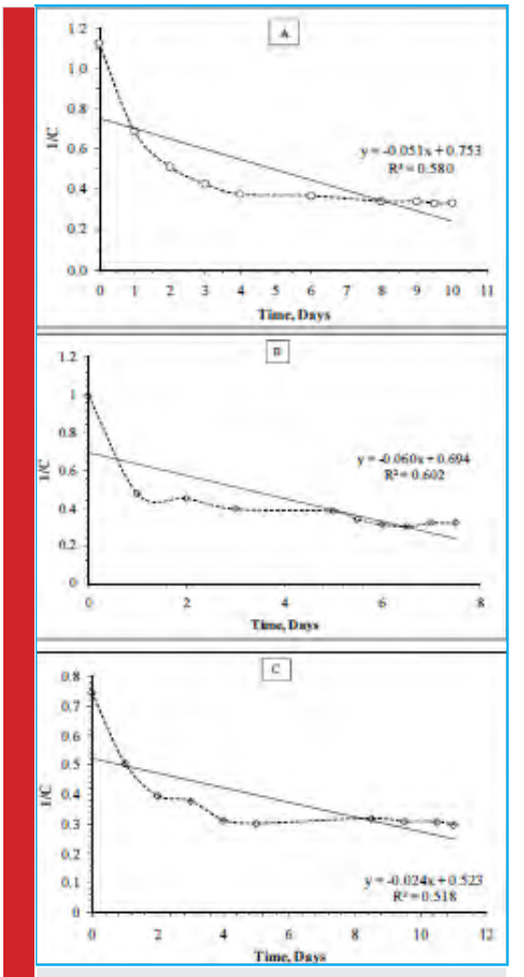

FIGURE 14. 2nd order reaction study of Copper bioleaching in all the experiments, A- CSGT 0.5h, B- CSGT 1h and C- CSGT 1.5h

1.5h was 77.8%, 84.7% and 86.6% (Figure 15). The Cu recovery was affected by the formation of a passivation layer of the oxidized iron species and grinding of copper slag. The lesser recovery of Cu in CSGT 0.5h was

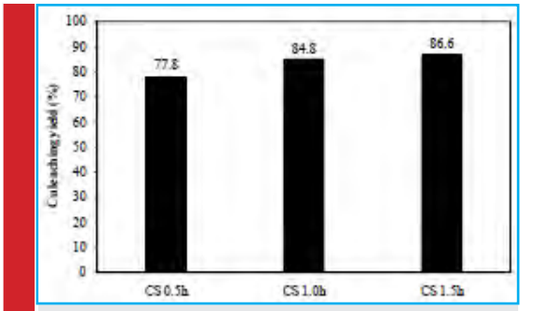

FIGURE 15. Comparative bar graph representing Cu leaching yield %

due to less grinding and larger particle size of the copper slag. Grinding of 30 minutes could not liberate the inbound copper inside the iron matrix completely. This iron matrix acted as a passivation layer which did not allow the lixiviant attack and Cu dissolution. In CSGT 1h and CSGT 1.5h, the Cu recovery was higher and almost similar due to their longer grinding times. The maximum Cu dissolution in CSGT 0.5h experiment was achieved in 8 days, in CSGT 1h and CSGT 1.5h Cu was dissolved in 6-7 and 4-5 days respectively.

CONCLUSION

The copper slag ground for 30, 60 and 90 mins and studied for copper bioleaching resulted with a highest copper recovery at 90 mins grinding time within 4-5 days leaching time. Copper leaching with different grinding time followed first order reaction kinetics with fastest leaching rate at 90 mins grinding time. The bioleaching of Cu followed diffusion controlled for all the experiments.

ACKNOWLEDGMENT

Authors are thankful to the Department of Science and Technology, Govt. India for funding the research from DST-SERB for Young Scientist YSS/2014/000895. Authors would like to thank UGC, DST Inspire and Central University of Rajasthan forproviding the scholarships. Authors would also like to be grateful to M/s Hindalco Industries Limited, India. The support in the XRD analysis of the samples by Department of Physics, Central University of Rajasthan is also highly acknowledged.

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Botanical
Communication

Biosci. Biotech. Res. Comm. 12(2): 523-530 (2019)

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Biochemical evaluation of chlorophyll content using different solvents in various plant species of Amravati, Maharashtra (India)

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ABSTRACT

Plants act as transducers of the solar light in the chemical energy because they contain chlorophyll molecules and are of utmost importance because of their light absorbing property. These pigment molecules are helpful in the measuring plant productivity, maintaining photostasis, protecting from excess sunlight and are also indicators of phototoxicity, pollution and environmental stress. In the present study, 40 plant species of District Amravati were evaluated for their chlorophyll content by Arnon's method of spectrophotometry, using 80% Acetone and 95% Ethanol as solvents. In case of 80% Acetone maximum chlorophyll-*a* concentration was found in *Psidium guajava* and *Polyalthia longifolia* and in case of 95% Ethanol maximum level of chlorophyll-*a* concentrations were found in eight species; *Psidium guajava, Bauhinia purpurea, Moringa oleifera, Murraya paniculata, Plumeria rubra, Terminalia catappa, Peltophorum pterocarpum* and *Delonix regia*. 80% Acetone based samples showed more difference in concentrations of chlorophyll-b with respect tochlorophyll-*a*. The maximum concentration of chlorophyll-*b* was found in *Psidium guajava, Murraya paniculata* and *Bauhinia purpurea* and in 95% Ethanol based samples chlorophyll-b concentration was maximum in Bauhinia purpurea. On the basis of the observation of all the results it was evident that the maximum and minimum values of chlorophylls are same in both the solvents but overall the 95% Ethanol showed higher concentration of both chlorophyll-*a* & *b*.

KEY WORDS: AMRAVATI, CHLOROPHYLL CONTENT, SOLVENTS, SPECTROPHOTOMETER

ARTICLE INFORMATION:

Corresponding Author: nasir.wagay1989@rediffmail.com
Received 1st April, 2019
Accepted after revision 15th June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
Crossref Clarivate Analytics
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/40

INTRODUCTION

The chlorophylls comprise a group of more than 50 tetrapyrrolic pigments with common structural elements and function (Scheer 2003). Pigments are functionally important molecules in photosynthetic organisms. They not only harvest the light energy necessary for carbon reduction but some serve to protect the organism from excess light. The balance of photosynthetic pigments is dynamic and contributes to the maintenance of photostasis within the cell (Huner *et al.* 1998). The ratio of chlorophyll-*a*, and chlorophyll-b in terrestrial plants has been used as an indicator of response to light or shade conditions (Porra, 1991; Vicas *et al.* 2010). The small proportion of chlorophyll-a/b is considered as sensitive biomarker of pollution and environmental stress (Tripathi and Gautam, 2007). The amount of extracted chlorophyll may provide information on the sensitivity of plants during cultivation and herbicide application, and even indicate the manner of phytotoxic activity of herbicides (Nikolić *et al.* 2007). In order to analyze and describe changes in the process of photosynthesis and detect stress in plants, various types of indicators of chlorophyll activity were used (Lichtenthaler, 1996). In tumor or cancer therapy chlorophyll or chlorophyll derivatives can be utilized as a photodynamic agent (Brandis *et al.* 2006). It can be studied, modified and synthesized in chemistry and physics disciplines for different applications i.e., electronic, photophysics, optoelectronic, electrochemistry etc (Nurhayati and Suendo, 2011).

The absorbance properties of pigments facilitate the qualitative and quantitative analysis of these molecules. Determination of the content of photosynthetic pigments in leaves is one of the key techniques in studying the process of photosynthesis and measuring plant productivity. Chlorophyll molecules absorb and re-emit light, a characteristic which was the basis for developing two basic methods: absorption and fluorescence monitoring of the optical activity of chlorophyll molecules. The method of chlorophyll fluorescence is used for monitoring photosynthesis *in situ* and *in vivo* and for estimating the impact of various stress factors (abiotic, biotic, xenobiotic) on this crucial process. It makes possible to differentiate plant genotypes resistant to the aforementioned stressful environmental factors, and also to assess the positive impact of various agricultural measures on plant health (mineral nutrition, use of herbicides, etc.). Methods for non-destructive quantification of chlorophyll in plant leaves are particularly valuable in assessing nitrogen content in plants because chlorophyll is one of the most important points of its accumulation, (Indira *et al.*, 2015).

The confirmation of the two forms of chlorophyll was practically confirmed by Tswett 1903 (Nešković *et al.* 2003). A hundred years later, we used spectrophotometry to analyze plant pigments extracted in various solvents. There is a trade-off between choosing the best solvent for efficient quantitative extraction of chlorophylls and use of a solvent best suited for spectrophotometric assay (James and Akaranta, 2011). The selection of solvent for extraction brings about a dilemma. A number of factors may affect the activity of a solvent: the time required for extraction, amount of plant material, percentage of moisture in plant material, preservation of extract in unchanged form, (Moran and Porath, 1980; Jelić *et al.* 1992), as well as the fact that the extraction of chlorophyll-*a* is a slower process than that of chlorophyll-*b*. Light, an important environmental factor, causes degradation of chlorophyll, so that extraction should be carried out in almost total absence of light (Jelić *et al.* 1992).

The absorbance of the chlorophylls is then quantitatively determined by spectrophotometry at the wavelengths of maximum chlorophyll-*a* and chlorophyll-*b* absorption i.e., λ = 647 nm and λ = 664 nm (Moran and Porath, 1980), while the actual content of photosynthetic pigments is calculated according to Wellburn's formulas (1994). This procedure is based on the Lambert-Beer law on linear relationship between absorbance and concentration of pigments within a certain range.To understand the variation in chlorophyll-*a*, chlorophyll-*b* and total chlorophyll content using different solvents, forty native species of tropical region were selected. Conclusions were drawn based on the variation in chlorophyll content values.

MATERIALS AND METHODS

Amravati is a district in the state of Maharashtra situated at 20˚55'33˚ N and 77˚ 45'53˚ E at 343m (1,125ft.) asl. The Amravati district has an area of 270 km^2. The study area has well demarcated four seasons as a hot summer, heavily raining monsoon, a brief autumn and a mild winter. The area has sub tropical and deciduous climatic conditions with ample rainfall in the monsoon resulting in a rich diversity of vascular plants (Figures 1, 2, and 3). 40 commonly grown plant species of Amravati district (viz., *Aegle marmelos, Acacia nilotica, Alstonia scholaris, Annona reticulate, Annona squamosa, Artocarpus heterophyllus, Anacardium occidentale, Azadirachta indica, Butea monosperma, Bauhinia purpurea, Buchanania lazan, Cassia siamea, Callistemon lanceolatus, Cinnamonum tamala, Citrus aurantium, Citrus limon, Dalbergiasisoo, Delonix regia, Eucalyptus globules, Ficus benghalensis, Ficus religiosa, Mangnifera indica, Moringa oleifera, Murraya paniculata, Murraya koenigii, Manilkara zapota, Peltophorum pterocarpum,*

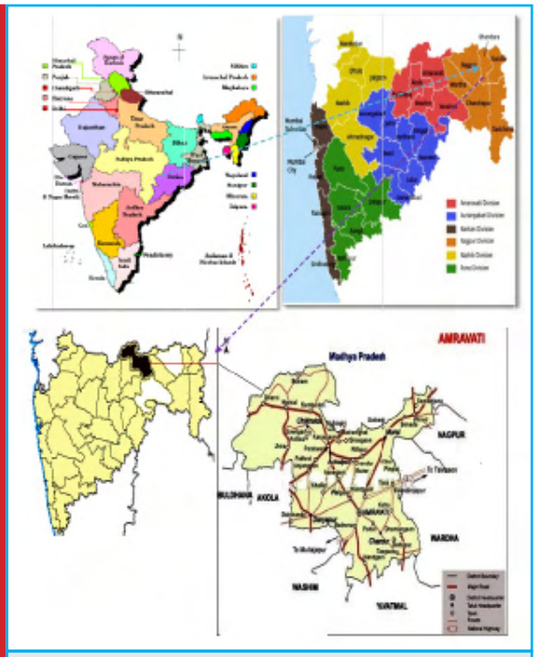


FIGURE 1,2 and 3. Map of India (Figure 1) showing Maharashtra State (Figure 2) in which study Area Amravati (Figure 3) has been shown.

Plumeria rubra, Pongamia pinnata, Psidium guajara, Polyalthia longifolia, Saraca indica, Santalum album, Syzygium cummini, Tectona grandis, Terminalia catappa, Tecoma stans, Thevetia peruviana, Tamarindus indica, Zizyphus mauritiana) were used for experimental purpose. The understudy plants were morphologically identified with the help of standard floras *i.e.*, Flora of British India (Hooker, 1876); and in Maharashtra collected and recorded by Cooke (1967); Naik (1998); and Singh and Karthekiyan (2001) and authenticated by taxonomist Professor Dr. S.P. Rothe. These species are mostly preferred to grow in tropical regions. Healthy and uninfected species were collected at their stage of maturity; and care was also taken during sampling of leaves to avoid mechanical injuries. Fresh leaf samples were washed thoroughly first in tap water followed by distilled water in the laboratory, kept to dry in room temperature (18°C) and analyzed for the determination of chlorophylls (chlorophyll-*a* and -*b*).

For the estimation of chlorophyll content the procedure given by Arnon (1949) with slight modifications was used. Leaves were cut into small pieces and major veins and tough fibrous tissue was discarded. 100 mg of material was used for grinding. 10 ml of 80% acetone (acetone:water; 80 : 20 v:v) was added. For complete pulverization of tissue few grains of sand was added. The homogenate was filtered through filter paper and retentate was discarded while the extract (filtrate) was collected in the test tube. Chlorophyll concentration was determined using spectrophotometer/ colorimeter. In this study, leaves of 40 different tree species commonly available in understudy region of Maharashtra were used for the quantification of chlorophyll content. Two different solvents 80% Acetone and 95% Ethanol were used separately for the extraction of chlorophyll. Absorbance readings of chlorophyll extracts were measured at two different wavelengths 645nm and 663nm respectively.

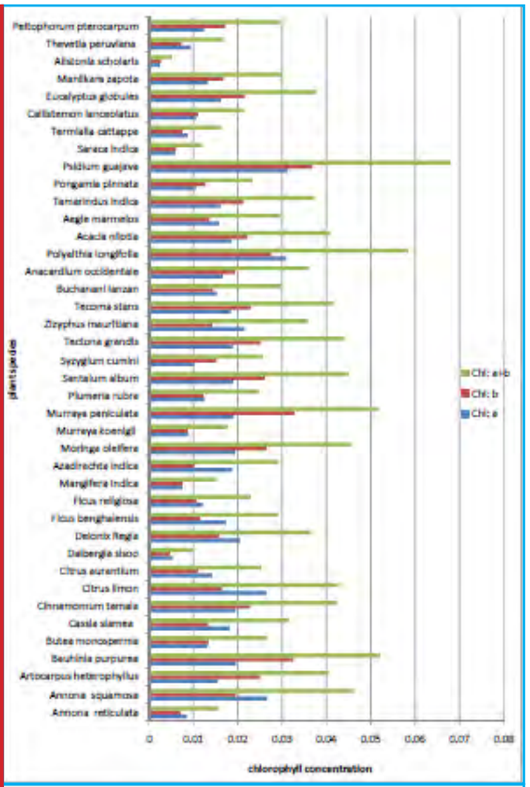
RESULTS AND DISCUSSION

Based on the absorbance value, calculations were made using Arnon's (1949) equation and the amount of chlorophyll-*a*, chlorophyll-*b* and total chlorophyll were estimated and tabulated. The results of Chlorophyll-*a*, chlorophyll-*b* and chlorophyll-*a*+*b* concentrations using 80% acetone as solvent are shown in Tabulated form (Table 1) and as well as in Graphical form (Graph 1). The maximum chlorophyll-*a* concentration was found in *Psidium guajava* and *Polyalthia longifolia* i.e., 0.03g/kg and minimum concentration was found in *Alstonia scholars* i.e., 0.0024 g/kg. In most of the species maximum concentrations of chlorophyll-*a* was found in the range between 0.005-0.02 g/kg. Concentration of chlorophyll-*b* showed much variation in comparison to concentration of chlorophyll-*a*. The maximum concentration of chlorophyll-*b* was found in *Psidium guajava, Murraya paniculata and Bauhinia purpurea* which was more than 0.03g/kg and the minimum concentration was found in *Alstonia scholaris* about 0.002 g/kg. Most of the concentrations were found in the range between 0.1-0.3 g/kg or more specifically between 0.1-0.2 g/kg. The maximum level of chlorophyll-*a*+*b* found in *Psidium guajava* was about 0.06 g/kg and the minimum concentration was found in *Alstonia scholaris*, 0.005g/kg. In most of the species chlorophyll-*a*+*b* concentration was found in the range between 0.02-0.04 g/kg. About nine species showed concentration of 0.02 g/kg and ten species showed concentration above 0.04g/kg.

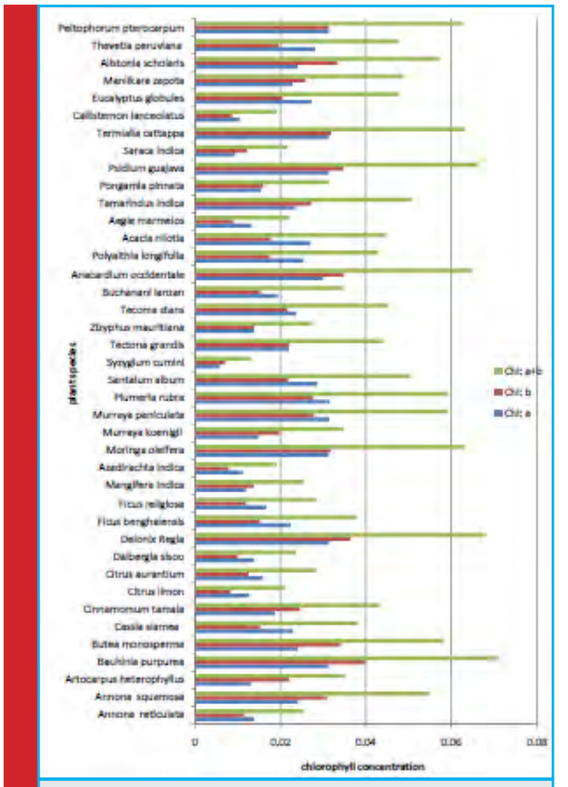
The results of Chlorophyll-*a*, chlorophyll-*b* and chlorophyll-*a*+*b* concentrations using 95% Ethanol as solvent are depicted in Graph 2 and in Table 2. The maximum level of chlorophyll-*a* concentrations were found in eight species such as (*Psidium guajava, Bauhinia purpurea, Moringa oleifera, Murraya paniculata, Plumeria rubra, Terminalia catappa, Peltophorum pterocarpum, Delonix regia)* in similar amounts 0.03 g/kg, and minimum level of chlorophyll-*a* was found in *Syzygium cumini*, 0.005 g/kg. In 12 species less than 0.015 g/kg concentrations were found. The remaining species showed the concentration more than 0.015 g/kg. The chlorophyll-*b* concentration was the maximum in *Bauhinia purpurea*, 0.039g/

Table 1. Chlorophyll-*a*, chlorophyll-*b* and chlorophyll-*a*+*b* concentrations using 80% acetone

S. No	Name of plant species	Chl-*a*	Chl-*b*	Chl-*a*+*b*
1	*Annona reticulate*	0.0085	0.0071	0.0157
2	*Annona squamosa*	0.0265	0.0195	0.0461
3	*Artocarpus heterophyllus*	0.0155	0.0250	0.0405
4	*Bauhinia purpurea*	0.0195	0.0325	0.0521
5	*Butea monosperma*	0.0131	0.0134	0.0265
6	*Cassia siamea*	0.0182	0.0133	0.0315
7	*Cinnamomum tamala*	0.0194	0.0228	0.0423
8	*Citrus limon*	0.0264	0.0165	0.0429
9	*Citrus aurantium*	0.0142	0.0110	0.0253
10	*Dalbergia sisoo*	0.0053	0.0047	0.0101
11	*Delonix regia*	0.0205	0.0159	0.0365
12	*Ficus benghalensis*	0.0174	0.0116	0.0291
13	*Ficus religiosa*	0.0121	0.0108	0.0229
14	*Mangifera indica*	0.0075	0.0076	0.0152
15	*Azadirachta indica*	0.0187	0.0102	0.0290
16	*Moringa oleifera*	0.0193	0.0264	0.0457
17	*Murraya koenigii*	0.0089	0.0087	0.0177
18	*Murraya paniculata*	0.0190	0.0328	0.0518
19	*Plumeria rubra*	0.0125	0.0122	0.0248
20	*Santalum album*	0.0189	0.0261	0.0450
21	*Syzygium cumini*	0.0103	0.0151	0.0255
22	*Tectona grandis*	0.0188	0.0252	0.0440
23	*Zizyphus mauritiana*	0.0215	0.0142	0.0358
24	*Tecoma stans*	0.0185	0.0230	0.0415
25	*Buchanan lanzan*	0.0153	0.0144	0.0298
26	*Anacardium occidentale*	0.0166	0.0193	0.0360
27	*Polyalthia longifolia*	0.0309	0.0275	0.0585
28	*Acacia nilotia*	0.0186	0.0222	0.0409
29	*Aegle marmelos*	0.0158	0.0136	0.0295
30	*Tamarindus indica*	0.0161	0.0212	0.0373
31	*Pongamia pinnata*	0.0105	0.0127	0.0233
32	*Psidium guajava*	0.0313	0.0368	0.0681
33	*Saraca indica*	0.0059	0.0060	0.0120
34	*Terminalia catappa*	0.0087	0.0075	0.0163
35	*Callistemon lanceolatus*	0.0105	0.0110	0.0215
36	*Eucalyptus globules*	0.0162	0.0216	0.0378
37	*Manilkara zapota*	0.0132	0.0168	0.0300
38	*Alistonia scholaris*	0.0024	0.0027	0.0051
39	*Thevetia peruviana*	0.0094	0.0073	0.0168
40	*Peltophorum pterocarpum*	0.0124	0.0172	0.0296



GRAPH 1. Concentration of chlorophyll-*a, b* and *a+b* using 80% acetone as solvent



GRAPH 2. Concentration of chlorophyll-*a,b* and *a+b* using 95% Ethanol as solvent

kg and minimum concentrations were found in about 6 species in the same amounts i.e., between 0.007-0.009 g/kg. In maximum species, concentrations were found in range of 0.03 g/kg. In ten species concentrations were found above 0.03 g/kg. The maximum level concentration of chlorophyll-*a+b* was found in *Bauhinia purpurea* i.e., 0.071g/kg and the minimum chlorophyll concentration was found in *Syzygium cumini* i.e., 0.013g/kg. In 7 species the concentrations were found more than 0.06 g/kg and in 12 species concentrations were found more than 0.05 g/kg. About 93% of species showed more than 0.02 g/kg chlorophyll- *a+b* concentration.

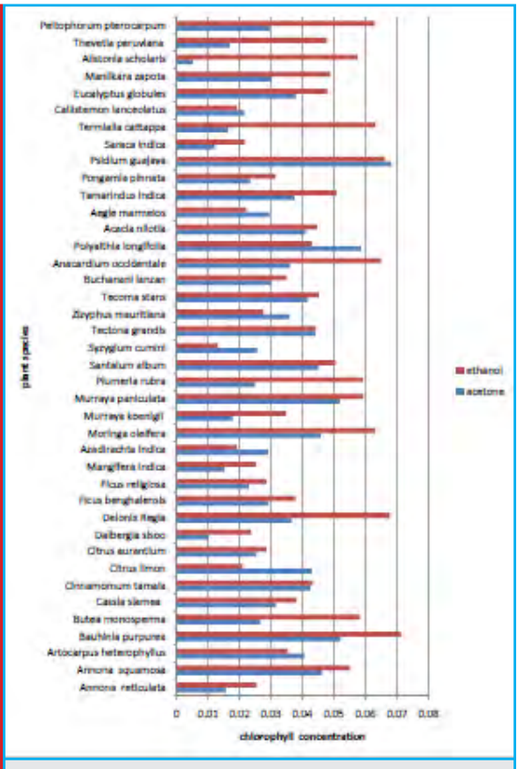
A graphical representation of comparison between chlorophyll *a+b* concentrations in these two different solvents; 80% Acetone and 95% Ethanol showed different results (Graph 3). Samples prepared using 95% Ethanol showed more concentrations of chlorophyll- *a+b* in most of the species. Only 8 species of 80% Acetone prepared samples showed slightly more chlorophyll- *a+b* concentration than 95% Ethanol prepared samples that too with minor differences. 95% Ethanol prepared samples showed very higher concentrations than 80% Acetone prepared samples in more than 25% species. In about 10 species both the solvents showed the same amount of chlorophyll- *a+b* concentration. *Alstonia scholaris*, *Terminalia catappa* were the two species which showed a huge difference of chlorophyll *a+b* concentrations in two solvents, in which 95% Ethanol showed the higher concentration then 80% Acetone.

As chlorophyll is one of the important attributes, the observations of chlorophyll-*a*, *b* and total chlorophyll-*a+b* in all the 40 species have been estimated and compared. A graphical representation on the basis of results have also been plotted in order to understand the variations and differences among different concentrations observed. Different solvents such as ethanol, acetone, DMSO etc, have been used by researchers from time to time, to estimate the chlorophyll concentration in plants. In the present study two solvents have been used: 80% Acetone and 95% Ethanol. The observations were taken using UV spectrophotometery at 663nm and 645nm and a variety of results were obtained.

In 80% Acetone maximum chlorophyll-*a* concentration was found in *Psidium guajava* and *Polyalthia longifolia* 0.03g/kg and minimum concentration was found in *Alstonia scholaris* 0.0024 g/kg and in 95% Ethanol maximum level of chlorophyll-*a* concentrations were found in eight species such as (*Psidium guajava,*

Table 2. Chlorophyll-*a*, chlorophyll-*b* and chlorophyll-*a*+*b* concentrations using 95% Ethanol

S. No	Name of plant species	Chl-*a*	Chl-*b*	Chl-*a*+*b*
1	*Annona reticulata*	0.0138	0.0115	0.0254
2	*Annona squamosa*	0.0241	0.0309	0.0550
3	*Artocarpus heterophyllus*	0.0130	0.0221	0.0352
4	*Bauhinia purpurea*	0.0312	0.0398	0.0711
5	*Butea monosperma*	0.0240	0.0341	0.0582
6	*Cassia siamea*	0.0228	0.0153	0.0381
7	*Cinnamomum tamala*	0.0187	0.0245	0.0432
8	*Citrus limon*	0.0127	0.0083	0.0210
9	*Citrus aurantium*	0.0159	0.0125	0.0284
10	*Dalbergia sisoo*	0.0137	0.0099	0.0236
11	*Delonix regia*	0.0313	0.0363	0.0676
12	*Ficus benghalensis*	0.0224	0.0152	0.0377
13	*Ficus religiosa*	0.0166	0.0119	0.0285
14	*Mangifera indica*	0.0120	0.0137	0.0253
15	*Azadirachta indica*	0.0112	0.0078	0.0191
16	*Moringa oleifera*	0.0312	0.0318	0.0630
17	*Murraya koenigii*	0.0149	0.0197	0.0347
18	*Murraya paniculata*	0.0314	0.0278	0.0592
19	*Plumeria rubra*	0.0314	0.0276	0.0590
20	*Santalum album*	0.0286	0.0218	0.0504
21	*Syzygium cumini*	0.0059	0.0071	0.0130
22	*Tectona grandis*	0.0219	0.0221	0.0441
23	*Zizyphus mauritiana*	0.0137	0.0138	0.0275
24	*Tecoma stans*	0.0237	0.0215	0.0452
25	*Buchanan lanzan*	0.0194	0.0153	0.0348
26	*Anacardium occidentale*	0.0300	0.0348	0.0649
27	*Polyalthia longifolia*	0.0253	0.0175	0.0429
28	*Acacia nilotia*	0.0270	0.0176	0.0446
29	*Aegle marmelos*	0.0132	0.0089	0.0221
30	*Tamarindus indica*	0.0234	0.0272	0.0507
31	*Pongamia pinnata*	0.0154	0.0160	0.0314
32	*Psidium guajava*	0.0313	0.0348	0.0661
33	*Saraca indica*	0.0094	0.0122	0.0216
34	*Termialia catappa*	0.0313	0.0318	0.0631
35	*Callistemon lanceolatus*	0.0104	0.0087	0.0191
36	*Eucalyptus globules*	0.0273	0.0204	0.0478
37	*Manilkara zapota*	0.0228	0.0259	0.0487
38	*Alistonia scholaris*	0.0240	0.0333	0.0573
39	*Thevetia peruviana*	0.0281	0.0196	0.0477
40	*Peltophorum pterocarpum*	0.0313	0.0313	0.0627



GRAPH 3. Comparison chlorophyll *a+b* concentration in 95% Ethanol and 80% Acetone

Bauhinia purpurea, Moringa oleifera, Murraya paniculata, Plumeria rubra, Terminalia catappa , Peltophorum pterocarpum, Delonix regia) in similar amounts 0.03 g/kg, and minimum level of chlorophyll-*a* was found in *Syzygium cumini* 0.005 g/kg. In both the solvents chlorophyll-*a* concentration was found to be almost same.80% Acetone based samples showed more difference in concentrations of chlorophyll-*b* with respect to chlorophyll-*a*. The maximum concentration of chlorophyll-*b* was found in *Psidium guajava, Murraya paniculata* and *Bauhinia purpurea* which were more than 0.03g/kg and the minimum concentration was found in *Alstonia scholaris* about 0.002 g/kg and in 95% Ethanol based samples chlorophyll-*b* concentration was maximum in *Bauhinia purpurea* 0.039g/kg and minimum concentrations were found in about 6 species in the same amounts in the range between 0.007-0.009 g/kg. *Alstonia scholaris* showed the least concentration of chlorophyll-*a* & *b* in 80% Acetone solvent.

On the basis of the observation of all the results it is evident that the maximum and minimum values of chlorophylls are same in both the solvents but overall the 95% Ethanol showed higher concentration of both chlorophyll-*a* and-*b*. Similar types of studies were also done by José Francisco *et al.,* (2008) in which they determined chlorophyll concentrations in tropical tree species by Portable Chlorophyll Meter with appropriate adjustment equations. Faisal and Anis *et al.,* (2006) reported higher amount of chlorophyll-*a* (0.91 ± 0.19 mg/g FW) and chlorophyll-*b* (0.61 ± 0.09 mg/g FW) in micro propagated plants of *Psoralea corylifolia* compared to chlorophyll-*a* (0.83 ± 0.31 mg/g FW) and chlorophyll-*b* (0.53 ± 0.14 mg/g FW) in seedlings. Dere (1998) have also investigated the level of chlorophyll-*a* in fresh water forms of some algal species. Indira *et al.,* (2015) have also estimated the chlorophyll content of *Tridax procumbens* grown in normal and polluted region in which they reported that the chlorophyll content in normal and polluted regions is 2.99mg/g and 2.56 mg/g respectively.

This method of chlorophyll quantification is reliable but time consuming and requires great precision. The main disadvantage of the method is that the process of extraction can result in erroneous qualitative and quantitative determination of the content of pigments (due to photochemical reactions, impact of ambient oxygen, chlorophyllase activity, pheophytinization caused by acids from plant tissue, etc (Jelić et al., 1992; Wellburn, 1994). Difficulties in comparing the results obtained by different extraction techniques sometimes raise the question of validity of research conclusions. A particular problem is posed by the fact that the amounts of chlorophyll measured after extraction with various solvents are hard to compare because different formulae are used for content calculation (Lichtenthaler, 1988). The defined absorption coefficients in these formulae are based on measurements made with outdated or imprecise spectrophotometers that are still in use. Therefore, the results obtained by different groups of researchers may differ, even when using the same extraction solvents, and be incomparable for several reasons: (I) differences in spectrophotometer resolutions in the range of red light wavelengths (II) the accuracy of readings of selected wavelengths, and (III) water content in analyzed tissues (Jelić et al., 1992).

CONCLUSION

Chlorophyll from 40 different tree species was extracted and estimated. Considering the results obtained in this work, chlorophyll content in *Psidium guajava* leaves was higher and almost similar in both the solvents with less variation followed by *Bauhinia purpurea, Murraya paniculata, Annona squamosa, Polyalthia longifolia, Acacia nilotica, Cinnamonum tamala.* Species like *Delonix regia, Anacardium occidentale, Terminalia catappa, Alstonis scholaris, Thevetia peruviana, Peltophorum pterocarpum* showed very high difference in their concentration in 95% Ethanol and 80% Acetone, it suggests that different

plants may need different solvents for isolation; it may be because of their biochemical constituents while as some plants showed a similar concentration in both the solvents. Chlorophyll-*a*, chlorophyll-*b* and total chlorophyll amount showed different values in the present study varying from 0.01 g/kg to 0.08 g/kg in these understudy tree species. By this we can conclude that different plant species have different requirements of photosynthetic pigments which have a direct relation to the photosynthetic activity and the rate of photosynthesis.

ACKNOWLEDGEMENTS

Authors are sincerely thankful to Professor S.P. Rothe (Ex-Head, Department of Botany, Shri Shivaji Science College, Akola) for his help in identification and authentication of several plant species. Authors are also thankful to Dr. V.R. Deshmukh (Ex-Head Department of Botany, Vidyabharati Mahavidyalya College, Amravati) for providing lab facilities during this study.

CONFLICT OF INTEREST

The authors declare no competing interests to any person, agency or institution.

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Pharmaceutical Communication

Biosci. Biotech. Res. Comm. 12(2): 531-538 (2019)

BBRC Bioscience Biotechnology Research Communications

Aloin from *Aloe vera* leaves: A potential natural aluminium detoxificant

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ABSTRACT

Aluminium is a known potent environmental nephrotoxin causing progressive biochemical changes in the kidney. The herb *Aloe barbadensis* is commonly known as *Aloe vera*, belongs to the family of *Liliacea*. It has been widely used in traditional system of medicines and its active compound has many therapeutic potentials. The present study has evaluated the nephroprotective effect of *Aloe vera* and aloin in aluminium sulphate exposed rats for a period of 45, 90 and 180 days. Aloin from *Aloe vera* leaf extract was isolated and characterised by HPTLC methods. Serum creatinine, urea and uric acid levels were found to significantly increased (p<0.05) after treatment of $Al_2(So_4)_3$ in group II compared to control group I animals fed with normal diet. Co treatment with $Al_2(So_4)_3$ and *Aloe vera* extract (group III) and $Al_2(So_4)_3$ and aloin (group IV) showed significant decrease (p<0.05) in creatinine, urea and uric acid. So, our present study has demonstrated that *Aloe vera* and aloin was effective in reducing Al toxicity in kidney. Hence, *Aloe vera* and its active compound aloin can be used as adjuvant therapy for the prevention and management of aluminium sulphate induced renal damage.

KEY WORDS: *ALOE VERA*, ALUMINIUM, CREATININE, UREA AND URIC ACID

INTRODUCTION

Heavy metals exist in our environment both naturally and from pollution. Some of them are very toxic and ranked as human carcinogens. Accordingly, Aluminium (Al) is a systemic toxic metal known for multiple domestic, industrial, medical and technological applications that contribute to its wide distribution in the environment. Aluminium exposure to human beings occurs through different routes. Common routes of exposure include inhalation, oral, and skin. Exposure is more common in people working in Al industries. The promi-

ARTICLE INFORMATION:

Corresponding Author: drshariqali@yahoo.com
Received 10th April, 2019
Accepted after revision 20th June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/41

nent use of Al cookware results in ingestion of some quantity of Al every day. Al is a component of some widely used medications including sucralfate, phosphate binders, and some vaccines. It is also found in preservative, emulsifying agents, colorants, and baking powders. Such widespread use of Al in consumable and non consumable items will eventually lead to entry and deposition in human body, (Gura, 2010; Mitkus *et al*., 2011; Riihimaki and Antero, 2012; Shaw *et al*., 2013; Kramer and Heath, 2014; Sjogren *et al*., 2015; Mahor and Ali, 2015; Exley, 2016; Jakkala *et al*., 2016; Weidenhamer *et al*., 2017; Gouda *et al*., 2018; Mahor and Ali *et al*., 2018;Yan *et al*., 2019).

Aluminium does not have any physiological role in the body but upon ingestion it gets stored in human organs such as liver, lungs, kidney and brain. Due to its atomic size and electric charge similar to important elements of our body like calcium, magnesium and iron, it acts as competitive inhibitors of them and causes severe damage. Additionally, it triggers generation of reactive oxygen species (ROS), and depletes the cellular antioxidant capacity. An imbalance of antioxidant pool affects cellular organelles, antioxidant enzymes, and damages membranes, DNA, proteins, and finally destroys the tissues. Therefore, exogenous administration of antioxidant substances would have a beneficial effect on the cells' antioxidant system to combat aluminium intoxication. In accordance, there are growing interests in using natural compounds to treat aluminium nephrotoxicity, (Garcia *et al*., 2010; Jing *et al*., 2011; Abdel Moneim, 2012; Shaw and Tomljenovic, 2013; Mardani *et al*., 2014; Exley and Mold, 2015; Jakkala and Ali *et al*., 2015; 2016; Tahir *et al*., 2016; Stahl *et al*., 2017; Khan and Strand, 2018; Haese *et al*., 2019 Mahor and Ali 2019).

Aloe vera L. (*Aloe barbendensis* Miller) is an important medicinal plant which belongs to the family *Liliacea*. *Aloe vera* plant grows readily in hot and dry climate but due to its cosmetic demand, it is cultivated on a large scale irrespective of climatic conditions. It is traded in medicinal drug market for an extensive range of therapeutic applications including wound healing effect, reduction of blood sugar, soothing burns, easing intestinal problems, reducing arthritis swelling. Many studies reports protective effect of *Aloe vera* and some of its bioactive compounds especially aloin, also called barbaloin is a bitter tasting yellow crystal found in *Aloe vera*. It is the most important anthraquinone glycoside claimed to be responsible for beneficial effects of *Aloe vera*, (Herrera *et al*., 2010; Yebpella *et al*., 2011; Ali *et al*., 2012; Lad and Murthy, 2013; Jakkala and Ali *et al*., 2015; 2016; Vieira *et al*., 2016; Minjares *et al*., 2017; Yavari *et al*., 2018; Mahor and Ali 2018, 2019; Shi *et al*, 2019).

To our knowledge, this is the first study to evaluate *Aloe vera* effects against aluminium induced nephrotoxicity in rat (*Rattus norvegicus*). Therefore, this study aims to investigate the potential protective effects of *Aloe vera* and its active compound aloin against kidney damage induced by subchronic administration of Aluminium sulphate.

MATERIALS AND METHODS

Chemicals and Drugs

In this study, Al-sulphate $(Al_2So_4)_3$ was purchased from Aldrich chemical Company (St. Lousis mo, USA) and Standard Aloin ($C_{21}H_{22}O_9$) was obtained from Sigma. The diagnostic kits required for enzymatic assays were purchased from Span Diagnostics. All other chemicals used in the experiment were of analytical grade. The dose of Al-sulphate $(Al_2So_4)_3$ was 98.3mg $(Al_2So_4)_3$ /L (1/25 using Probit analysis based LD_{50}). The dose of A.vera extract and Aloin were 100 mg/kg bw.

Collection and identification of plant material

The fresh leaves of *A.vera* (*Aloe barbadensis*) were collected from the Minor Forest Produce Processing and Research Centre (MFP-PARC) Van Parisar, Barkhera Pathani, Bhopal, (M.P.) India. The plant was authenticated by Dr. Zia-Ul-Hassan Head of the Department of Botany at the Saifia College of Science Bhopal, (M.P.) India and the voucher specimen (403/Saifia/Bot/16) has been deposited at the Herbarium of the Saifia Science College, Bhopal, (M.P.) India.

Preparation of extracts

After collection and weighing, fresh leaves of *Aloe vera* were washed with distilled water to remove dirt and dried under shade separately. The extraction of *A. vera* leaves was done according to the method (Kumar and Muthuselvam, 2009). Slight modification, Skin of the leaves were pealed and the gel inside was used for extraction. 100 gm of the gel was added to 250ml of ethanol and extracted using the Soxhlet assembly. Later on, the solvent of the extracted material was removed at low temperature in a rotary vacuum evaporator and the resulting dried extract was lyophilized in a freeze dryer.

Quantitative estimation of aloin in Aloe vera extract

Chromatographic separation of extracts of *A. Vera* was performed on 20 cm x 10 cm aluminium backed HPTLC plates coated with 200 µm layers of silica gel 60F254 (E. Merck, Darmstadt, Germany). Before use, the plates were pre washed with methanol and activated at 110°C for 5 min. Both test and standard samples (5µL each) were applied on to HPTLC plates as 6 mm wide bands and 12 mm apart from middle of bands by spray-on technique along with nitrogen gas supply for simultaneous drying

of bands, by means of a Camag Linomat V auto sample applicator fitted with a 100 µL syringe (Hamilton, Bonaduz, Switzerland).

A constant spot application rate of 150 nL was used. Plates were developed to a distance of 165 mm, in the dark, with 30 mL ethyl acetate, methanol and water (10:1:4:1) for aloin, as mobile phase. Before development the chamber was saturated with mobile phase for 15 min at room temperature (25 ± 2°C) and 50% relative humidity. Chromatography was performed in Camag's twin-trough chamber. Wavelength for detection of aloin was evaluated from complete UV spectrum of aloin. To calculate the concentration of aloin in each sample loaded, following equation was used as developed by Sharma *et al.*, (2012).

Volume made x concentration x total solubility/ weight of dried extract x sample loaded x 1000.

Maintenance of animals and approval of protocol

Healthy adult male albino rats (*Rattus norvegicus*) weighing 120-150g were used for the present investigation. They were housed in a clean polypropylene cage and maintained in an air-conditioned experimental room at 12-hour light: dark cycles. The animals were acclimatized to laboratory condition for one week prior to experiment. Standard pellets were used as a basal diet during the experimental period. The control and experimental animals were provided with purified drinking water *ad libitum*. The animals were maintained in accordance with the "CPCSEA guidelines for laboratory animal facility" (Committee for the Purpose of Control and Supervision on Experiments on Animals) and the approval number is CPCSEA Registration number SSC/06-06-22/CPCSEA, dated 26/10/2006. Before starting the experiment the animals were carefully marked on different parts of their body, which was later used as identification mark for a particular animal, so that the response of a particular mouse prior to and after the administration could be noted separately.

Acute oral toxicity studies

A.vera extract at the dose range of 100–2500 mg/kg body weight were administered by oral gavage method on different group of mice comprised of 6 rats in each group. Animals were kept under close observation for 4 hours after administering the fraction for behaviour, neurological and autonomic profile and then observed for any change in the general behaviour and physical activities; mortality was recorded within 72 hours. Acute toxicity was determined according to the method of Lorke, (1983).

Animal Grouping /Induction of Toxicity/Experimental design

A total of 24 male (2 months old) Albino rats (*Rattus norvegicus*) weighing 120-150g were used for the present investigation. The animals were divided into four groups (6 rats/ group): Group I:-was kept as control without giving any treatment. Compared to adult controls, Group II: - animals in this group were given 17±6 ml of water supplemented with Al-sulphate to consume, corresponding to 98 mg of Al per day (Jakkala and Ali *et al.*, 2015; 2016) for 45, 90 and 180 days. Group III: - This group animals were fed with normal diet and received aluminium sulphate (98 mg/ kg body weight) and *Aloe vera* extract (100mg/kg body weight) for 45, 90 and 180 days. Group IV: - these group animals were fed with normal diet and received aluminium sulphate (98 mg/kg body weight) and Aloin (100mg/kg body weight) for 45, 90 and 180 days.

Collection of Blood Sample

Blood samples were collected by orbital sinus puncture method (Hui *et al.*, 2007). Serum was separated by following procedure. Blood samples were withdrawn from orbital sinus using non heparinised capillary tubes, collected in dried centrifuge tubes and allowed to clot. Serum was separated from the clot by centrifuged at 3000 rpm for 15 min. at room temperature.

Biochemical Assays

Determination of Serum Creatinine, Urea and Uric acid

Serum Creatinine, Urea, Uric acid levels were assayed using reagent kits purchased from Biosystems (Spain), following methods of Young (1995); Kaplan (1984) and Fossati *et al.*, (1980) respectively.

STATISTICAL ANALYSIS OF DATA

Statistical analysis was performed using Graph Pad Prism 5 software (Graph Pad Software, San Diego, CA). All parameters results were expressed as mean ± standard error (SEM) and all the statistical comparisons were made by means of the one-way ANOVA test, followed by Turkey's test post hoc analysis. A P value <0.05 was considered significant.

RESULTS

In the chromatogram of *Aloe vera* extract, many well resolved spots were observed, out of these spots one spot matched with the Rf value shown by standard aloin (0.76). The results of percentage of aloin found in samples are shown in Table 1.

It was observed that all four groups of rats received the following treatment schedule: shows the significant change in all three parameters discussed here. After 45 days (Group II) showed a significant ($P<0.05$) increase in the level Creatinine, Urea, Uric acid to Al toxicity compared to group I. whereas significant ($P<0.05$) decrease

Table 1. Percentage of aloin found in *Aloe vera* extract

S. No.	Sample	Rf	Amount of sample applied (ng/spot)	Amount of aloin (%)
1	Aloe vera extract	0.76	600	44.41
2	Aloe vera extract	0.76	800	59.31
3	Aloe vera extract	0.76	1000	63.10
4	Aloe vera extract	0.76	1200	65.56

in Creatinine, Urea and Uric acid level was reported in group III and group IV (Fig: 1).

After 90 days (Group II) showed a significant ($P<0.05$) increase in the level of Creatinine, Urea, Uric acid to Al toxicity compared to group I. whereas significant ($P<0.05$) decrease in Creatinine, Urea and Uric acid level was reported in group III and group IV, (Fig: 2).

It was observed that Al toxicity enhances compared to 45, 90 days. It means Al on long term exposure induces toxicity in group II whereas *A. vera* extract and aloin was also effective in reducing Aluminium sulphate toxicity, significant ($P<0.05$) decrease in kidney function test (Creatinine, Urea and Uric acid) studied after 180 days and last study (180 days) show that in group II Creatinine, Urea and Uric acid level significant ($P<0.05$) increase compare to normal (control) group I and whereas group III and group IV showed significant ($P<0.05$) decrease, (Fig.3).

DISCUSSION

Aluminium is one of the trace elements with toxic effect on living organism. However, in recent years, increased attention is being focused on possible adverse effects of aluminium on human health. The present study reveals that the administration of aluminium sulphate significantly ($P<0.05$) enhanced the levels of creatinine, urea and uric acid. Significantly ($P<0.05$) elevated creatinine, urea and uric acid were observed in aluminium sulphate fed rats (group II) when compared with control (group I). The rise in creatinine, urea and uric acid may indicate aluminium toxicity in kidney function. This is in

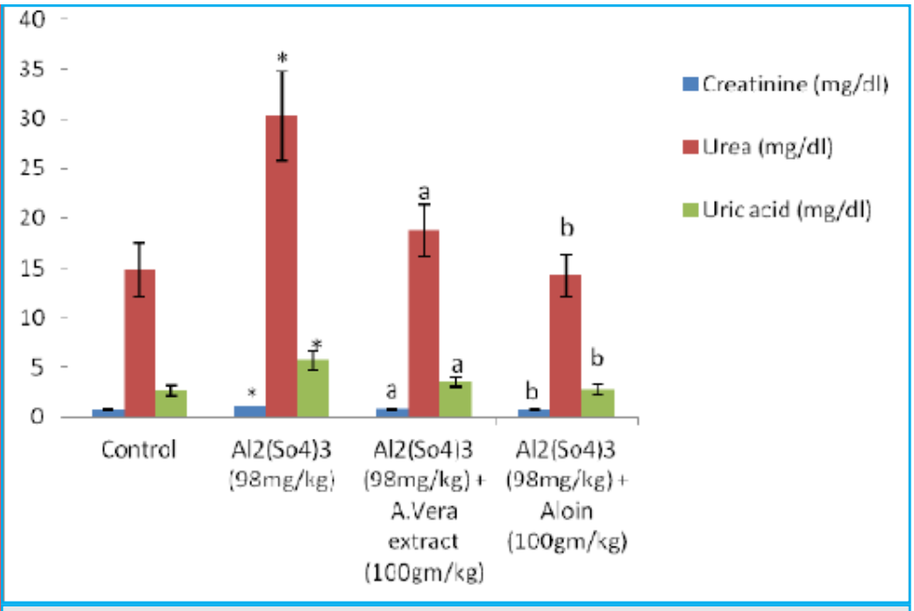

FIGURE 1. Graph showing variation in different parameters level of Creatinine, Urea and Uric acid against Aluminium sulphate (98mg/kg/bw) induced toxicity after 180 days.
Results are expressed as mean ± S.E., $P<0.05$ was considered to be statistically significant.
* Significantly different form control group.
a Significantly different form Aluminium sulphate treated group.
b Significantly different form Aluminium sulphate + *Aloe vera* extract treated group.

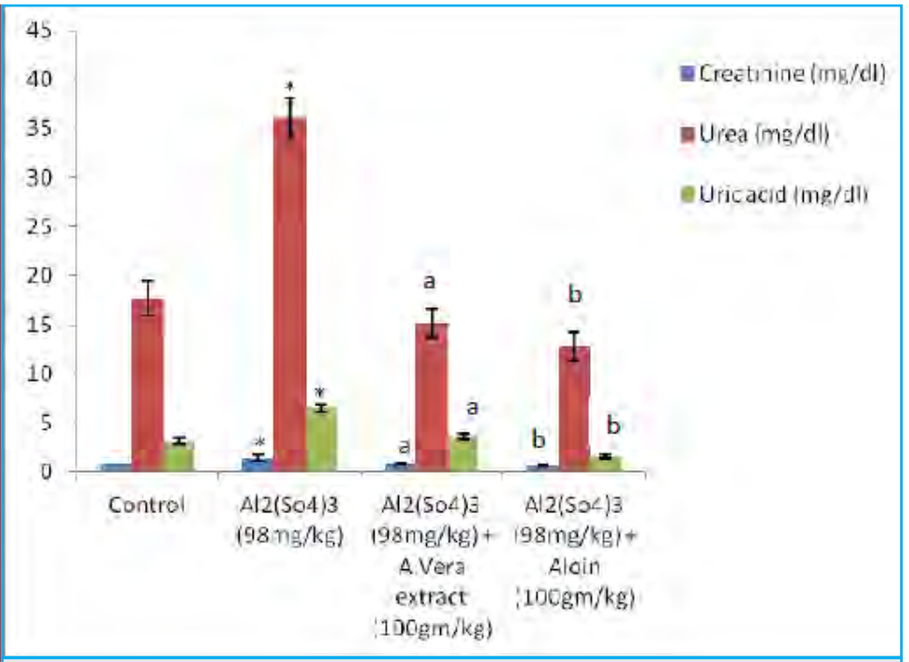

FIGURE 2. Graph showing variation in different parameters level of Creatinine, Urea and Uric acid against Aluminium sulphate (98mg/kg/bw) induced toxicity after 180 days.
Results are expressed as mean ± S.E., $P<0.05$ was considered to be statistically significant.
* Significantly different form control group.
a Significantly different form Aluminium sulphate treated group.
b Significantly different form Aluminium sulphate + *Aloe vera* extract treated group.

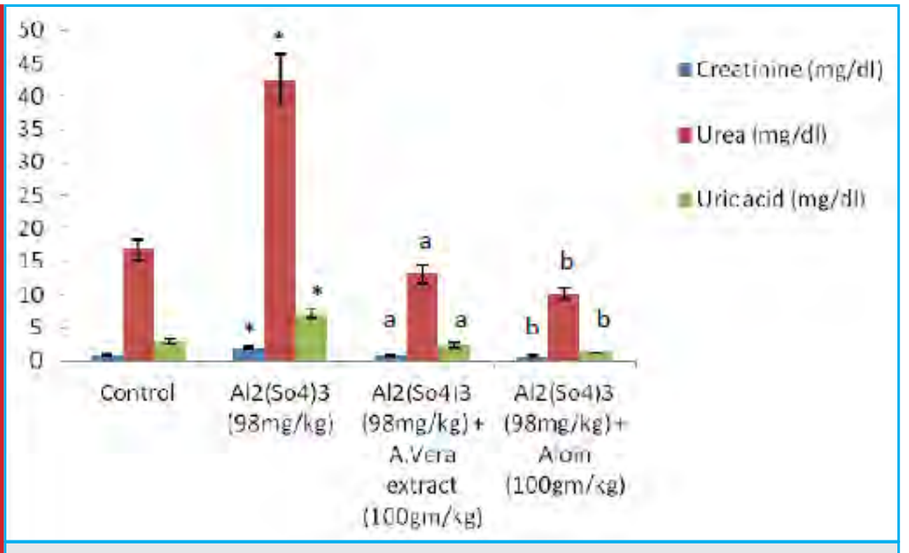

FIGURE 3. Graph showing variation in different parameters level of Creatinine, Urea and Uric acid against Aluminium sulphate (98mg/kg/bw) induced toxicity after 180 days.
Results are expressed as mean ± S.E., $P<0.05$ was considered to be statistically significant.
* Significantly different form control group.
a Significantly different form Aluminium sulphate treated group.
b Significantly different form Aluminium sulphate + *Aloe vera* extract treated group.

consonance with the recent investigation of Ajibade *et al.*, (2019) and Yousef *et al.*, (2019) in which there were biochemical changes observed in the kidney of adult Wistar rats when fed with aluminium chloride. Significant increase ($p<0.05$) in serum urea and creatinine were observed.

In the present study, significant decreased ($p<0.05$) level of creatinine, urea and uric acid were observed in aluminium sulphate and *Aloe vera* extract fed rats (group III) when compared to group II animals. These results are in agreement with the study of Belaid-Nouira *et al.*, (2013; Miraj *et al.*, 2015) using other plant, they have found that fenugreek seeds showed effectiveness in restoring normal plasma values of urea, creatinine in the kidney injured by aluminium chloride. Several other studies using *Aloe vera* plant, also support our findings concluded that *Aloe vera* extract showed nephroprotective effect against heavy metal toxicity (Iftikhar *et al.*, 2015; Hussain *et al.*, 2016). In our study, administration of aloin showed significant decreased ($p<0.05$) level of creatinine, urea and uric acid when compared to group III animals. This is analogous to the study of Al Dera, (2016) who has proposed that standard resveratrol when administered with aluminium chloride showed significant ($p<0.05$) decreased in serum creatinine and urea. In our previous study it has been showed that aloin also significantly ($p<0.05$) reduced in Total cholesterol, triglyceride, HDL and LDL (Mahor and Ali, 2018).

In agreement with previous studies, results from this study revealed that aluminium induced nephrotoxicity is indicated by significant ($p<0.05$) increase in creatinine, urea and uric acid. But, co treatment of aluminium sulphate and *Aloe vera* extract and Aluminium sulphate and aloin showed significant ($p<0.05$) reduction in creatinine, urea and uric acid. This suggests that *Aloe vera* and its active compound aloin is very potent in preventing aluminium toxicity in kidney.

CONCLUSION

Based on the findings of present work, it can be concluded that the *A. vera* extract and aloin was effective in reducing Al toxicity in kidney function tests (Creatinine, Urea and Uric acid). Deeper study is needed using histological analysis for gaining better pharmacological information and intervention.

CONFLICTS OF INTEREST

The authors have no conflict of interest to declare.

ACKNOWLEDGEMENT

GM is thankful to UGC, Delhi for awarding RGNF, Vide File No. 2014-15-SC-MAD-67686/2014.

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Technological Communication

Biosci. Biotech. Res. Comm. 12(2): 539-547 (2019)

BBRC Bioscience Biotechnology Research Communications

Smart Irrigation Control System Using Internet of Things: An Empirical Study in Kingdom of Saudi Arabia

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ABSTRACT

Saudi Arabia is one of the countries that suffer from the scarcity of water resources in the world. Due to the misuse of water resources, some sectors of our country face the danger of droughts. One such is agriculture. Plants require enough water resources to grow healthy and be fecund. Most previous researchers have focused on how to water plants depending on the time without checking whether the plants need watering or not. With the evolution of information technology, the Internet of Things (IoT) is one new technology, which can assist our country to reduce the overall impact of wrong water management in the agricultural sector. In this research, we focused on developing a smart irrigation control system (SICS) that only waters the plants when they need it, and can determine the exact amount of water consumed after each irrigation process. This system aims to help the homeowner by facilitating the process of watering the garden without involving any manpower.

KEY WORDS: SOIL MOISTURE SENSOR; WATER FLOW SENSOR; ARDUINO; IRRIGATION SYSTEM; RELAY; IOT

INTRODUCTION

Home gardens play an important role and contribute significantly to giving an aesthetic and cultural appearance to the home. Studies have proven that just looking at plants will improve heart activity, muscle tension, blood pressure, and electrical brain activity. In addition, through colors and textures, a homeowner can feel calm, happy and satisfied (Siswazah, 2012). Furthermore, plants have evolved to be a form of treatment for the soul and body (Siswazah, 2012). One of the most important benefits of the home garden is increasing income, if it is exploited in a perfect and correct way. It gives homeowners immediate access to fresh fruit and vegeta-

ARTICLE INFORMATION:

Corresponding Author: halbaity@ksu.edu.sa
Received 10th April, 2019
Accepted after revision 20th June, 2019
BBRC Print ISSN: 0974-6455
Online ISSN: 2321-4007 CODEN: USA BBRCBA
Thomson Reuters ISI ESC / Clarivate Analytics USA
NAAS Journal Score 2019: 4.31 SJIF: 4.196

Online Contents Available at: http//www.bbrc.in/
DOI: 10.21786/bbrc/12.2/42

bles, therefore, they do not need to visit a grocery store or farmers market to buy them. Water and irrigation are vital factors for preserving the home garden's success. Currently, water availability and irrigation management are challenges in the agricultural sector, especially in the Kingdom of Saudi Arabia. Saudi Arabia is located in one of the driest and hottest regions in the world. Furthermore, Saudi Arabia lacks permanent rivers and surface water. In addition, it suffers from climatological conditions, which are not environmentally ideal for agriculture (Roy & Ansari, 2014). Thus, to schedule irrigation properly, a homeowner must be aware of the environmental demands for surface water, which causes watering losses and consumes a lot of time and effort when processed manually.

Irrigation has been defined as "replenishment of soil water storage in plant root zone through methods other than natural precipitation" (Baig, et al., 2012). It began almost simultaneously in Egypt and Mesopotamia using the Nile river and Tigris and Euphrates rivers (Alkolibi, 2002). Irrigation is important in maintaining the landscape and reducing the effect of inadequate rainfall (Buechley & Hill, 2010). There are two kinds of irrigation: manual and automatic. Manual irrigation refers to a system that has the ability to water plants with human intervention and without technical equipment. Automatic irrigation is defined as a system that has the ability to water plants without human intervention and with technical equipments, (Li, et al., 2015 Javed 2016., Chandak et al., 2017).

Irrigation has been an important factor in agriculture since old times, where people used flooding rivers and tides to irrigate the plants. With the rapid growth of the population and the evolution of culture, these resources are no longer sufficient to meet the needs of human communities. Thus, they started to develop other irrigation methods that are based on enhanced techniques. With the evolution of information technology, the Internet of Things (IoT) is found in every industry with a variety of applications, one such is irrigation.

The IoT is defined as a technology that is used to connect things or objects with each other and allows the objects to collect and exchange data. The things or objects can be instruments, Radio-Frequency Identification (RFID) tags, cars, homes, sensors, and various artificial intelligence tools (Javed, 2016; Melorose, et al., 2015). The Internet of Things semantically means, "A worldwide network of interconnected objects uniquely addressable, based on standard communication protocols" (Melorose, et al., 2015). Many people think that the term "Internet of Things" refers to a new technology, however, the concept of IoT was developed from M2M communication (machine-to-machine communication), which was first used in telecommunications in 1999 by Kevin Ashton (Javed, 2016; Yao, et al., 2010). Currently, the IoT is widely used in various fields from remote health management, alarm systems, transportation systems, and home automation, to smart cities and industrial IoT (Javed, 2016). One of the most important fields that utilize the IoT is agriculture, where it is used to connect objects with each other to water crops remotely in order to save time and effort (Yao, et al., 2010).

The purpose of this work is to develop a cost-effective Smart Irrigation Control System (SICS) to manage and organize water consumption, improving the effectiveness of soil protection by monitoring soil moisture.

The remainder of this paper is organized as follows. In Section 2, we present an overview of the previous works related to solving the problem of irrigation systems. Section 3 describes the proposed methodology. Section 4 discusses how SICS was implemented. Section 5 provides a detailed description of the proposed approach and experimental results. Finally, Section 6 concludes this paper and discusses potential future work.

RELATED WORK

A review of the literature shows that many studies have been conducted in the field of irrigation systems. Those studies were conducted using different approaches in order to provide more efficient and appropriate solutions to the irrigation problem. These approaches are: data Mining and multidisciplinary. The taxonomy below shows the classification we will follow in our review of the literature.

A. Data Mining Approach

The authors in (Yao, et al., 2010: Chen & Yue, 2011) aimed to apply the data mining algorithm Fuzzy Neural Network (FNN) to the irrigation system to determine the exact quantity of water required by different crops. FNN consists of fuzzy logic, which is used to provide thinking based on fuzzy rules for the neural network, and a neural network, which is used to provide a connection structure and learn-

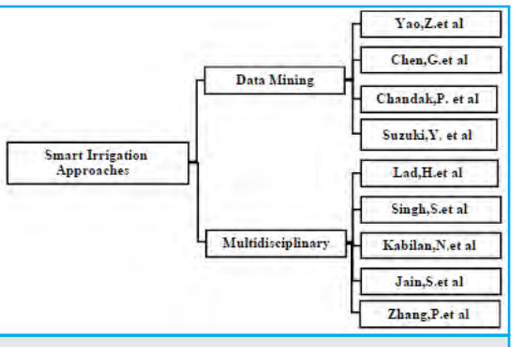

FIGURE 1. Taxonomy of Irrigation System Approaches

ing capability for fuzzy logic. The irrigation system works as follows: first, the system collects information about water and transmits this to a processing unit through the sensors. This information is then divided into 'hunger' and 'thirst rate' and transmitted to the fuzzy controller. The neural network performs self-learning on the results and specifies the water needs of the crops. The system has achieved high levels of success in determining the exact water needs of crops.

Chandak and Student (2017), have designed and developed a smart farming system based on data mining clustering techniques. The motive behind this study was to provide a better solution for growers to achieve high productivity. The data was gained from various resources such as satellite information, the internet, and soil testing reports. A clustering algorithm was used to partition the dataset into homogeneous groups based on similarities and dissimilarities, and decisions are based on awareness of weather changes. The use of this system was found to be effective at increasing the productivity of fields. Another study by (Suzuki, et al., 2013) focused on how to help novice users irrigate plants correctly. The authors proposed an agricultural cloud support system based on support vector machine (SVM), whereby data was collected for a week using different sensors: temperature, humidity, soil moisture, and sensing time. The data gained from the sensors was collected for six days for training phase and one day for testing phase. Then, SVM was applied for decision-making.

B. Multidisciplinary Approach

Lad.et al (2014) designed automated embedded systems and distributed them to different farms before developing a Controller Area Network (CAN) to connect these systems in order to improve the productivity of each crop. This system achieved a high level of success for establishing communication between data in distributed nodes. Singh, et al., (2016) proposed a system that can be used to monitor greenhouses in India. The system depends on wireless sensor network, which is used to transfer, control and monitor greenhouses. It used Yuktix IOT and CDAU (central data acquisition unit) to observe the internal conditions of greenhouses locally and remotely. Yuktix cloud was used, via a web- and android-based application, to store data and use it for deep analysis, such as statistics regarding the mean temperature and humidity throughout the year. This system showed the ability to monitor the greenhouse remotely with some limitations that require improvements.

Kabilan & Senthamil Selvi, (2016) developed an irrigation system that is based on IoT techniques. First, a database was created with attributes acquired from images of the plant and the soil, including training samples related to moisture content, plant leaf condition, soil type, temperature, humidity level and the amount of water flow required. Second, Fisher's linear discriminant analysis (LDA) was used to retrieve the soil images and color of the plants, which can accurately determine the water requirements. A classification algorithm called transductive support vector machine (TSVM) was then applied for classification and quantification. Finally, with this system, the necessary irrigation level of the plants can be specified, and the water supply can be automatically regulated with less cost.

Some of the authors of like, Jain, et al., (2016) have described the development of a smart wireless sensor network with two parts, a transmitter and receiver, for monitoring agricultural and environmental conditions. The transmitter consists of various sensors placed in the farm to transmit data to the receiver, which is a computer user. Soil moisture sensors are used to measure the quantity of moisture in the soil. In this multidisciplinary study, different approaches are combined to enhance the irrigation system such as network, cloud computing, data mining and IoT. In this approach, improvements are added to irrigation systems. These improvements include transmitting data to the microcontroller. The values are transmitted to the receiver via RF protocol and the results determine the soil's need of water. The system improves upon the traditional method of agricultural irrigation by monitoring the crops in a wireless manner. Zhang, et al., (2017) designed and implemented a smart water-saving irrigation system based on the IoT and big data. The system used IoT technologies to monitor and collect data related to crop growth. This was uploaded to and processed by the Shandong Agricultural University big data central target database to establish a crop growth model based on big data for determining the timely and prosperous irrigation of crops.

C. Discussion of Related Work

We have summarized different approaches for irrigation system developments from 2010-2017. The data mining approach employs the different data mining techniques and algorithms used in irrigation development, such as fuzzy logic and neural networks, clustering, and SVMs (Yao, et al., 2010: Chen & Yue, 2011:Chandak and Student 2017: Suzuki, et al., 2013). Data mining techniques help irrigation systems by improving water utilization, increasing the productivity of fields, providing accurate results, and assisting with decision-making. Fuzzy logic and neural network techniques were the most widely used since they provide accurate results, as shown in (Yao, et al., 2010: Chen & Yue, 2011). They accurately determine water requirements, work without the need for human intervention, monitor crops locally and remotely, improve the productivity and quality of the crops, and make decisions efficiently.

The most widely used method in the multidisciplinary approach is the wireless sensor network, since it improves productivity and quality of crops by transferring data among devices efficiently and accurately as shown in (Kabilan & Senthamil Selvi, 2016: Jain, et al., 2016). It is followed by data mining approach, which determines the water requirements more accurately as shown in (Kabilan & Senthamil Selvi, 2016: Zhang, et al., 2017) and then cloud computing is used to help monitor greenhouses as shown in (Singh, et al., 2016).

In conclusion, from this review of the literature, it can be seen that despite the good results obtained using the data mining approach, it still has considerable drawbacks, such as complex methodologies, computational intensity, time-consuming, and a costly process of collecting training data. Although the multidisciplinary approach can produce impressive results, it is complicated to implement, time-consuming and requires considerable effort, such as big data pre-processes that extract useful information to establish a model. It also evinces a high level of jitter and noise, through using a wireless sensor network at long distance.

The best choice for our work is the IoT. We will use an Arduino Uno microprocessor for our research since it has powerful, easy to use features, a low cost, and can be adapted to any environment. From our review of the literature, we noticed that most of the existing irrigation systems water plants according to a specific predefined time without checking whether the plants need watering or not. In this research, SICS will not be triggered unless the plants need watering. Moreover, most of the previous studies are only prototypes and have been done in India. Our research will be a real application and applied in particular in Saudi Arabia.

METHODOLOGY

1. Hardware and Software System Components

In this section, we will describe the basic hardware components and software required for developing SICS.

The additional hardware Components that have been used with Arduino are the following:

a. Half Breadboard

A breadboard is a widely used tool for designing and testing circuit as shown in Figure 2. In our study, the breadboard is used to connect the ground (GND) pin from Arduino to its row

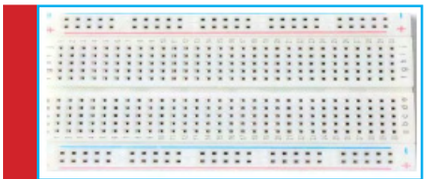

FIGURE 2. Half Breadboard

b. Soil Moisture Sensor

The Soil Moisture Sensor (SMS) is connected to Arduino UNO to measures soil moisture content. As shown in Figure 3, SMS consists of two probes via which current will move into the soil, and then reads the resistance of the soil. This will help reading the current moisture level in the soil.

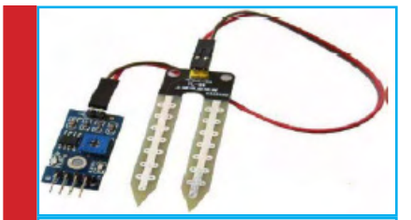

FIGURE 3. Soil Moisture Sensor

c. Relay

Relay, as shown in Figure 4, is used to control the water pump.

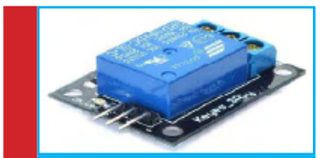

FIGURE 4. Relay

d. Submersible pump

Figure 5 shows the Pump, which used to transmit water into soil.

FIGURE 5. Submersible Pump

e. Jumper cables

Figure 6 shows the cables used to connect Arduino with sensors and relay.

f. Data logger shield

The data logger shield. as shown in Figure 7, is used with Arduino Uno for logging sensor data into Secure Digital card(SD).

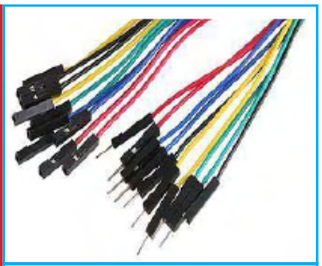

FIGURE 6. Jumper Cables

FIGURE 7. Data Logger Shield

g. GSM SIM800L

The GSM SIM800L, as shown in Figure 8, is used with Arduino for sending text messages to homeowner's mobile phone.

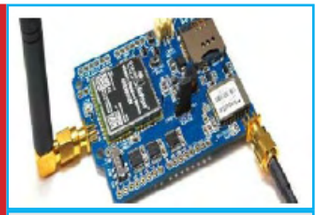

FIGURE 8. GSM SIM800L

h. Water Flow Sensor

The water flow sensor, as shown in Figure 9, is used to measure the flow of water during the irrigation process. It consists of a plastic valve body, a water rotor, and a hall-effect sensor. Also, the water flow sensor has different measurements (water pressure (MPa) and flow rate (L/m)). In our study, we are concerned with the flow rate (L/m) measure.

FIGURE 9. Water Flow Sensor

The block diagram in Figure 10 shows how the system's components are connected. The Arduino is connected to the soil sensor through soil moisture sensor pins in a way that the VCC, GND, and A0 of the sensor are attached to 5V, GND, and pin A0 of Arduino respectively. The relay has three low voltage pins (-, +, and S) .These pins are connected to Arduino in order to operate or stop the pump according to the moisture value. The pump is connected with relay to pass water to the soil .Also we have used water flow sensor wires to connect them to the pump and Arduino (VCC to VCC, GND to GND, and pulse to D2) to measure the amount of water consumption during irrigation. GSM SIM800L pins are connected to Arduino for sending text messages to the homeowner informing him/her about the irrigation time and the amount of water consumption during irrigation. Where data logger is used to log data (soil sensor's readings) to the text file for future use.

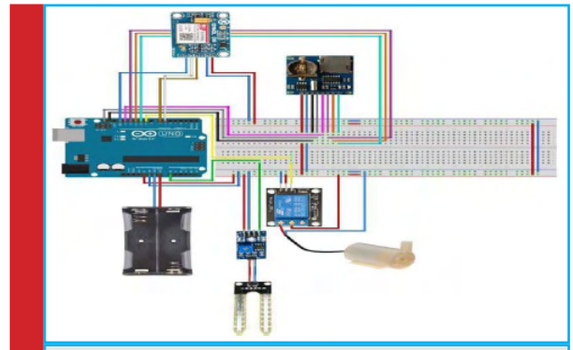

FIGURE 10. Block Diagram of the Hardware Components of the Proposed System (SICS)

Arduino software IDE is used as our experimental platform since it uses a simplified version of C++, which makes it easier to learn and program.

2. System Design

In this part, we will walk through the key steps required to develop SICS.

Step1: Connect the soil sensor to Arduino via breadboard to monitor the Soil moisture continuously.

Step 2: Connect the GSM SIM800L to Arduino to send text messages to the homeowner.

Step 3: Connect the data logger shield to Arduino via breadboard to log the sensor's data in the SD card.

Step 4: Connect the relay to Arduino via breadboard and the pump to the relay to control the water.

Step 5: Connect the pump to the water flow sensor to measure water flow.

Step 6: Connect the buttery to Arduino to increase power supply.

Step 7: Connect Arduino to the PC to use the Arduino interface.

Step 8: Download Arduino software from Arduino's official site to write the code.

Step 9: Write the code to implement the system.

3. Algorithm of SICS

Step 1: Initialize SD.

Step 2: Initialize (pumpRelayPin =0, sensor Pin=0, sensor Value=0, pump Status = 0, watering Delay=1000, sensor Pin W=2

Step 3: Read from the soil moisture sensor the moisture level

Step 4: Watered= False

Step 5: While soil moisture level < threshold value

Step 6: Start water pump to water the plant

Step 7: Watered=True

Step 8: Read from the soil moisture sensor the moisture level

Step 9: EndWhile

Step 10: If Watered

Step 11: Notify the homeowner

Step 12: Read from water flow sensor the amount of water passed in millilitres and save in data log

Step 13: Watered= False

Step 14: EndIf

Step 15: End

The system begins by checking the soil moisture level using the soil sensor. According to the level of the soil moisture, the system valve will turn ON or OFF automatically. Basically, the required level of the soil moisture of the planet will act as the threshold in our experiment. If the soil moisture is below the threshold value, then the water pump will start and a notification message will be sent to the homeowner. The threshold value is set according to the soil moisture level required for the plant. After reaching the required threshold value, the irrigation system will stop automatically.

SYSTEM IMPLEMENTATION

SICS was developed in two phases: the system hardware development phase and the system software development phase.

A. System Hardware Development

Figure 11 illustrates how we connected Arduino to all other hardware components in the system.

B. System Software Development Phase

First, we have included the libraries for the SD card and Real-Time-Clock (RTC). Then we defined multiple variables for reading and writing from/to Arduino pins.

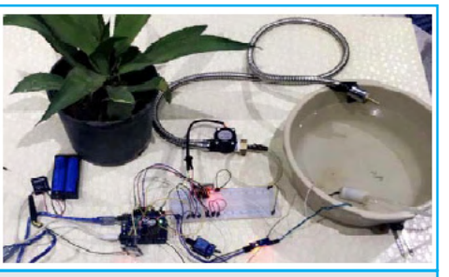

FIGURE 11. Interfacing Arduino with all Hardware Components of the System

1. Soil Moisture Sensor Function

We take moisture readings from the soil via the soil sensor. Then, we map the output values from 0–100, since the moisture is measured in percentage. For instance, the soil moisture percentage that is required for the cactus plant is 90% (Jain, et al., 2016). The readings taken from the cactus dry soil were less than 90%, and in wet soil the readings were more than 90%. We compare the moisture reading with 90% and, based on the comparison result, the relay water pump will turn ON or OFF. Finally, the pump state will be printed on the serial monitor.

2. Water Flow Sensor Function

We use the water flow sensor to calculate the water consumption after completing the irrigation process. In order to determine how many milliliters of water passed through the sensor in a one-second interval, we divided the water flow rate by 60 and then multiply it by 1000.

C. Experimental results and Discussion

In order to conduct experiments and test SICS performance; we developed a mini garden of certain plants as shown in Figure 11.

1. Experiment 1

This experiment was conducted on two plants; rose and cactus. The season when the experiments were conducted was Spring and the temperature was around 33 °C.

Table 1 illustrates the results of the experiment. According to a previous study (Jain, et al., 2016), the required soil moisture level for the rose plant is 40% and for the cactus plant is 90%. The observations in our experiment showed that the rose plant was watered daily and it required 1-4 liters per week, while the cactus plant was watered twice a week and required 2-3 liters per week. In addition, we noticed that the period of time that the irrigation system requires to pump sufficient water to the soil of the rose plant was 1-2 minutes whereas for the cactus plant it was five minutes. The state of both plants was good. By good we mean that the plant

Table 1. Results of Testing Water Consumption of the Cactus and the Rose Plants

Days	Moisture level (%)		Execution time (Minutes)		Water Consumption (ML)		Plant state	
	Rose	Cactus	Rose	Cactus	Rose	Cactus	Rose	Cactus
1	40%	90%	1-4	5	567	1000	good	good
2					934			
3					1332			
4					1852	2203		
5					2445			
6					2932			
7					3545			

Table 2. Results of Testing Water Consumption in Multiple Ranges

	Moisture level ranges	Irrigation Scheduling	Watering Execution Time (Minutes)	Water Consumption (ml)	Total of Water Consumption (two days)	Plant status
Rose Plant	35-40	6h	2m	500	48÷6 = 8 8*500 = 4000 ml 4000÷1000 = 4 Litters	Good
	30-40	11h	4m	1000	48÷11 = 4 4*1000= 4000 ml 4000÷1000 = 4 Litters	Good
	25-40	17h	7m	2000	48÷17 = 2 2*2000 = 4000 ml 4000÷1000 = 4 Litters	Good

looks alive, hydrated and in a healthy manner without any deficiencies. We assessed the overall health status of the plant through observing the plant growth when the experiment was conducted for a period of five weeks. This experiment shows that the rose plant consumes more water than the cactus plant.

2. Experiment 2

This experiment was conducted on the rose plant and lasted for two days to measure water consumption under multiple sensor's reading (as ranges) in order to determine the optimal range that saves water and plant. As mentioned earlier, the required soil moisture level for the rose plant is 40% (Jain, et al., 2016), therefore, when the soil moisture is under 40%, the water pump will start pumping water to irrigate the soil. The ranges that we tested were as follow: 35-40, 30-40, and 25-40. Table 3 demonstrates the results of the experiment. It was observed that in range 35-40, the time interval between two consecutive irrigation processes was six hours and the maximum amount of water consumed during irrigation was 0.5 liters. In range 30-40, the time interval between two successive irrigation processes was 11 hours and the maximum amount of water consumed

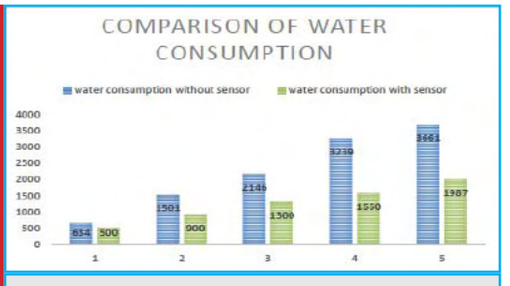

FIGURE 12. Graphical Representation of Water Consumption with and Without Sensor on Mint Plant

FIGURE 13. Dry and Wet Soil

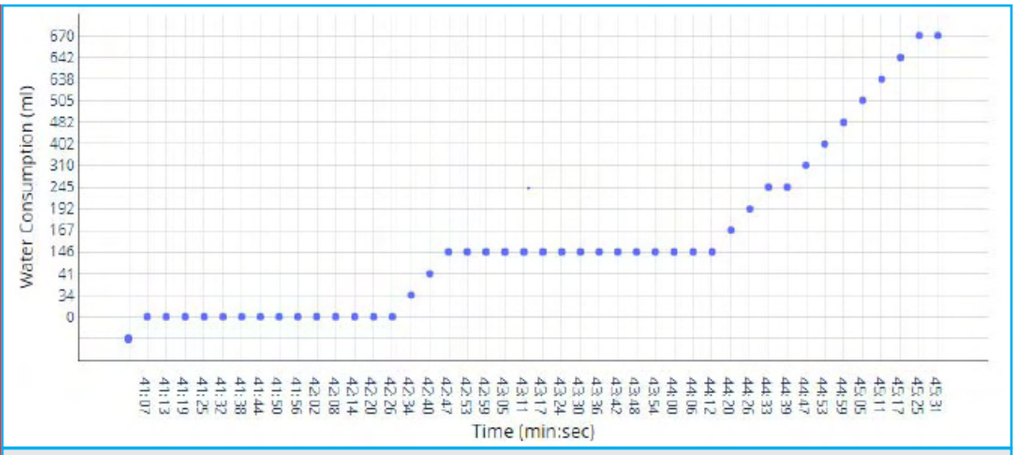

FIGURE 14. Time Series of the Water Consumption before and after Plant Watering

during irrigation was 1 liter. Range 25-40 shows that the time interval between two irrigation processes was 17 hours and the maximum amount of water consumed during irrigation was 2 liters. Although the time interval between two consecutive irrigation processes was different in the tested ranges (6h,11h, and 17h), the water consumption of each irrigation process was different (500ml,1000ml, 2000ml respectively). However, there is no difference between the ranges in terms of the total amount of consumed water and plant status as shown in Figure 12. Therefore, none of the ranges was better than the others as all ranges eventually consumed the same amount of water (4000 ml) and the status of the plant was good in all cases.

3. Experiment 3

For the sake of comparison and to clarify the impact of using SICS, our approach was compared to the automated irrigation approach, which relies on the irrigation timer. In order to perform the comparison, we collected the readings of water consumption (in millimeters) for each system for five days on mint plant. It can be observed from Figure 12 that the automated system consumes more water. This means that SICS provides the appropriate amount of water by monitoring the level of the soil moisture. As such, there is no wasted water.

SYSTEM EVALUATION

A. Functional Testing

The aim of this testing is to assess the system behavior by evaluating the functionality of SICS and determining whether it is working (functioning) as expected or not. From the conducted experiments, we can say that SICS was able to sense the soil moisture, start watering the plant when needed, send a notification to the homeowner, log the sensor data in the text file, determine the water consumption, and stop pumping water when the required soil moisture level is reached.

To assess system reliability, SICS was tested in two different soil conditions (dry and wet) as shown in Figure 13. We observed that only in the case of dry soil the pump operates and the irrigation system starts watering since the soil sensor can determine if soil needs watering. Figure 14 shows the time series of the irrigation process before and after the system starts watering

CONCLUSION AND FUTURE WORK

The aim of our study was to design and develop a smart irrigation control system, which will further enhance the irrigation system in the KSA by optimizing water utilization, reducing the manpower, and providing an ideal environment for plants. SICS was successfully designed and tested. After conducting several experimental tests, we noticed that the system provides good results as expected, in terms of conserving the plants and watering them only when necessary by monitoring the amount of moisture in the soil. In addition, the system succeeded in quantifying the amount of water consumption through the irrigation process. As a result of our monitoring, we noticed that the plant maintained its balance in a desired, systematic and healthy manner without any deficiencies. For the future work, we plan to extend our experiments to include large areas of gardens with different plants. In addition, we would like to investigate and examine the behavior of the soil moisture sensor under salinity and fertilizer conditions. We also plan to use the readings collected from the soil moisture sensor as a reference for further development of new methods for the irrigation system, such as constructing a classification model for predicting soil status (dry or wet).

ACKNOWLEDGEMENTS

This work has been supported by a grant from the Research Center of the Female Scientific and Medical Colleges, Deanship of Scientific Research, King Saud University.

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