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Continued Inside Cover

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Continued From Back Cover

Bioscience Biotechnology Research Communications
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About the Journal

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Exosomes influence the engraftment of tumor cell lines in athymic mice BALB/c nude

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ABSTRACT

The strain of mice with immunodeficit provides a popular experimental model in oncological, immunological, and transplantation studies. Athymic nude mice (Nudes) are usually used to explore the engraftment, growth, invasive infiltration, and potency to develop metastases for different types of cancer, as well as to test in preclinical assessment new anti-tumor medical products. Nude-mutation in *Foxn1* gene results in the congenital thymus dysgenesis, and, as a consequence, in the lack of T-lymphocytes. In its turn, it leads to the suppression of the immune functions which involve T-cells. It is generally accepted that Nudes' immune deficiency provides an opportunity to engraft in them immunologically incompatible human tumors that are xenogeneic for mice.In our study we have shown that there are some types of tumors that cannot be engrafted in BALB/c Nude mice. In particular, we have discovered that tumor cells deficient in p53 gene are not able to grow in Nude mice.Furthermore, we have used *in vitro* model system to demonstrate that the exosomes released by the fibroblasts of BALB/c Nude mice suppress the growth of the very same tumor cell lines that cannot be engrafted in these mice, but the exosomes do not affect the growth of the tumor cells that can be engrafted successfully. One of the possible mechanisms of tumor growth suppression in BALB/c Nude mice is the transfer of the exosomes containing wild type p53 protein from the surrounding tissues to tumor cells with gene p53 damage.

KEY WORDS: BALB/C NUDE MICE, CELL LINES, EXOSOMES, PROTEIN P53

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INTRODUCTION

Experimental models of human diseases play an important role both in understanding what biological and genetic factors influence phenotypic characteristics of the disease and in developing the strategy for its treatment.When using *in vitro* cell models for human tumor research one should take into consideration that, though these studies are necessary, they are reductionist, as often the parameters of the cell culture cannot be equated with the tumor growth inside the host's body where the tumor has specific micro-environment. Modeling human cancer with the help of genetically flawed mice is an alternative way to study carcinogenesis *in vivo*. It provides an opportunity to detect cell and molecular changes taking place at the initiation of cancer and during its further progression (Stakleff, 2003, Shultz, 2014; Boone, 2015).

One of the mechanisms preventing formation and growth of the malignant tumors in human body is immune system, and in particular, cell immunity based on the activity of T-lymphocytes attacking tumor cells. To research anti-tumor activity of the immune system the mice with deficient T-cell immunity are widely used. Athymic mice line BALB/c Nude is generally accepted as a model for this kind of experiments. The main advantage of this experimental model is natural immunodepression resulting from the functional deletion in *Foxn1* gene. As the result of the deletion, thymus is either deteriorated or absent, and, as a consequence, the number of T-cells is negligible, and, therefore, cell immunity is inhibited (Pignata,1996; Frank, 1999; Zuklys, 2016). In cancer research a large number of various tumor cell lines are used for inoculation hence, it is comparatively easy to engraft a tumor being studied in the mice just inoculating the host-animal with the tumor cells. It is believed that BALB/c Nude mice can host even immunologically incompatible, xenogneic for mice human tumors, (Liebman, 2007; Zeineldin, 2014; Conrad, 2015).

However, in our study we discovered that not all tumor grafts could grow in BALB/c Nude immune deficient mice. We were not able to reach a positive result of inoculation with primary glioma lines developed in our laboratory. The fact led us to the idea of an alternative mechanism of tumor growth inhibition – the mechanism which did not involve the immune system. If the mechanism exists, then even though Nude mice lack T-lymphocyte mediated cell immunity, the mechanism will account for inhibition of the growth of inoculated human tumor cells.Taking into consideration the growing amount of the data that point to participation of exosomes in formation, development and inhibition of cancer (Webber, 2015; Yu, 2015), we suggested that the discovered phenomenon of tumor growth inhibition in BALB/c Nude mice was possibly a result of the influence of the exosomes formed in the tissues surrounding the tumor.

Exosomes are extracellular membrane nano vesicles that are released from multivesicle bodies into extracellular space through exocytosis (Huang, 2013). The ability of the exosomes to be efficient transporters of exogenous proteins and RNA into cells-recipients has been reported (Shtam, 2013; Haney, 2015; Srivastava, 2016). Earlier in our experiments *in vitro* we demonstrated that the exosomes released from the cells, which contained wild type of the p53 protein, were able to inhibit the growth of the cancer cells lacking the same protein. Furthermore, the exosomes both released by the cell lines and isolated from human blood plasma carried one of the main tumor suppressors - protein p53 (Burdakov, 2017; Jorgensen, 2015, Burdakov, 2017).

In this study we now present some arguments in favor of our assumption that one of the factors inhibiting inoculated tumor growth in BALB/c Nude athymic mice may be negative effect of exosomes on tumor cells. The effect may be based on the reaction of the inoculated tumor cells deficient in *p53* gene to the wild type p53 protein, which exists in exosomes released by the surrounding tissues.

MATERIALS AND METHODS

CELL LINES AND CULTIVATION CONDITIONS

In our study we used both transplantable and primary human cell lines: HT-1080 (fibrosarcoma), Gl-V, Gl-R, Gl-Sh (primary cultures of glioma cells, developed in our laboratory), transplantable culture of rat cells C6 (rat brain glioma), and fibroblasts of BALB/c Nude mice. Cells were cultivated in DMEM/F12 medium (Biolot, Russia) supplemented with 10% fetal bovine serum (Biolot, Russia), without antibiotics, under 5% CO_2, at 37°C. When tumor cells of each of the lines were cultivated with isolated exosomes, the exosomes had final concentration in the culture of 10^{13} – 10^{14} exosomes/ml.

Isolation of exosomes in the systems in vitro

Conditional medium was collected from growing cell culture. Then, it was consequently centrifuged at 2,000 g and at 20,000 g to eliminate dead cells and their fragments. The procedure was repeated until the amount of the purified conditional medium reached 500 ml. This amount of collected purified conditional medium was used to isolate exosomes by ultracentrifuging. Beckman coulter ultracentrifuge was used (45Ti rotor) at 100,000 g for 2 hours. After that, the residue was suspended in 100 ml of PBS and underwent the second ultracentrifugation at the same conditions. The exosomes obtained as the result of the second sedimentation were used in the experiments.

Evaluation of the cell survival rate

The cells were suspended in Versene/Trypsin solution (Biolot, Russia), and the survival rate was evaluated through direct calculation of the cells with the help of vision-based automated cell counter Scepter (Millipore, USA). To visualize cell survival, the cells of Gl-V, Gl-R, Gl-Sh and C6 lines were seeded into 12-well plates in the concentration equal for all the cell lines (10,000 cells per a well). After that we added equal amounts of the exosomes harvested from the conditional medium of the fibroblasts of BALB/c Nude mice to all the cell lines. As the control cells formed a monolayer, all the cells in the well plates were stained with Crystal Violet (Fluka AG, Germany). The data were analyzed by Student's t test. Data are shown as the mean ± SEM (standard error of the mean).

Animal care and engraftment rate evaluation

Adult BALB/c Nude mice, 7-8 weeks of age and 18-20 grams of bodyweight, were purchased from the laboratory animal breeding center "Pushchino" (Pushchino, Russia). All procedures for mouse care and use were conducted in accordance with the National Standard of the Russian Federation GOST R 53434-2009: Principles of good laboratory practice (introduced 01.03.2010 by Federal Agency for Technical Regulation and Metrology, published by Standardinform, Moscow, 2010). The protocol was approved by scientific committee of the Division of Molecular and Radiation Biophysics of National Research Center "Kurchatov Institute" B.P.Konstantinov St Petersburg Nuclear Physics Institute (Gatchina, Russia).

Animals were housed in groups of three per cage (males and females were housed separately) with ad libitum access to food and water in standard polycarbonate cages (width: 300 mm, depth: 400 mm, height: 200 mm) and maintained under standard conditions of temperature (22 ± 2°C), humidity (55 ± 10%), artificial 12-hour light-dark cycle (lights on at 8:00 A.M., lights off at 8:00 P.M.). The mice were fed on the nutrient extruded and granulated food designed for experimental rodents (LLC «Laboratorkorm», Russia).

Tumor inoculations were performed through intramuscularly injections of 150,000 to 1,000,000 tumor cells. To estimate the engraftment rate for each tumor cell line, not less than 20 BALB/c Nude immune deficient athymic mice were used.

RESULTS AND DISCUSSION

Evaluation of engraftment in BALB/c Nude immune deficient athymic mice for tumor cell lines

To evaluate the engraftment rate we took 5 different tumor cell lines, which were cultivated in our laboratory. Two of them were standard transplantable cell lines: HT-1080, C6. The rest three lines (GL-V, GL-R, GL-Sh) were primary lines of human gliomas, developed in our laboratory. In the experiments with each of the cell lines not less than 20 BALB/c Nude immune deficient athymic mice were used. In the first experiment engraftment was performed through intramuscularly injections of 150,000 tumor cells per animal. As the result there were two polar outcomes: transplantable tumor lines HT-1080 and C6 engrafted in 100% of the animals; tumor lines GL-V, GL-R did not engraft at all (fig. 1). Engraftment rate of the latter lines (GL-V, GL-R) did not change with the increase in the amount of inoculated cells up to 1,000,000 cells per animal.

Analysis of survival rate of the tumor cell lines after treatment with the exosomes from the fibroblasts of BALB/c Nude mice

We assumed that negative results of engraftment of some of the tumors in BALB/c Nude athymic mice stemmed from the ability of exosomes to inhibit tumor cell growth. To confirm the assumption, we decided to compare the engraftment rate obtained in the experiments with the survival rate of the same tumor cell lines after they had been treated with the exosomes from the fibroblasts of BALB/c Nude mice. To carry out the comparison, we used standard ultracentrifugation methods described previously, (Lässer et al., 2012 and Shtam et al 2013) to isolate exosomes from the condensed cultural medium after growing fibroblasts of BALB/c Nude mice in it. Cells from the analyzed lines were seeded onto 24-well plates in the equal concentration. Then, equal amounts of the isolated exosomes were added to all of the tested tumor cell lines. In 10 days after the exosomes had been added, the number of living cells was calculated for each of the studied tumor cell lines.

The results of the calculation are presented in fig. 2. Addition of the exosomes isolated from the fibroblasts of BALB/c Nude mice did not affect the survivability of the tumor cells HT-1080 and GL-Sh. However, exosome addition to the cells of the tumor lines GL-R и GL-V resulted in significant decrease in their survivability (fig. 2). Survivability of the glial cells of GL-R cell line decreased by almost three times, and survivability of the cells of the cell line GL-V decreased by more than 10 times. The obtained results point to the ability of the BALB/c Nude cell exosomes to inhibit the growth of the target cells GL-R and GL-V in *in vitro* experiments. It correlates with the negative results of *in vivo* inoculation of the cells of these tumor lines to BALB/c Nude immune deficient mice.

Exosomes, participating in inter-cellular communication by transporting RNA and specific proteins between cells, are able to cause both functional and epigenetic

a)

Cell line	Description	Engraftment rate, %
HT-1080	Fibrosarcoma	100
C6	Rat brain glioma	100
GL-Sh	Primary human glioma cell culture	100
GL-V	Primary human glioma cell culture	0
GL-R	Primary human glioma cell culture	0

b)

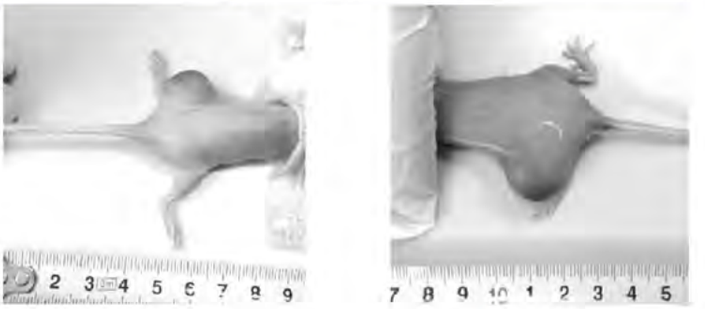

FIGURE 1. Engraftment rates of the tumor cell lines in BALB/c Nude immune deficient athymic mice
(a) Table of engraftment rates of the tumor cell lines C6, HT-1080, GL-Sh, GL-R и GL-V.
(b) Examples of the engrafted tumors HT-1080 and GL-Sh.

changes in the recipient cells (Grange, 2011; Kobayashi, 2014). Both discovery of p53 oncosupressor in the exosomes of the human blood plasma and their ability to affect proliferation of p53-negative cells confirm their important role in control over oncogenesis (Burdakov, 2017; Jorgensen, 2015). Protein p53 is the main participant of many signaling pathways through which cell reactions to stress are regulated. The protein is able to stop cell-division cycle and/or to cause apoptosis, thus preventing uncontrollable cell division – reproduction of the cells with damaged genome. In human malignant tumors p53 gene is the one that most often is found mutated

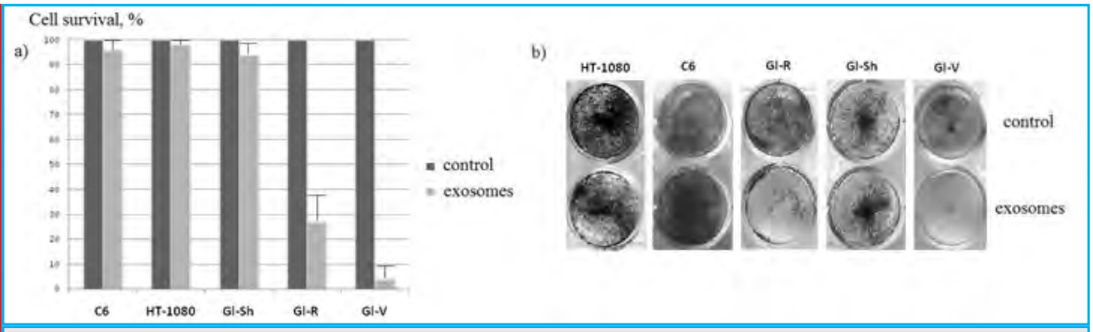

FIGURE 2. Survival rates of the tumor cell lines C6, HT-1080, GL-Sh, GL-R and GL-V after treatment with the exosomes from the fibroblasts of BALB/c Nude mice
(a) Histograms of survival rates of the tumor cell lines after treatment with the exosomes from the fibroblasts of BALB/c Nude mice.
(b) Survival of the cell lines C6, HT-1080, GL-Sh, GL-R and GL-V after coculturing with the exosomes from the fibroblasts of BALB/c Nude mice demonstrated by means of Crystal Violet staining.

(Levine, 2009; Vogelstein, 2013; Kandoth, 2013). There are a number of mouse model studies demonstrating that restoration of the function of wild type p53 may result in tumor regression (Ventura, 2007; Xue, 2007).

We assume that negative effect of the exosomes containing wild type p53 protein on inoculated tumor cells may be one of the factors which prevent the engraftment of the tumor in BALB/c Nude athymic mice.

It is a common knowledge that any experimental model based on the usage of the athymus Nude mice has its restrictions: immunodeficiency is severe, but not absolute (there still exists humoral adaptive immune system and undamaged congenital immunity). Hence, despite almost complete lack of functioning T-lymphocytes, both congenital immune response and high activity of NK-cells are able to restrict the speed of engraftment and to decrease metastatic potency of majority of tumors (Shultz, 2005; Shultz, 2014; Szadvari, 2016).

However, the data on the engraftment rate of different tumor cell lines we present in this study point to a clear division of the tumors into two groups: the tumors engrafting in BALB/c Nude mice and the tumors which cannot be engrafted in BALB/c Nude mice. Basing on the vast statistics we discovered no variations in the engraftment rate for each of the tumors used in the study (fig.1a). These facts cannot be explained only by existence of the rudimentary immunity in immune deficient mice. We assume that one of the possible mechanisms of the engraftment inhibition involves systemic influence of mouse's own exosomes on the inoculated tumor cells. It is necessary to remark that the cells with the wild type of p53 protein (HT-1080) had 100% engraftment rate, while GL-V cells lacking endogenous p53 protein (Kovalev, 2015) could not be engrafted at all.

Then we examined the sensitivity of all the tumor cell lines used for inoculation to the influence of the exosomes isolated from the fibroblasts of BALB/c Nude mice. The results we received in the experiments *in vitro* showed that cells of the GL-R cell line and especially p53-negative GL-V line were sensitive to such influence: the cells from abovementioned tumor cell lines died as the result of addition of the exosomes from the fibroblasts of BALB/c Nude mice to the tumor cell culture.The results obtained support the hypothesis that exosomes circulating in the body are able to perform a defense function controlling oncogenesis through onco suppressor p53.

Conflict of interest disclosures: We have no conflict of interest in any part of this article.

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Features of soil renaturation: an application for ecological rehabilitation of disturbed lands

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ABSTRACT

Land reclamation is disproportionate of areas that are diverted to mining, despite the requirements of environmental legislation. This creates an arena for the process of mine dumps overgrowth, which is similar to the renaturation abandoned sites. In a variety of the environmental conditions and with a wide amplitude of changes in factors, young ecosystems on the mine dumps and abandoned sites (after 30-70 years) form a spectrum of development pathways, which is characteristic of the adaptive self-organization of complex natural systems. Studying the various environmental restoration pathways of disturbed lands gives an understanding of the renaturation stages and rates, which allows moving to a controlled technology of plant and soil cover reproduction. The organic carbon accumulation values comparison in a comparable soil thickness showed that the efficiency of soil restoration in the forest-steppe with the postindustrial mine dumps renaturation compared to the restoration of the abandoned sites in the steppe zone were twice as high. This is due to the higher bioclimatic potential of the renaturation conditions; in particular, the sum of annual precipitation in the forest-steppe is more by 275 mm than in the steppe. However, the quantitative and qualitative indicators of pedogenesis (morphological structure, accumulation of organic carbon) show themselves similar to various bioclimatic conditions both in the renaturation of post technogenic and in the lands' ordinary mechanical disturbances, which would help to implement the universal technologies for the controlled renaturation of the disturbed lands. The established rates for the environmental rehabilitation of degraded land will be useful in implementing an irrecoverable conservation scenario where the most necessary types of ecosystems and aesthetic rehabilitation badlands can be formed.

KEY WORDS: MINE DUMPS, ABANDONED SITES, LAND RECLAMATION, ENVIRONMENTAL RECLAMATION, SOIL RESTORATION

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INTRODUCTION

The post-technogenic landscapes formation occurs under the combined influence of both natural and technological factors. The mining technology is connected with the formation of a technogenic relief, placement and rock-to-earth ratio. Natural factors include the geographical location of the mine excavations area, their background landscape-ecological surroundings, habitats-sources availability and the age of technogenic complexes. Violated reclamation lands during the development of the mineral deposit and in connection with the industrial facilities construction, include the technical stage of the works. This stage creates a geomorphological and lithogenic basis for the formation of post-technogenic landscapes. At the same time, land survey work is usually carried out to smooth out the open pit sides, to create terraces on slopes at the mine dumps, planning recultivated surfaces for antierosion purposes, chemical reclamation (if necessary) of toxic rocks, or to apply a high-velocity layer from a potentially fertile rock. In the operation of ore mining and processing facilities, wastes are moved to tailing dumps, for which prevention activities or reduction of damage from environmental contamination are very important .The environmental reclamation of a mine dumps can be defined as the general process of repairing disturbed, damaged, degraded, or destroyed land with respect to its former or other productive uses, (Favas, 2017; Rafkatovich and Mironova, 2018).

The final part of the technical stage of reclamation – moving the fertile soil layer out of the temporary mine dumps and applying it to the prepared surface – can be replaced with land renaturation, which was used, for example, in land reclamation for forestry use. Other methods of biological reclamation are now practiced plant growing (cultivation of agricultural crops); agromelioration (special methods of soil treatment); landscaping; agroforestry (protective forest belts); phytoreclamation (cultivation or maintenance of natural plant communities); bioremediation. The environmental reclamation is a broad concept encompassing all the other terms commonly referred to (restoration, rehabilitation, replacement, remediation, mitigation), used alone or combined (Gilland and McCarthy, 2014; Ngugi et al., 2015; Zenkov, 2016; Zamotaev et al., 2017; Favas, 2017).

Lands, where the methods of the biological reclamation are not involved and the process of mine dumps overgrowth is under way, can be under the controlled process of reproduction of plant and soil cover. The fact is that the rate of soil formations in overgrowth is small: only after 10 (12) years and more on the destroyed lands do the differentiation of plant species composition becomes dominant and a stable plant cover with layers and seasonal dynamics is formed (Golovanov et al., 2009). If the surrounding ecosystems have a low regeneration potential, which is typical for internal areas of the technogenic landscapes, then the targeted construction of the ecosystem renaturation is the most rational way. The soil development depends on the hydrothermal and geochemical processes that cause differentiation of the landscape conditions both in the natural setting and on artificial structures (Lisetskii et al., 2016). Geochemical features of the technogenic substrates are determined by a more complex mineralogical composition (Alekseev et al., 2008) and high content of heavy metals (Akbari, 2016).

Formation of soil cover has a subordinate character with respect to the development of phytocenoses, although it proceeds syngenetically (Goleusov and Lisetskii, 2008). Technologies of renaturation should be based on observing the sequence of the main stages of succession of phytocenoses: pioneer groupings (1-3 years) – simple groupings (3-8 years) – complex groupings (8-15 years). In the case of favourable edaphic and soil-forming properties of the substrate, accelerated renaturation scenarios are possible: the community's formation from "climax" plants species (Goleusov, 2003; Tokhtar and Martynova, 2015).

The bioclimatic potential study of the territory and the renaturation's natural mechanisms potential make it possible to outline effective optimal rehabilitation methods of disturbed lands. This study aims to assess the soil renaturation results of the destroyed lands during their formation by different types of anthropogenic transformation from the lithogenic basis under contrasting bioclimatic conditions.

MATERIALS AND METHODS

Study area: The study was carried out at two test sites: in the forest-steppe (Belgorod Oblast, career in the extraction of iron ore) and steppe (Crimean Peninsula, flat part). The area of Belgorod Oblast is relatively small (27.1 thousand km^2), but over 200 kinds of useful minerals have been identified in this territory. The Kursk Magnetic Anomaly (KMA) is a large iron-ore province with a total area of 125 thousand km^2 that extends from south-east to north-west at 625 km and a width of up to 250 km (Kornilov et al., 2014; Petin and Ignatenko, 2016). The territory of Belgorod Oblast includes 14 of 18 explored iron-ore mineral deposits of the KMA basin. The balance iron ore reserves in the Belgorod Oblast reach 51.32 bn t (78% of all KMA reserves or 51.2% of the reserves of Russia), and in addition the region contains 97.4 % All-Russian stocks of rich ores (with Fe content 53-62%) (Petin and Ignatenko, 2016).

In addition, Belgorod Oblast has 328 quarries of common minerals (chalk, clay, sand) on an area of about 1.5 thousand hectares. Among the study subjects in the

KMA region, there are mine dumps of various types: excavating, car dumps, hydro dumps, where was not carried out reclamation by applying a fertile soil layer. The KMA region is located in the forest-steppe, where the climate is moderately continental with an average annual temperature of 5.4 °C, and an annual precipitation of 616 mm.

The objects of steppe region study were the abandoned settlements territories and earth mounds of World War II in the so-called belligerent landscapes. The steppe study area is located 700 km south-west of KMA and here the climate is very arid with mild winter, and the average annual temperature is higher by 4.6 °C and an annual precipitation is less by 275 mm.

Data used: The empirical basis of the study rests on the previously created databases in MS Access format, which contain author's pedochronological data (morphology, properties) in forest-steppe and steppe soils (numbers of security documents RU2010620190 and RU2016621001, respectively). The computer program, which is a database management system (DBMS) of the different-aged soils, allowed selecting data with comparable history. The objects of study in 2003-2016 were young different-aged soils (30-70 years old) and their parent rocks in the Belgorod region (KMA) and Steppe Crimea. If in the KMA forest-steppe area the study subjects are mining dumps that for 32-35 years have passed restoration, then in the steppe region, these landscapes age 68-73 years, which are formed on the destroyed lands as a result of the termination of human activity lands (abandoned sites, hills of ash, military trenches). Survey targets comparison in two regions reveals the role of bioclimatic conditions and the significant differences in the chemical composition of parent rocks.

Methods: The rate of humus horizon formation (accumulative (A) and transitional (AB)) is taken as a relative indicator of soil formation effectiveness. The choice of an adequate mathematical model was based on the assumption that the model should reflect the rate of pedogenesis gradual deceleration, corresponding to the established organic matter equilibrium in the maximum concentration zone of soil biota in the renaturation horizon. Therefore, we chose the group of S-shaped growth models and in particular, a Gompertz function. Licensed software STATISTICA 10.0 was used for the modelling of the changes in the thickness of humus horizon (sum of A and AB, if it is formed). Chemical analyses for soil horizons A and AB included the following standard procedures: the content of CO_2 in carbonates measured by acidometry, bulk nitrogen content (N) as determined by Kjeldahl's procedure, and pH_{H2O}.

The determination of Corg in the soil was performed by oxidation of the organic substance with a solution $K_2Cr_2O_7$ in sulfuric acid until the formation of carbon dioxide. The quality of the organic substance can be estimated based on the atomic ratio of C:N, which characterizes the degree of nitrogen enrichment of humus: if C:N < 5, then very high, if C:N < 14, then very low. Most of the humus horizons of the zonal-genetic row have a high and medium degree of enrichment of humus with nitrogen at C:N ratio of about 5-8 and 8-10, respectively. If using the index Corg for every 10 mm of soil thickness (C/10 mm), then the data is comparable in the analysis of individual objects. This is due to the fact that after the initial accumulation of soil organic matter in horizon A with the highest concentration of roots, the humus is moved down the profile as the pedogenesis time increases. Concentration of 22 macroelements and trace elements within the soils were determined by technique of measuring metals mass fraction and oxides in powder samples using the method of X-ray fluorescence analysis on the spectrometer (Spectroscan Max-GV). With weathering of iron-containing minerals, amorphous iron compounds are formed, which were extracted by Tamm's reagent (oxalate buffer solution ($H_2C_2O_4$*$2H_2O$+$(NH_4)_2C_2O_4$*H_2O) with pH=3). Soil colours (dry and moist) were described using the Munsell color system (Munsell, 1994).

RESULTS AND DISCUSSION

Renaturation of the soil-plant cover

Land reclamation involves the application of a fertile soil layer on a post-technogenic surface. However, when applying the layer of minimum thickness (15 cm), the payback period (upon the return of the used land to arable land) with the increasing delivery distance using scrapers from 300 to 1000 m will increase from 3 to 7 years. The land reclamation in the KMA is not carried out in due scope due to the lack of financial resources and insufficient technical equipment of mining companies. The annual increase in the area of destroyed land is comparable to the area of land, where reclamation was carried out. Therefore, Belgorod Oblast needs more than 100 years to fully reclamation of all disturbed lands, if the same reclamation rates continue in the future. The reclamation biological stage economic costs during formation of the post-technogenic landscape can be significantly reduced by controlling the restoration process.

Therefore, it is necessary to use the results of empirical studies that will allow selecting the most effective restoration trajectories and self-sustaining native ecosystem restoration. The soil-plant cover renaturation is a complex ensemble of natural reproduction processes of the biotic and abiotic geosystems components and the anthropogenic geosystem natural evolution, in which

the economic activity was completed. A characteristic feature of technogenic geosystems is the high heterogeneity of their lithological base made of rocks that were moved in mine dump using the dry method or in the slurry form in the hydraulic filling process. And it is important to point out that these rocks belong to different geological formations and are often not parent rock for soil cover of the background landscapes.

In polycomponent technogenic mixtures, their environmental favourableness for renaturation is determined by the rocks ratio and, as a result of their mixing; the emergent properties of technogenic substrates appear. The morphology of the newly formed soil profiles of the technogenic landscapes strongly depends on the parent rock type. The soil profile is best formed in loamy and sandy-loamy rocks, soils on sands, clays, chalk and dense crystalline rocks are less developed. The biogeochemical cycle plays a determining role in the soil reproduction, including the process of complexes with organic residues, their transformation, mineralization, and humification (Lisetskii, 2012). Loamy soils better fix the organic matter.

With renaturation, aggregation processes are underway and the structure provides optimal water-air and microbiological regimes and favourable conditions for nutrient entry into the plants, improves anti-erosive soil stability in difficult terrain conditions (Bulygin and Lisetskiy, 1992). Biocenoses regeneration in ecotopes with a favourable substrate for the settlement of higher plants occurs at an accelerated rate, which determines the most active transformation of the mineral part within the rhizosphere area, where maximum organic matter accumulation is observed. Therefore, the maximum humus horizon formation rate (1.5-2 mm yr^{-1}) was noted with a soil aged of about 30 years.

Soil restoration on the mine dumps

In the forest-steppe, the results of renaturation postindustrial mine dump after 32-35 years (Table 1) are characterized by the following indicators of pedogenesis: the thickness of humus horizon is 43.3 ± 2.9 mm, the content of humus reaches 3.42 ± 0.29%, N = 0.26 ± 0.02%. These fertility indicators are formed under conditions when the content of Fe is 1.50 ± 0.30%, the soil solutions have a medium alkaline reaction (pH_{H2O} = 8.24 ± 0.07), which is due to the carbonate content (CO_2 = 3.69 ± 0.73%).

With renaturation postindustrial mine dumps, the processes of humus horizon formation and humus synchronously accumulates in the first three-four decades as a whole. Soils reproduction in technogenic landscapes under conditions of mine dumps overgrowth with overburden rocks occurs under rather extreme conditions; however, in most cases by the age of 30-40 years' young soils have a well-defined morphological profile.

Table 1: Basic indicators of primary pedogenesis in renaturation postindustrial mine dumps in forest-steppe conditions

Object (FS)	Age (t)	PR[a]	VC[b], Number species	Horizon	H, mm	Munsell color moist dry	CO_2, %	Fe, %	Corg	C:N	Vh, mm yr^{-1}	Vc, % yr^{-1}	C/10 mm, %
1	35	LM	PE 15	A	16±0.3	10YR3/2 10YR4/2	4.50	0.31	3.07	7			
				AB	29±1	10YR3/3 10YR4.5/2	4.27	0.23	1.91	8	0.83	0.07	0.88
5	33	LL + RF	HG 43	A	23±1	10YR3/2 10YR3/3	2.00	0.76	3.07	11			
				AB	51±1	10YR2/2 10YR3/2	2.10	0.69	2.32	10	1.55	0.08	0.52
7	35	RF	CM 26	A+AB	52±2	10YR2.5/2 10YR4/3	3.52	0.56	1.92	13	1.49	0.05	0.37
8	35	RF + LM	CE 26	A	35±1	10YR3/1.5 10YR3/2	4.38	0.42	2.24	13			
				AB	74±1	10YR3/3 10YR3/2	3.94	0.50	1.26	7	2.11	0.05	0.23
9	34	RF	W+G 15	A+AB	31±0.4	7.5YR3/3 5YR4/3	1.58	3.70	1.94	8	0.91	0.06	0.63
10	34	RF + LL	C+MA 6	A	27±1	10YR2.5/2 10YR3/3	1.08	2.10	2.98	12			
				AB	81±2	10YR2.5/2 10YR4/3	0.81	2.65	2.40	6	2.38	0.08	0.46
11	32	Sca	PA 16	A+AB	56±2	2.5YR6/3 2.5Y7/2	12.08	0.05	2.27	81	1.75	0.07	0.41

Note: [a]Parent Rocks: LM, Loam Medium; LL, Light Loam; RF, Rock (Ferruginous); Sca, Carbonate Sand. [b]Vegetation Communities: PE, Poa-Euphorbia community; HG, Heterogeneous group (43 species of different coenotypes); CM, Calamagrostis-Medicago association; CE, Calamagrostis-Elytrigia community; W+G, Woody vegetation (5 species) and a rare grassy tier; C+MA, Cornus sangguinea and Medicago-Achillea community; PA, Poa-Artemisia grouping.

The greatest development among soil genetic horizons is obtained by humus – accumulative (A) and transitional (AB or AC (in the early stages)) horizons. The dependence of the total thickness (A+AB(AC)) of humus horizon (H (t), mm) on the time (t, years) in the early stage of pedogenesis in the technogenic landscapes of the forest-steppe has the form: H (t) = 200 exp (–exp (0.923-0.025 t)). Using this model, it was found that the average rate of humus horizon formation of Chernozem-like soils (Vh) in the first 35 years is 2.00 mm yr^{-1}.However, in the various substrates and phytocenotic conditions of the technogenic landscapes, significant differences in Vh (n = 22) from 0.83 to 2.38 mm yr^{-1} are noted with a coefficient of 36% variation. During this same time, the average rate of Corg accumulation is 1.86 ± 0.15% with a coefficient of 38% variation. As shown in Table 1, with the exception of soil on sands, all other soils when achieving an average content of Corg = 2.2% acquire a characteristic soil colour (dry) dark grayish brown (10YR4/2).

The maximum growth rates of the humus horizon thickness were noted, when rocks (Ferruginous) were mixed with loams, although the plant cover features were not a factor of pedogenesis rate differentiation. Nevertheless, the presence of woody vegetation (such as *Cornus sangguinea*) stimulated both soil growth downwards and Corg accumulation with a high degree of humus enrichment with nitrogen. As shown earlier (Goleusov, 2003), in the forest-steppe conditions, there are no significant differences in the pedogenesis efficiency under grass and tree vegetation in the early stages of renaturation, but at a later stage, the organic carbon accumulation rate in grassy communities becomes higher.

High Corg accumulation rates do not depend on the content of carbonates in parent rock and they are not hindered by the increased content amorphous Fe in the rocks of mining dumps. The lowest rates of Corg accumulation were noted on ferruginous rock and more favourable parent rocks (loam and loamy sand) were a more significant factor pedogenesis, than the diversity of plant species and type of vegetation. The high degree of humus enrichment with nitrogen (C:N=5-8) is always accompanied by high rates of humus horizon (Vh), but the ratio C:N=8 was also noted in cases where the maximum values of C/10 mm were recorded.

At the quartzite mine dumps (KMA), vegetation in the early stages of recovery is very poor, but at the foot of the slopes where the meadow-steppe vegetation with ruderal species concentration, 58 species of plants are noted (Kornilov et al., 2008). At the age of young geosystems from 35 to 50 years and beyond, there comes a stage of sustainable geosystems operation where the structure and regeneration processes stabilize. The rate of soil reproduction decreases; the species composition in a phytocenosis gradually stabilizes; the share of ruderal species decreases.

Soil restoration on the abandoned sites

With mechanical destructions of land and the human activity termination, semi-natural landscapes (post-settlement, fortification, etc.) were formed with a variety of conditions on lithology and vegetation composition. In such conditions (Table 2), with an arid steppe climate, the average rate of humus horizon formation (Vh) for the first 70 years ranged from 1.1 to 2.3 mm yr^{-1} with a variation coefficient of 28%. During the same time, the average rate of Corg accumulation was 0.04% yr^{-1} with a variation coefficient of 23%, i.e. is identical to the accumulation of carbon on mine dumps KMA. In this way, judging by the values of the indicator C/10 mm, the pedogenesis efficiency (with respect to the accumulation of organic substance in a comparable thickness) during renaturation of the postindustrial mine dumps (0.50% on 10 mm) compared with the usual mechanical violations of land in the steppe zone was twice as high.This is due to the higher bioclimatic potential of renaturation conditions, which was typical for mining facilities of KMA in the forest-steppe. Consequently, we can note the higher species diversity and vegetation productivity during the overgrowth of mine dumps, despite the lesser age of ecosystems restoration in the forest-steppe compared with the steppe. In comparison with the young forest-steppe soil, colour is lighter in the steppe soils, which is mainly determined by the presence of such parent rock as ash.

Table 2 includes only the nine macroelements and trace elements that belong to the group “plant nutrients”, and among them there are those that showed a significant variation (V>20%) among the subjects studied. A comparison of the soil quality values that were obtained by calculating the geometric means for nine indicators (Table 2) showed that the richest soil (in biogeochemical terms) was formed in seven decades under conditions, when the parent rock was ash, although the plant cover was not characterized by maturity and diversity. Scenarios of ecological renaturation pathways are more diverse under overgrowth conditions of the postindustrial mine dumps in comparison with the restoration of the abandoned sites (settlements and fortification objects). It can be assumed that this is facilitated by more favourable climatic conditions of the forest steppe in comparison with the steppe. Moreover, the narrower scope of implemented pathways of renaturation of steppe ecosystems is due to the greater influence of adjacent ecosystems due to their territorial proximity. The important role of habitats-sources for the successions acceleration was shown in the definition of the kurgans vital role in steppe restoration (Deák et al., 2016). In areas where the

Table 2. Features of the chemical composition of soils formed during the first 70 years in the conditions of the steppe zone

Object		S1/1	S1/2	S2	S3	S4	S5	S6	Average
PR[a]		A	A	T	T	T	BL	A	–
V[b]		C	C	HG	GMG	MG	W	W	–
T	yrs	70	70	70	68	68	73	73	–
Horizon, depth	mm	A, 0-162	AB,162-280	A, 0-95	A, 0-93	A, 0-122	A, 0-81	A, 0-98	–
Munsell color	dry	10YR 6/2	10YR 6/2	10YR 7/3.5	10YR 5/3	10YR 7/3	10YR 6/3.5	10YR 5/2.5	–
Corg	%	3.29	2.59	3.46	2.66	2.16	1.98	2.43	2.65
P_2O_5	%	1.33	1.39	0.28	0.15	0.21	0.30	0.49	0.59
K_2O	%	3.00	3.32	1.84	1.90	1.23	1.10	1.83	2.03
Fe	%	2.15	2.08	2.00	3.10	1.62	1.47	1.92	2.05
SiO_2	%	45.8	44.6	37.7	46.3	20.0	21.6	34.0	35.71
CaO	%	16.9	17.7	21.8	6.5	36.0	28.1	16.5	20.51
MgO	%	2.5	2.5	2.3	1.3	3.3	2.8	1.9	2.38
Cu	ppm	20.8	21.4	24.7	43.1	35.0	38.9	42.1	32.29
Zn	ppm	143.8	123.6	72.0	81.6	67.9	59.6	78.1	89.51
Vh	mm yr^{-1}	–	2.31	1.36	1.37	1.79	1.11	1.34	1.55
Vc	% yr^{-1}	0.05	0.05	0.05	0.04	0.03	0.03	0.03	0.04
C/10 mm	%	–	0.20	0.36	0.29	0.18	0.24	0.25	0.25

Note: [a]Parent Rock: BL, Building Layer; A, Ash; T, Ejection Parent Rock (Trenches). [b]Vegetation: W, Weeds; C, Cereals; HG, Herb-Grass; GMG, Grassy-Motley Grass; MG, Motley Grass. Ppm = mg kg^{-1}

ratio of natural habitats is high, we can expect a fast recovery of semi-natural habitats (Valkó et al., 2017).

CONCLUSION

The type and geochemistry of parent rock are the determining factors of pedogenesis and makes the main contribution to the soil properties variation that is close in age. Young ecosystems, that were formed in a variety of conditions (relief, lithology, climate, vegetation), through the environmental factor amplitude of oscillations could, in the self-organization process, enter one, but a development pathways spectrum, since they had many degrees of freedom. The range of the environmental renaturation efficiency in the forest-steppe under the recent pedogenesis conditions in postindustrial mine dumps is diagnosed to the greatest extent by the Corg/10 mm index and Vh is inferior to it. The small differences (with varying types of rocks and vegetation) are typical for the average rate of Corg accumulation. In the steppe zone, the range of values is less than in the forest-steppe and the manifestations of the processes that are reflected in indicators C/10 mm and Vh and the average rate of Corg accumulation is inferior to them. Thereby, in various bioclimatic conditions, indicators of the pedogenesis efficiency on renaturation post-technogenic and of conventional mechanical disorder lands manifest themselves in a similar way. This allows using the comprehensive technology of controlled renaturation of the disturbed lands. We attribute this type of renaturation to irrecoverable conservation, whose goal is to form the most "scarce" ecosystems with the selection of tolerant species, the creation of natural reserves and the badlands aesthetic rehabilitation. It is necessary to provide system solutions, since environmental renaturation in post mining sites includes stabilization of the relief, primary and recent pedogenesis, self-purification of natural environments (restoration of the geochemical balance), primary and regenerative biota successions (revitalization of anthropogenic surfaces), intercomponent geosystem interactions restoration.

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Cross cultural validation of functional autonomy measurement system (SMAF) into Arabic geriatric rehab-culture

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ABSTRACT

The Functional Autonomy Measurement System (FAMS) measures functional ability in Five domain: mobility, activities of daily living, instrumental activities of daily living, mental function and communication. FAMS however was validated into many languages such are English, Spanish, Japanese and Dutch, but it was not validated into Arabic. So, it may not suit Arabian cultures and society. Thus, the validation of FAMS into Arabic is vital. The aim therefore was to validate FAMS into Arabic culture. This is a prospective cross-sectional design. The study was conducted in Riyadh at King Abdul-Aziz Medical city (KAMC), in King Fahad Hospital (KFH) /in rehabilitation department. The FAMS was translated from original English version into Arabic. Translated Arabic version was distributed to 30 health professionals who work in rehabilitation department to check it for contents and accordingly FAMS was modified. Then, the final modified version of Arabic FAMS was distributed to 30 Saudis elderly who are 60 years old and above to test the question applicability and clarity. The results showed a measure of the internal consistency of our study by using Cronbach's alpha. The optimal value of this test is 0.7 and above. So, we found the value of our study was more than 0.7. The value was 0.9 for the 5 domains (ADL, Mobility, Communication, Mental function, IADL) which means it reliable for Arabic patients in Arabic cultures. The result revealed a new Arabic version of important assessment tool in the field of rehabilitation for the first time ever. The internal consistency of the FAMS assessment tool is in line with other previous similar validation research globally. Conclusion and clinical implications: The new translated Arabic version of FAMS was produced with Arabic cultural modifications. Further studies are needed to test this important assessment tools with larger population of Arab patients.

KEY WORDS: SOCIAL FUNCTIONING, FUNCTIONAL AUTONOMY, INSTRUMENTAL ACTIVITIES, OLDER PEOPLE, VALIDITY, ARABIC CULTURE

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INTRODUCTION

The Functional Autonomy Measurement System (FAMS) is a tool which developed in 1984 by a team from the Community Health Department at Hôtel-Dieu in Lévis to measure the demands and needs of the elderly handicapped (Hébert, Guilbault, Desrosiers, & Dubuc, 2001; Hébert, Carrier, & Bilodeau, 1988). SMAF quantifies the functional ability on 29-items scale which developed by World Health Organization's classification of impairments, handicaps and disabilities (Hébert, Robichaud, Roy, Bravo, & Voyer, 2001). It measures functional ability in 5 sectors: mobility [6 items], activities of daily living (ADL) [7 items], instrumental activities of daily living (IADL) [8 items], mental functions [5 items] and communication [3 items] (Hébert, et al, 2001). These items were standardized using four-level measurement scale. Level 0: autonomous, Level 1: needs supervision or stimulation, Level 2: needs help, and Level 3: dependent (Hébert, et al 2001; Hébert et al, 1988; Hébert, et al, 2001). According to the occupational therapy practice framework: Doman & process, activities of daily living (ADLs) are activities that are specialized toward taking care of one's own body. Which are bathing, showering, toileting and toilet hygiene, dressing, swallowing/eating, feeding, functional mobility, personal device care, personal hygiene and grooming, and sexual activity (Amini, D. A., Kannenberg, K., Bodison, S., Chang, P., Colaianni, D., Goodrich, B., & Lieberman, D., 2014). Not all the ADLs covered in the SMAF. The seven items included in SMAF are eating, washing, dressing, grooming, urinary continence, facial continence, and toileting (Hébert, Guilbault et al 2001).

Instrumental activities of daily living (IADLs) as in occupational therapy practice framework are "activities to support daily life within the home and community" (Amini et al, 2014) Care of others, care of pets, child rearing, communication management, driving and community mobility, financial management, health management and maintenance, home establishment and management, meal preparation and cleanup, religious and spiritual activities and expression, safety and emergency maintenance, and shopping are all considered IADLs" (Amini et al, 2014)

IADLs in the SMAF are meal preparation and cleanup, financial management, shopping, health management and maintenance, driving and community mobility, home establishment and maintenance (cleaning the house, and doing the laundry), and communication (Hébert, et al, 2001).

Disability is any restriction or lack (resulting from an impairment) of ability to perform an activity in the manner or within the range considered normal for a human being (World Health Organization,1980) World Health Organization classified the disability into 9 categories (World Health Organization,1980). The first one is behavior disabilities which mention to a person's awareness of himself, others, their roles and occupations, places, and time (World Health Organization,1980). The second one is communication disabilities that refer to person's ability to communicate with other such as disability in speaking, listening, seeing, and writing (World Health Organization,1980). The third is personal care disabilities which mean the person's ability to take care of himself, his personal hygiene, dressing, feeding, and getting to bed (World Health Organization,1980). The fourth is locomotor disabilities which are disability in the walking, climbing stairs, running, transfer, transport, and lifting (World Health Organization,1980). The fifth is body disposition disabilities that mean the person's ability to carry out his own activities such as preparing food, cocking, serving food, reaching, and kneeling (World Health Organization,1980). The sixth is dexterity disabilities that refer to skill body movement such as modify the environment, moving and handling objects, fine motor control, and body control (World Health Organization,1980).

The seventh is situational disabilities that are disability in dependence, endurance, and disability in tolerance in the temperature, noise, and work stress (World Health Organization,1980). The eighth is particular skill disabilities that is disability in behavior such as intelligence, motivation, perception, learning, orientation, and concentration, and disability in achievement the tasks such as problem solving, adaptability, and accuracy (World Health Organization,1980). The last one is other activity restrictions; this section has been developed, in order to develop some of the categories that were not mentioned in the previous classifications (World Health Organization, 1980).

FAMS was validated into many languges such as English, Spanish, Japanese and Dutch, but it was not validated into Arabic. So, it may not suit our cultures and society (Desrosiers et al., 1995). Thus, the validation of FAMS into Arabic is vital (Desrosiers et al, 1995). It would help occupational therapists who work with people with disabilities and elderly who live in Arab counteries and speak Arabic languges in evaluating the areas of disability in them (Desrosiers et al, 1995).

Validation and realibility are independent on each other. A measurement maybe valid but not reliable, or reliable but not valid. Relibility is the reapition of findings (Kimberlin, & Winterstein, (2008). Validity of a test is that it measure what it is supposed measures (Kimberlin et al, 2008). We conducted this study to valiate SMAF culturally into Arabic culture. We choose prospective cross-sectional design to insure the willing of the participants.

Literature Review

In 2009 Yount and Sibai found that By 2045-2050, the researchers expected that most (20 of 23) Arab populations are have at least 10 percent of population aged 60 and older. Hence, the Arabic country which have a largest number of elderly presently lived in it is Egypt (Yount & Sibai, 2009). The epidemiological research in Arab countries has been focused on non–communicable disorders and mostly on those of the circulatory system (Yount et al, 2009). The differences in Physical dependence with elderly are noted through out Arab countries with a high prevealance of ADL, IADL difficulties (Yount et al, 2009). The highest rates of limitations in ADL were shown in Tunisia, Egypt, Jordan and Lebanon (those reported between 25 and 38 percent) these countries followed by the UAE and Saudi Arabia which have between 17-19 percent (Yount et al, 2009). The limitations in ADL and IADL due to disability among women more than among men. However, there is lacking of measurement tools to assess functional limitations resulting In over-reporting of disabilities by the persons with disability (Yount et al, 2009). Based on that the women have shown higher difficulties in performing physicals tasks than men in Egypt, Tunisia, Jordan and Lebanon, but Egypt was reported as highest rates of restriction in performing physical tasks (PT) by 71 percent in men and 88 percent in women (Yount et al, 2009). Yount et al opined that capability to perform physical tasks (PT) is the most commonly used measurement to evaluate disability among elderly (Yount et al, 2009). "This measure is presumably independent of one's physical and social environment (e.g., cultural and gender roles), which would increase its validity and reproducibility and thereby make it preferable to measures of ADL and IADL disability" (Yount et al, 2009)

In two Canadian's studies used the SMAF as a measure using same study designs. Both studies used qusi-experimental design with elderly group patients aged 75 years. The first study included 151 elderlies from the geriatric day hospital and the second study included 1,501 persons identified at risk of functional decline (Tousignant, et al 2003; Hébert, et al 2009).

In another study using FAMS, they find that this tool was important tool to reduce elderly functional decline by identifying functional limitation at early stage, and Similarly Hébert Brayne, and Spiegelhalter used SMAF tool to measure functional movement, this helped identify risk factors of dependency and this helped to reduced the period of dependency in elderly group (Hébert, R., 1997; Hébert, R., Brayne, C., & Spiegelhalter, D, 1999).

Other functional studies carried out with elderly agreed that SMAF is very important tool and instrument synthesizes and systematizes the various scales proposed to measure the functioning of the elderly or disabled (Mercier, et al, 2001; Pinsonnault, et al, 2003; Wales, et al, 2016). In All previous studies, they recruited similar age groups of fifty-five to seventy fife years old. All these studies were helped to evaluate the needs of individuals (elderly) by measuring the disabilities and the handicaps with which they are afflicted. These studies indicate that the FAMS is vital tool to measure the functional movement and independency in elderly groups as well as in people with disabilities. These very important in occupational therapy practice, make it worth studying it and translated and validated for people from other cultures rather than only people from western culture.

Additionally, another study aimed to apply the ISO-SMAF classification to funding long-term care facilities in one area of the Province of Quebec and to compare the results of this new funding methodology to the formal methodology (Desrosiers, et al, 2004).

FAMS was validated into many languges such are English, Spanish, Japanese and Dutch, but it was not validated into Arabic. So, it may not suit our cultures and society (Desrosiers et al 1995). Thus, the validation of SMAF into Arabic is vital. The aim of this study is to valiate SMAF into Arabic culture. It would help occupational therapists who work with people with disabilities and elderly who live in Arab counteries and speak Arabic languges in evaluating the areas of disability in the Arabic elderly (Desrosiers et al, 1995). In the future, when there is an Arabic version, the method of evaluating elderly will be easy for the specialist and for patients. Also on the specialists' side it will be comfortable and easy for them because they will not make much effort to explain the point to patients, and it will save a lot of their time and effort (Desrosiers et al, 1995).

METHODS

The study was about cultural validation of Functional Autonomy Measurement System (FAMS) into Arabic culture. The study was conducted in Riyadh at King Abdulaziz Medical city (KAMC), in King Fahad Hospital (KFH) specifically in rehabilitation department. The capacity of beds in KAMC is 1501 beds. In 2003, the rehabilitation unite was only for neurological rehabilitation. The neurological rehabilitation unite expanded to be a rehabilitation department at 2004. Now, the rehabilitation department involve occupational and physiotherapy unites, In vitro fertilization (IVF), OB, medical imaging, laboratory, pharmacy, and continues renovation of patients' wards. (Ngha.med.sa. 2018). The Arabic version of FAMS was administered among 30 health providers in rehabilitation team including occupational therapists, physical therapists, physicans, nurses and 30 elderley patients 60 years and over after following the inclusion and exclusion criteria. The inclosion cri-

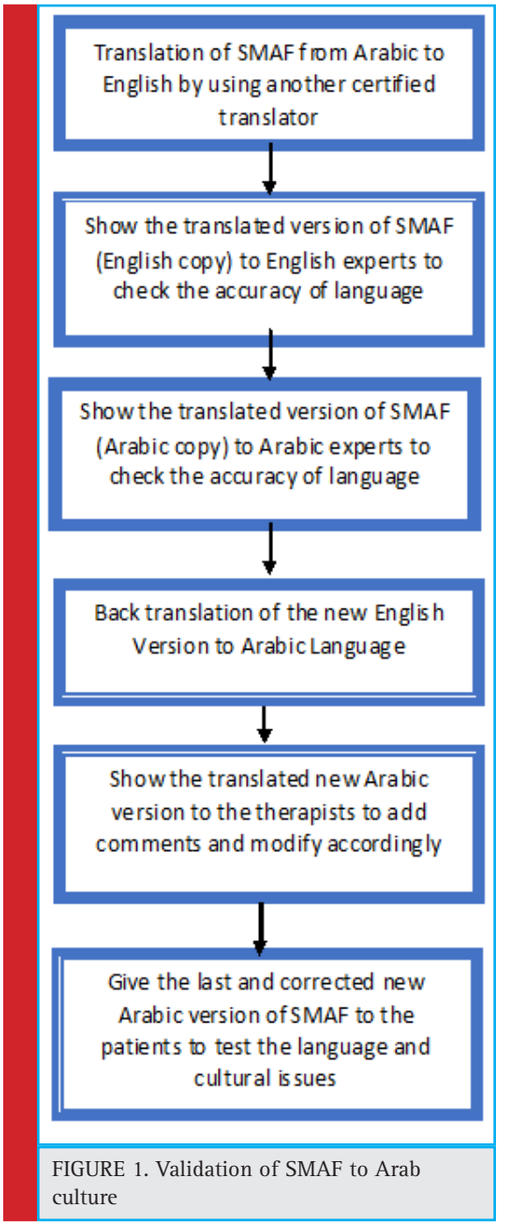

FIGURE 1. Validation of SMAF to Arab culture

teria of this stude was the elderly should be 60 year-old and above, assess both genders and for those who have loss or limited of function. Howevere, participants who were below 60 year-old and anyone who have cognitive impairment were excloded from this study. The design for this study was prospective cross sectional. The sample size of this study was determined by the previous validation process in previous studies as listed in the reference list, (Beaton et al, 2000, Elboim-Gabyzon, et al 2015).

So, the estimated sample size for health providers who work in rehabilitation department in KAMC were 30 but we found only 19 health profissionals in rehabilitation team who are Arabic native speaker and for Saudi elderly clients over 60 years the sample size were 30 also. There is no randomization needed for this study. Purposeful sampling was used which means to select people who are willing to participate and available. The steps involved in the validation of SMAF to Arabic culture is given in Figure 1 and detailed as below.

Stage I & II: Initial Translation

The first and second stage in adaptation were the forward translation. Two forward translations were made by two different certified translators. One certified translator was translated from English language into Arabic language, and then second translator was translated the tool from the new Arabic version into English language.

Stage III & IV: Synthesis of The Translations

The third stage was to compare between the old English and the new English version to find any discrepancies and modify them. The fourth stage was to give the new Arabic version to Arabic language expert to check it for errors.

Stage V: Back Translation

The fifth stage was to translate the new English into Arabic language; This is a process of validity checking to make sure that the translated version is reflecting the same item content as the original versions.

Stage VI: Expert Committee

The sixth stage was to consult therapists about the new Arabic version and to receive their comments on it without using survey. Based on the comments from expert committee, modification was done on the Arabic version of FAMS.

Stage VII: Test of the Pre-Final Version

The final stage of adaptation process was the pretest. This field test of the new questionnaire seeks to use the pre-final version in subjects. Ideally, 30 persons were tested.

Accordingly, the final Arabic version of FAMS was edited and finalized and released to be used in upcoming studies.

Ethical consideration and data management/ statistical plan:

Ethical consideration:

Approval from IRB (King Abdullah Medical Research Center) was sought. An informed consent was completed prior to data collection. The researchers were safeguard the confidentiality of participants of the study. Personal data was stored on a computer with accessibility only to

the researchers. Subject data was coded and names were not used in any of the documents related to this study.

Data management/ statistical plan:

Data was analyzed using SPSS statistical software, version 22. Descriptive statistics of means was used. Response frequencies and means or medians for the survey items were determined and displayed in tabular formats. After we completed the pilot study with 30 patients, the reliability of the new questionnaires items was tested using Cronbach's alpha.

RESULTS

The first stage was the distribution of the Arabic version of FAMS to nineteen native Arabic speakers both female and male health professionals who work in rehabilitation team to make sure that it is clear and valid for Arabic culture. After two weeks, we received the distributed FAMS. Following analysis of the data, twelve of them were satisfied and happy with the objective and identify that the objective of Arabic version is the same as the objective of original version. However, some of the participants suggested that for cultural purpose this tool needs to be completed by face to face interview instead of filling the form subjectively. The second step, after taking the opinions of experts into consideration and making modifications for the tables arrangement and sentence structure, we distributed the Arabic FAMS to thirty female and male Saudi elderly. The age group of the validation of FAMS differ from country to another, in Arabic culture elderly stage starts at sixty which is the retirement age in Arab countries. The mean of elderly participants age was 71.6 as seen in table 1.

The participants in this research were representative for Arabic culture. They have different level of education; Master degree, Bachelor degree, high school, middle school, elementary school, and illiterate. Those participants differ in health status, some of them were in good health, however; others have chronic condition such as Diabetes, Irritable Bowel Syndrome, Hypertension, Osteoporosis, and Rheumatoid Arthritis and sever health conditions such as Hemodialysis, Breast Cancer, and heart disease. The female participants were twenty-one and male participants were nine as seen in table 2.

Table 1. mean of elderly age

Statistics		
age		
N	Valid	30
	Missing	0
Mean		71.6667

The result of distributing the FAMS to elderly in Arab culture was clear, simple, and easily understood for the participants. They satisfyingly answered all the questions of the Arabic version of FAMS. As a result, the Arabic version of FAMS is valid to be used with elderly in all Arab countries.

We used Cronbach's Alpha to measure the validity of the tool. The value of activity of daily living (ADL) was 0.914. And the value of mobility was 0.914. Also, the value of communication was 0.918. The value of mental function was 0.917. The value of instrumental activities of daily living (IADL) was 0.913. The optimal value of this test is 0.7 and above. The result that we found for all items in SMAF was 0.9 as seen in table 3.

DISCUSSION

The suitability of the content of FAMS for elderly people was reliable and valid and widely used in many countries. however the application of this scale in other non-English speaking made the outcomes nonspecific and weary. In the Arab countries a notable boundaries were addressed by patients and families during the application of the FAMS in English version. the variation in the language interpretation and diversity in the meaning between Arabic and English was a real barrier. therefore this study was of extreme importance and the optimization of interpretation of the FAMS was the real challenge.

In this study, we validate the Arabic version of the functional Autonomy measurement system for the result, we have carried this study through two stages. In stage one, we distributed the Arabic version of FAMS to nineteen Arabic health professionals in rehab rehabilitation team. They were satisfied about it but they have some comments regarding the sentence structure. Based on their comments we modified the Arabic version to be

Table 2. number of male and female who are participate in research.

gender					
		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	male	9	30.0	30.0	30.0
	female	21	70.0	70.0	100.0
	Total	30	100.0	100.0	

Table 3: shows the value of cronbach's alpha for ADL, Mobility, Communication, Mental function, IADL.

Item-Total Statistics					
	Scale Mean if Item Deleted	Scale Variance if Item Deleted	Corrected Item-Total Correlation	Squared Multiple Correlation	Cronbach's Alpha if Item Deleted
activities_of_daily_living_eating	93.6667	929.333	.699	.	.915
activities_of_daily_living_bathing	93.6000	916.938	.835	.	.914
activities_of_daily_living_dressing	93.6667	922.368	.851	.	.914
activities_of_daily_living_grooming	93.7000	916.631	.886	.	.913
activities_of_daily_living_urinary_function	93.9333	935.237	.767	.	.915
activities_of_daily_living_bowel_function	94.1000	954.231	.631	.	.917
activities_of_daily_living_toiletting	93.9000	941.610	.604	.	.916
Mobility_TRANSFERS	93.8333	934.144	.756	.	.915
Mobility_WALKING_INSIDE	93.6667	927.540	.678	.	.915
Mobility_PROPELLING_A_WHEELCHAIR_INSIDE	93.9000	931.059	.760	.	.915
Mobility_NEGOTIATING_STAIRS	92.9667	900.930	.810	.	.913
Mobiliy_MOVING_AROUND_OUTSIDE	93.2333	901.151	.760	.	.913
communication_vision	93.8667	962.051	.578	.	.918
comunication_hearing	94.1333	966.602	.603	.	.918
communication_speaking	94.2000	969.407	.569	.	.919
mental_function_memory	93.8667	957.844	.687	.	.917
mental_function_orientation	94.0667	952.685	.808	.	.917
mental_function_COMPREHENSION	94.1333	962.740	.733	.	.918
mental_function_JUDGMENT	93.9000	941.128	.749	.	.916
mental_function_BEHAVIOUR	94.0333	968.723	.553	.	.919
INSTRUMENTAL_ACTIVITIES_OF_DAILY_LIVING_HOUSEKEEPING	93.0000	898.897	.812	.	.913
INSTRUMENTAL_ACTIVITIES_OF_DAILY_LIVING_MEAL_PREPARATION	92.6000	902.041	.755	.	.913
INSTRUMENTAL_ACTIVITIES_OF_DAILY_LIVING_SHOPPING	93.0333	894.378	.829	.	.912
INSTRUMENTAL_ACTIVITIES_OF_DAILY_LIVING_LAUNDRY	92.7333	894.616	.769	.	.913
INSTRUMENTAL_ACTIVITIES_OF_DAILY_LIVING_TELEPHONE	93.1667	912.833	.835	.	.913
INSTRUMENTAL_ACTIVITIES_OF_DAILY_LIVING_TRANSPORTATION	93.1667	906.695	.894	.	.913
INSTRUMENTAL_ACTIVITIES_OF_DAILY_LIVING_MEDICATION_USE	93.2333	906.875	.805	.	.913
INSTRUMENTAL_ACTIVITIES_OF_DAILY_LIVING_BUDGETING	93.0667	898.133	.803	.	.913

more suitable Setubal and understandable for the Arabic speakers. The second stage, we distributed the modified Arabic version to 30 individuals from a representative elderly group. They were able to understand and answer all the questions with no difficulty and help.

This study considered to be the first study that validate FAMS to Arabic culture, which is one of the strength of this cross-sectional study. Another strength of this study, is that by now this version of FAMS is available to be used by all Arabic countries with different dialects. However, this study have some weakness. Although, the validation of FAMS into Arabic version has been completed, the sample size was small. Despite that we try to make it representative for older people living in Saudi Arabia in term of social-economic status, ethnicity, level of education, age, and health status. Thus, future research is needed to increase the sample size and better sampling technique in order to obtain valid information and to recheck our validity. The internal consistency for the study is 0.9 which consider a high consistency. In a study carried out by Desrosiers, et al., (1995) they used test re-test to measure the intraclass correlation reliability. While, in our study we used the Cronbach's alpha to measure the reliability, despite this the finding from previous study were similar to the present study (Desrosiers et al, 1995). In terms of ADL our present study was in agreement with Desrosiers, et al (1995) study. For the mobility part, present study was also in line with both Desrosiers et al. (1995) study and Desrosiers et al. (2004) study (Desrosiers et al, 1995; Desrosiers, et al, 2004).

However, for communication part, our study was in line with Desrosiers, et al. (1995); but was not in agreement with Desrosiers, et al (2004). The possible explanation could be because the 2004 study internal consistency for this part was different from our study which was range from 0.3 to 0.4 the same as the mental function. For IADL, our study In line with Desrosiers et al. (1995) study and Desrosiers et al. (2004) study (Desrosiers et al, 2004). By using Cronbach's alpha, the reliability and validity of the Arabic FAMS was measured. The internal consistency is used to measure the reliability. Therefore, we found that the Arabic version of FAMS is valid to be used in Arabic culture. However, further study to implement SMAF as measurement tools in Arab culture elderly will strengthen the tools and increase its reliability.

CONCLUSION

This research revealed that it is possible to validate western based rehabilitation tool into Arabic culture in order to use reliable measure. The new Arabic SMAF version is valid and reliable measure and culturally adapted tool to test ability of elderly people in Saudi Arabia and widely in Arab population. For the first time ever we introduced rehabilitation specialists with new tool appropriate for Arab culture and resources.

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Comparative analysis on the levels of some bioactive constituents of Asian and African garlic types

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ABSTRACT

The present study investigates the comparative levels of some bioactive constituents of the important plant *Allium sativum* (garlic). Comparisons have been made on the levels within three garlic types, one type from Asia (Chinese garlic), and two African garlic types from Sudan. Results showed that the garlic from South-western Sudan (*Zalenge* region) had higher levels concerning most of the constituents, particularly it contains 38.17±1.26 g/100g of carbohydrate, 2.53±0.45 g/100g fat, 110.00±2.45 mg/100g phosphorus, and 185.22 ±2.47 mg/100g calcium as compared to 29.00±1.00 g/100g, 1.23±0.25 g/100g, 90.22±1.66 mg/100g, and 176.60±1.20 mg/100g, respectively of the Northern Sudan garlic, and 23.97±1.06 g/100g, 0.57±0.21 g/100g, 99.11±3.72 mg/100g, and 148.21±3.28 mg/100g, respectively of the Chinese garlic. The variations were almost due to the enriched soil of that region of Sudan which is known as a source of many plant products, either cultivated or naturally grown. The justification for the uncertainty of results of allicin levels in garlic samples had been discussed.

KEY WORDS: *ALLIUM SATIVUM*, CHINESE, CONSTITUENTS, GARLIC, NORTHERN SUDAN, *ZALENGE*

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INTRODUCTION

The botanical name of garlic is (*Allium sativum* L., Amaryllidaceae) (Cruz and García 2007, Block 2010). Common garlic is classified as *Allium sativum*, British wild garlic as *Allium oleraceum*, and American wild garlic as *Allium candense* (Block 2010). The plant is likely to have originated from Central Asia, USA, Central America, Iran, and Egypt. It is now cultivated worldwide, but China provides 80% of the total world production (Cruz 2007). The garlic plant is one of the most popular herbs (traditional medicine) and spices (food flavoring agent) in the world, (Butt et al. 2009). There are more than 70 garlic varieties. Garlic is a bulb-shaped plant belongs to the onion family, it is close relative to the onion and resembles it in size and growth habit. The plant grows as a vegetable rosette close to the ground, (Block 2010, Renton 2013 and FAOSTAT 2017).

Garlic (*Allium satvium* L.) is an important vegetable crop in the Northern, Central, and Western Sudan (River Nile State and Darfur State) during the winter season, (October – March) (Ahmed et al. 1984, Nouria 1994, Mohammed Ali and El-Sayed 1999, Factfish website 2017). However, the production of garlic in Sudan is humble as compared to its enormous agricultural potential, it is ranked as the 22nd in the world, (Factfish website 2017). Sudanese variety of garlic is preferred for its strong odor and flavor compared to that imported from South Asian region, although the latter is bigger in size and easy in peeling.Usually, the garlic bulb (head) consists of (6 to 35) discrete bulblets called cloves. In *Allium sativum* there are 10–12 cloves per bulb and *Allium porrum* is a single clove type of garlic. Each garlic clove is made up of just one leaf base, unlike onions, which are composed of numerous leaf layers, (Shah and Qudry 1996 Muhammad Ibrahim et al. 2018).

The storage part of the garlic plant is the clove and not the leaves (Libner Nonnecke 1989). The characteristic pungent odor, the unique flavor, aroma, and biological effects of garlic are generally attributed to its organosulfur components (particularly allicin) (Block 2010, Shah and Qudry 1996), in addition to diallyl trisulfide (DATS) and diallyl disulfide (DADS) and derivatives which are released from garlic upon their processing (mincing, chewing and etc.) (Makoto et al. 2006 Block 2010).

The allicin generated from garlic is unstable so it quickly changes into a series of other sulfur-containing compounds such as diallyl disulfide (by the enzyme alliinase), therefore the garlic should be consumed immediately after crushing (Cavallito et al. 1944). Allicin has a very short half-life breaking down within 16 hours at 23 °C (Hahn 1996), and it is very volatile, so it takes a lot of garlic to gain those benefits (Lanzotti 2005). Harris et al., stated that allicin decomposes rapidly and some smaller metabolic breakdown products also exert strong antimicrobial effects (Harris et al 2001). Alliin is a derivative of the amino acid cysteine, and it is not present in garlic unless tissue damage occurs, (Iberl et al., 1990).

Alliin transforms to allicin on crushing the natural garlic cloves or bulb, so the commercial garlic preparations may contain no allicin. Allicin first reported by Cavallito and Bailey in 1944, and then Cavallito first noted its potent antimicrobial activity (Cavallito et al. 1944). Many researchers found that the allicin quantity depends on the cultivation region and harvest date, and fertilization especially with sulfur (Block 2010). But Baghalian reported no significant correlation between the ecological condition and the allicin content (Baghalian et al. 2005). Out of different garlic extracts, the "Aged Garlic Extract" (AGE) is the prominent one, with the highest antioxidant activity, even more than fresh garlic and other commercial garlic supplements. AGE contains phytochemicals (tannins, flavonoid, Saponin and glycoside) and also contains phenolic compounds, alkaloids, terpenoids and fatty acids (Butt et al. 2009, Shah and Qudry 1996). Crushed raw garlic is high in allicin, containing 37 mg/g. Allicin content found ranged from 0.16–13.0 mg/g in Iranian garlic ecotypes, measured by HPLC method (Baghalian et al. 2005).

Differences in the concentrations of organosulfur compounds in different garlic types may affect the medicinal properties of the garlic (Block 2010, Hassan Khalid et al. 2012, Huzaifa et al. 2014). In addition to these organosulfur compounds, fresh garlic is a source of numerous vitamins like vitamins B-6 and C, and minerals although their quantities may vary depending on the type of soil on which they are cultivated (Stephen & John 2000).

The long history of the medicinal use of garlic is well-documented. Since the time of Louis Pasteur (1858) and Lehmann (1930), garlic was used as an antibacterial agent, and till recent time studies were conducted on garlic as an inhibitor of bacterial growth (Alejandra et al. 2010). The properties of garlic against atherosclerosis, coronary thrombosis, myocardial infarction, and its inhibition of platelet aggregation and the proliferation of cancer cells had been reported (Lawson 1992). Traditionally garlic is known as an important antiseptic, and it has hypotensive, anthelmintic, chloretic and expectorant properties, it shows a hypoglycemic activity (Beretz and Cazenave 1991, Lawson, Ransom and Hughes 1992, Mostofa et al. 2007). It is used to treat intestinal infection (Cavallito et al. 1944), and treats ailments like diarrhea, headache, and dysentery and showed to have antifungal (Saha and Bandyopadhyay 2017), antibacterial, antiviral, antiprotozoal, and antifungal activity. Garlic inhibits the growth of both bacteria and fungi (Saha and Bandyopadhyay 2017, Emad Mohamed Abdallah 2017).

It has antioxidant properties, increases the levels of antioxidant enzymes of the liver, so reduce inflammation (Sha Li et al 2015, Hassan Khalid 2012). Garlic can help to chelate heavy metals and promote their excretion by the body (Ashraf Nasr 2014). The effectiveness of garlic may be prevention rather than therapy, thus it may need long-term supplementation (Leyla Bayan et al 2014). The mechanism by which allicin treats infections in people seems to be unclear. This study compares the levels of some bioactive constituents of three types of garlic, Chinese garlic (the most produced Asian garlic), and the Sudanese garlic (African garlic type) which had been represented by two types, one from Northern Sudan, and the second type is from South-western Sudan, a region called *Zalengei* located in Darfur State. This last garlic type would be mentioned as "*Zalengei* garlic" throughout this study, as it is a common local name in Sudan.

MATERIAL AND METHODS

Samples of three types of garlic were collected, two types were from Sudan (One from Northern Sudan and the other from South-western Sudan - *Zalengie* -), the third type was a Chinese garlic purchased from Omdurman local market. These types had been confirmed and authenticated by a collective agreement of specialized Doctors and technicians of the concerned laboratories and herbarium section, in addition to the collective opinions of the importing and exporting experts working in this field.A series of laboratory experiments were undertaken at the College of Agricultural Studies, Sudan University of Science and Technology at Shambat. Garlic fruits were then crushed using grinding machine.

Extraction method: 100 grams of each sample were weighted into 1000ml conical flask using sensitive balance; Samples extracted using of Ethanol 70% (500 ml) in a shaker 200 rpm for 24h at room temperature and subsequently filtrated under suction (reduce pressure using vacuum pump), extract samples were transferred to ice form using (-20C˚) refrigerator and dried using freeze dryer machine (-50 C˚ for 48 h). Dry samples were collected and kept in vials till used.

The **moisture** content was determined according to of the Association of Official Analytical Chemists (AOAC, 2008). Two grams were weighed into a pre-dried and tarred dish. Then, the sample was placed into an oven (No.03-822, FN 400, Turkey) at 105 °C±1 °C until a constant weight was obtained. After drying, the covered sample was transferred to a desiccator and cooled to room temperature before reweighing. Triplicate results were obtained for each sample and the mean value was reported according to the following formula;

Calculation:

$$\text{Moisture content (\%)} = \frac{W1 - W2}{Wt1} \times 100$$

Where;
W1= Sample weight before drying
W2 =Sample weight after drying
Wt1=initial sample weight

The **ash** content was determined according to the method described by Pearson (1981). Five grams were weighed into a pre-heated, cooled, weighed and tarred porcelain crucible and placed into a Muffle furnace (No.20. 301870, Carbolite, England) at 550 to 600 °C until a white-gray ash was obtained. The crucible was transferred to a desiccator then allowed to cool to room temperature and weighed. After that, the ash content was calculated as a percentage based on the initial weight of the sample.

Calculation:

$$\text{Ash \%} = \frac{[(\text{Wt of crucible} + \text{Ash}) - (\text{Wt of empty crucible}] \times 100}{\text{Initial weight (Wt)}}$$

Crude fiber content was determined according to the official method of the AOAC (2008). Two gram of a defatted sample was placed into a conical flask containing 20m1 of H_2SO_4 (0.26 N). condensed and allowed to boil for 30 minutes. the digest was filtered (under vacuum). rinsed and boiled in 20 ml NaOH (0.23 N) solution for 30 min under reflux condenser and the precipitate was filtered, rinsed with hot distilled water, 20m1 ethyl alcohol (96%) and 20 ml diethyl ether. Finally, the crucible was dried at 105 °C (overnight) to a constant weight, cooled (in a desiccator), weighed, ashed in a Muffle furnace (No.20. 301870, Carbolite, England) at 550-600 °C until a constant weight was obtained and the difference in weight was considered as crude fiber.

Calculation:

$$\text{Crude \%} = \frac{[\text{Dry residue crucible Cal}] - [\text{ignited residue} + \text{crucible (g)}] \times 100}{\text{Sample weight}}$$

The **protein** content was determined in all samples by micro-Kjeldahl method using a copper sulphate-sodium sulphate catalyst according to the official method of the AOAC (2008). Two gram sample was transferred together with 4g Na_2SO_4 of Kjeldahl catalysts (No. 0665, Scharlau chemie, Spain) and 25m1 of concentrated sulphuric acid (No. 0548111, HDWIC, India) into a Kjeldahl digestion flask. After that, the flask was placed into a Kjeldahl digestion unit (No. 4071477, type KI 26, Gerhardt, Germany) for about 2 hours until a colourless digest was obtained and the flask was left to Cool to room temperature. The distillation of ammonia was carried out into

25m1 boric acid (2%) by using 20m1 distilled. water and 70m1 sodium hydroxide solution (45%). Finally, the distillate was titrated with standard solution of HCI (0.1N) in the presence of 2-3 drops of bromocresol green and methyl red as an indicator until a brown reddish colour was observed.

Calculation:

$$\text{Nitrogen\%} = \frac{\text{Titre volume x HC1 (N) x Nitrogen equivalent weight x 100}}{\text{Sample weight x 1000}}$$

Crude protein% = Nitrogen% x Protein conversion factor (6.25)

Total and available **carbohydrates** were calculated by difference according to the following equations:

Total carbohydrates = 100 - (Moisture + Protein + Fat + Ash)

Available carbohydrates = Total carbohydrates – Crude fiber.

Fat content was determined according to the official method of AOAC (2008).

Samples had been put in extraction thimble , then about 100 ml hexane were attached to the extraction unit (Electrothermal, England), after 16 hr extraction process , the solvent was redistilled, put in an oven at 105 . for 3 hr, cooled in a desiccator, reweighed and the dried extract was registered as fat content according to the following formula;

Calculation:

$$\text{Fat content (\%)} = \frac{(W2 - W1)}{W3} \times 100$$

Where;

W2 =Weight of the flask and ether extract

W1 =Weight of the empty flask

W3=initial weight of the sample

To analyze the minerals content, Samples were placed into a muffle furnace (No.20. 301870, Carbolite, England) at 550 to 600 °C. the ash content was cooled and 10 ml of HCI (2.0N) was added. the concentrations of Minerals were determined using, Atomic Absorption Spectrophotometer (3110-Perkin Elmer. USA).The investigation of alliin and allicin (thiosulfinates) concentrations in garlic bulbs were determined using HPLC methods according to Iberl (1990).

RESULTS AND DISCUSSION

The physical appearance and the size of bulbs (and cloves) variations between the tested three samples were shown in **(Fig 1).**

Apparently and physically, the Zalenge garlic has more flavor, stronger taste, and it has smaller blub and cloves and darker color than both the Northern-Sudan garlic and the Chinese garlic **(Fig 2).**

Regardless of these results, the flavor is very subjective and personal, and there is not truly accurate scientific measurement for it. *Zalenge* garlic is denser and heavier (more solid). It is rarely found in the national markets because of its low production in that region of the Sudan which is suffering under national war and social conflicts.

Our findings of the measured parameters of the three garlic types, North-Sudan garlic, *Zalenge* garlic, and Chinese garlic, were shown in **(Table1)**.

The low moisture content of *Zalenge* garlic (40.97%) compared to the other garlic samples and other garlic types reported by previous studies may lead to higher concentrations of the garlic clove constituents, i.e. create more dense clove. The two Sudanese garlic types (South-Sudan, and *Zalenge*) had an almost similar protein content (6.50g/100g, and 6.73g/100g, respectively), but *Zalenge* garlic had a higher carbohydrate content (38.17g/100g), and fat content (2.53g/100g). The levels of the phosphorus and calcium were higher in the *Zalenge* garlic (110.00 mg/100g, 185.22 mg/100g, res.) than levels we found in North-Sudan garlic, but they were simi-

FIGURE 1. Appearance and size of the studied garlic types [The largest: Chinese garlic; The medium: Northern Sudan garlic; The smallest: *Zalenge* garlic]

FIGURE 2. Appearance and size of the bulb (left) and cloves (right) of *Zalenge* garlic

Table 1. Levels of some parameters of the North-Sudan garlic, *Zalenge* garlic, and Chinese garlic

parameter	Results of Samples*		
	Chinese garlic	North- Sudan garlic	Zalenge garlic
Moisture%	55.57 ± 0.21	50.30 ± 0.44	40.97 ± 0.21
pH	6.05 ± 0.06	5.13 ± 0.12	5.83 ± 0.06
Crude ash%	4.50 ± 0.06	4.53 ± 0.3	3.77 ± 0.25
Crude fibre g/100g	2.04 ± 0.06	4.03 ± 0.35	4.03 ± 0.45
Crude protein g/100g	5.00 ± 0.60	6.50 ± 0.40	6.73 ± 0.50
Carbohydrate g/100g	23.97 ± 1.06	29.00 ± 1.00	38.17 ± 1.26
Fat g/100g	0.57 ± 0.21	1.23 ± 0.25	2.53 ± 0.45
Phosphorus mg/100g	99.11 ± 3.72	90.22 ± 1.66	110.00 ± 2.45
Calcium mg/100g	148.21 ±3.28	176.60 ±1.20	185.22 ± 2.47

*Mean ± SD of triplicate trials (n=3) on dry weight (DW) basis.

lar to, or lower than levels reported by some previous studies, Rossella et al. (2016) reported 180mg/100g of Ca, besides other studies reported higher Ca levels. A notable variation was observed in the concentration of the mineral Ca between the two Sudanese samples from one hand and the Chinese garlic from the other hand. The amount of trace minerals contained in garlic is a function of their presence in the soil on which they were grown (Stephen & John 2000).

We couldn't obtain precise and certain values of thiosulfinates in the different trials of the three garlic types. The non-precise thiosulfinates results may be due not only to the technical uncertainty of our laboratories and the unavailability of the pure standards, but may also be due to the unstable nature of the allicin compound, that (thiosulfinates) were converted giving rise to a wide variety of derived sulfur compounds (Lanzotti 2005). The allicin content of the Iranian garlic ecotypes ranged from 0.16–13.0 mg/g (Baghalian et al. 2000). Stephen & John (2000) reported that allicin content of California garlic is higher than in the Chinese garlic (Alejandra 2010).

Although Eugeniusz (2007) couldn't detect allicin or allin in powdered garlic, he found 11.12 of allin, and 4.91 of allicin in the raw garlic bulbs. In contrast, Miron (2004) found a level of 10g/kg alliin at most in powdered garlic. Others reported a range of 2.5 - 5.1mg/g as a total allicin-yield of fresh, crushed garlic, but Miron found higher allicin levels at 37mg/g in crushed raw garlic. Iberl (1990) stated that, upon crushing, allin quantity is about double of the allicin of the raw garlic bulb.Although our study preferred to neglect to report these uncertain results, we expect the existence of a high total allicin-yield of *Zalenge* garlic more than other garlic types because *Zalenge* garlic had higher contents of most of the natural constituents, higher density, flavor, and stronger taste.These variations were explained by the difference in the genetic variations of the garlic types, differences in the environmental conditions and soil ecology.

CONCLUSION

Results revealed the variations on the levels of some active constituents of the three garlic tested types. Particularly, *Zalenge* garlic (grown in South-Western Sudan) was heavier, denser, and had more flavor than Chinese garlic which had bigger bulbs and easily peeled. *Zalenge* garlic contains higher levels of carbohydrate, fat, phosphorus, and calcium than the imported Chinese garlic, these higher concentrations may improve the taste and flavor of *Zalenge* garlic. Determination of physicochemical properties is a necessary step to identify and obtain data on the nutritional value of each garlic type. This study remains unfortunately insufficient for characterization of different African garlic types, which need to be completed by a further quantitative analysis. The chemistry behind garlic health-promoting effects was not fully understood, and the safety of allicin to treat infections in people was still unclear.

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Standardisation of recipe for batter and breaded product Acetes cutlet from Jawala, *Acetes indicus*

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ABSTRACT

Acetes is a small sized shrimp, locally known as 'Jawala' or 'Kolim' is landed along the north-west coast in the states of Gujarat and Maharashtra, India. At present the fresh utilization of Acetes is very limited. One of the most important food product is battered and bearded product. A standardized recipe of batter and breaded Acetes product is mentioned in the paper. Acetes cutlet having a standard size of 2.0-3.5 cm in length cm diameter, 1 cm thickness and 0.2-0.5 g weight Acetes cutlets were standardized by using different ingredients viz., cooked potatoes, salt, green chilly (C) and ginger (G). Prepared of cutlets were standardized by organoleptic evaluation. It was found that, Acetes cutlet prepared with the ratio of 75:100 (w/w), 20:100 (w/w), 5.5:100 (w/w), 5.5:100 (w/w) and 2.5:100 (w/w) for potato: Acetes mince, onion: Acetes mince, green chilly: Acetes mince, ginger: Acetes mince and table salt: Acetes mince respectively, ratios showed highest score as compared to other ratios tried in the experiments.

KEY WORDS: ACETES, BATTER AND BREADED PRODUCT, INGREDIENTS, SENSORY EVALUATION, VALUE ADDED PRODUCTS

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INTRODUCTION

Acetes is widely distributed in different parts of the world with dominance along the coast of many countries bordering the Indian Ocean. Acetes is a small sized shrimp, belongs to family Sergestidae, genus Acetes and scientifically named as *Acetes indicus*. In India, a substantial quantity of this group locally known as 'Jawala' or 'Kolim' is landed along the north-west coast in the states of Gujarat and Maharashtra (Zynudheen, 2004; Mahakal *et al.,* 2016). At present the fresh utilization of Acetes is very limited and people locally people consume Jawala or Kolim regularly either in fresh or dried form in the preparation of day-to-day food dishes. Acetes is consumed mostly in dried form due to its availabity throught out year in market. But consumption of dry Acetes is more during non-fishing season when fresh fish is not available (Zynudheen *et al.,* 2004; Mahakal *et al.,* 2016; Shaikh *et al.,* 2017).

Now a days consumer's particularly urban area are showing more and more interest in seafood products which are available in ready-to-eat or ready-to-cook in convenience form. Recent scenario peoples both man and women are engaged in jobs and they hardly find time to cook food. This leads to reasonably increase in their income and they become more depend on ready-to-eat, ready-to-cook and ready-to-fry types of products available in market (Balachandran, 2001; Pagarkar *et al.,* 2011). One of the most important foods in ready-to-eat and ready-to-cook group is battered and breaded products. The process of coating with batter and bread crumbs increases the bulk of the product, thereby reducing the content of costly fish (Pagarkar *et al.,* 2012). Keeping this in view, the present research work was undertaken to develop a battered and breaded product from low cost shrimp locally called as Jawala or Kolim (*Acetes indicus*).

MATERIAL AND METHODS

Acetes (*Acetes indicus*) procured from fish market of Alibaug, district Raigad of Maharashtra coast of India, was brought to processing hall under iced condition. Acetes were washed with potable water, cleaned and cooked in boiled water for 15 min., the cooked Acetes mince was prepared using a grinder. Prepared Acetes mince was placed in polythene bags then it was frozen and stored in deep freezer at a - 20°C temperature until further use. As and when required, Acetes mince was taken out and thawed before use. Cooked potatoes were peeled and mashed and made into a fine paste. Chopped onions were fried in sunflower oil till they became brown. Bread crumbs were used as a coating material.

Standardization in the basic recipe (Pawar, *et al.,* 2012) as shown in Table 1 was used by varying the ratios of different ingredients. Firstly the Acetes mince was mixed with boiled potatoes, turmeric powder and salt, a mixture of these was kept aside, then chopped onions were fried in oil until brown in colour then mixed with green chilly, ginger and garlic paste followed by the powdered spices (Turmeric powder, pepper powder, cinnamon powder and clove powder) then mixing of mince base with the fried spices and cooked. Then cutlets of 40 g were taken and flattened to 1cm thickness, dipped into batter mix (Pawar, 2011) rolled over bread crumbs and fried in sunflower oil till they became brown in colour then were subjected to sensory evaluation.

Acetes cutlet were prepared keeping the constant level of oil 10 ml (w/w), powdered spices (Turmeric powder 0.2g (w/w), pepper powder 0.3g (w/w), cinnamon powder 0.2g (w/w), clove powder 0.3g (w/w)] and bread crumbs 20g (w/w) while varying quantities of cooked potatoes, onion, green chilly (C), ginger (G), and table salt, were used for standardising the recipe. The fried cutlet were subjected to organoleptic evaluation by a group of ten trained panellist using 9 point hedonic scale (ISI, 1975) on the attributes such as colour, taste, texture, odour and overall acceptability. Potato was standardised in basic recipe and Acetes cutlet was prepared with incorporation of different ratios of potato to Acetes mince such as 60:100 (T1), 65:100 (T2), 70:100 (T3), 75:100 (T4) and 80:100 (T5) (w/w). All other ingredients were kept constant. Organoleptic evaluations were carried out to find best ratio.

Onion was standardised in basic recipe and Acetes cutlets were prepared keeping the standard ratio of potato obtained from its standardisation 75:100 (T4), with different ratios of onion to Acetes mince, such as 15:100 (T1), 20:100 (T2), 25:100 (T3), 30:100 (T4) (w/w) and 35:100 (T5) (w/w). All other ingredients were kept constant and prepared Acetes cutlets were organolpetically evaluated for the best combination. Raios of green chilly (C) was standardised in basic recipe and Acetes cutlet was prepared keeping standard ratio of potato 75:100 (T4) and onion 20:100 (T2) with different ratios of green chilly (C) to Acetes mince such as 4:100 (T1), 4.5:100 (T2), 5:100 (T3), 5.5:100 (T4) (w/w) and 6.0:100 (T5). All other ingredients were kept constant. Prepared cutlets were organoleptically evaluated for the best combination.

Ratio of ginger (G) was standardised in basic recipe and Acetes cutlets were prepared keeping standard ratio of potato 75:100 (T4), onion 20:100 (T2) and 5.5:100 (T4) with different ratios of ginger (G) to Acetes mince such as 4:100 (T1), 4.5:100 (T2), 5:100 (T3), 5.5:100 (T4) (w/w) and 6.0:100 (T5). All other ingredients were kept constant. Prepared cutlets were organoleptically evaluated for the best combination.Table salt was standardised as basic recipe and Acetes cutlet was prepared keeping the standard ratio of green chilly and ginger obtained from its standardisation 5.5:100 (T4), with different ratios of salt to Acetes

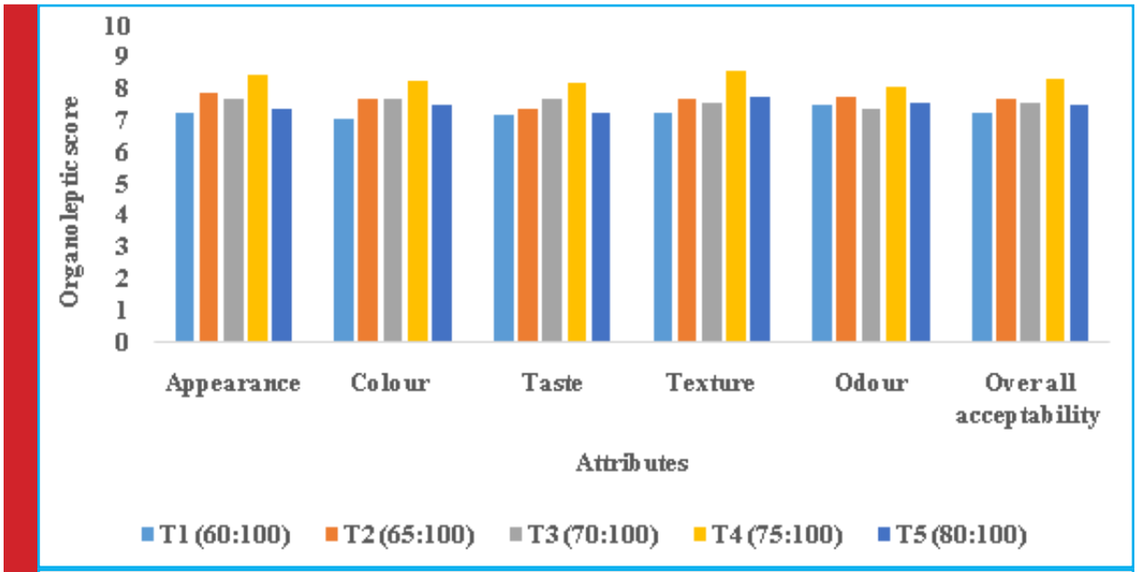

FIGURE 1. Organoleptic evaluation of Acetescutlet prepared with different ratios of potato

mince, such as 2:100 (T1), 2.5:100 (T2), 3:100 (T3) and 3.5:100 (T4) (w/w) and 4:100 (T5). All other ingredients were kept constant and prepared cutlets were organoleptically evaluated for the best combination.

Finally, Acetes cutlets were prepared using standardised recipe (Table 1) keeping the standardised ratios of potato 75:100 (T4), onion 20:100 (T2), ratios of green chilly 5.5:100 (T4), ginger 5.5:100 (T4) and salt 2.5:100 (T2). Proximate composition viz. moisture, crude protein, crude fat, ash and carbohydrate (CHO) content of Acetes cutlets were determined following standard methods (AOAC, 2005).

RESULTS AND DISCUSSION

Standardisation of different ingredients viz., potato 75:100 (T4), onion 20:100 (T2), chilly 5.5:100 (T4), ginger 5.5:100 (T4) and table salt 2.5:100 (T2), showed better organoleptic values compared to other ratios (Fig. l, Fig. 2, Fig. 3, Fig. 4 and Fig. 5). The organoleptic evalua-

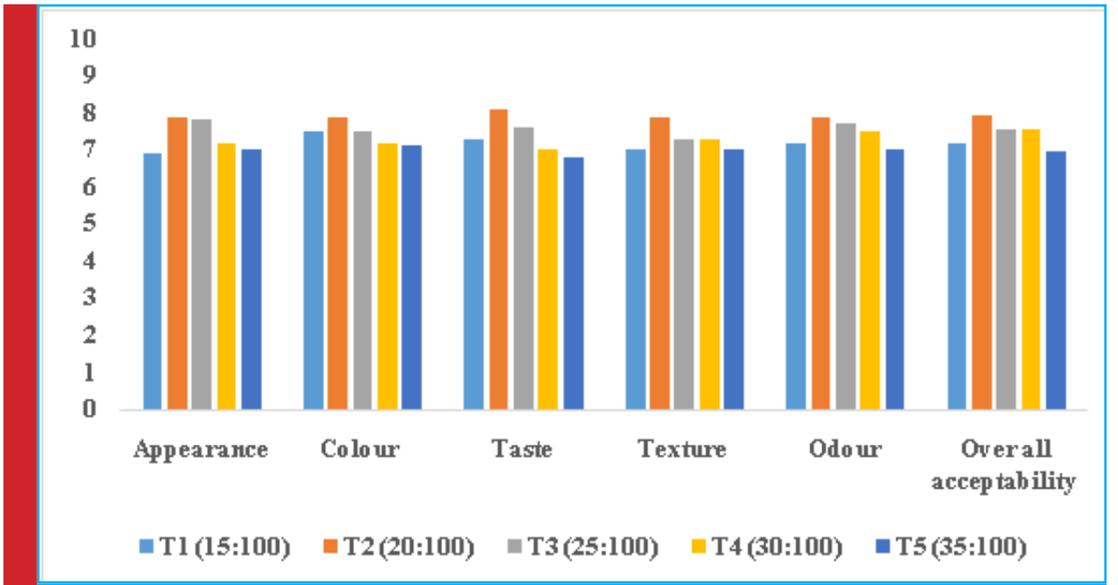

FIGURE 2. Organoleptic evaluation of Acetes cutlet prepared with different ratios of onion

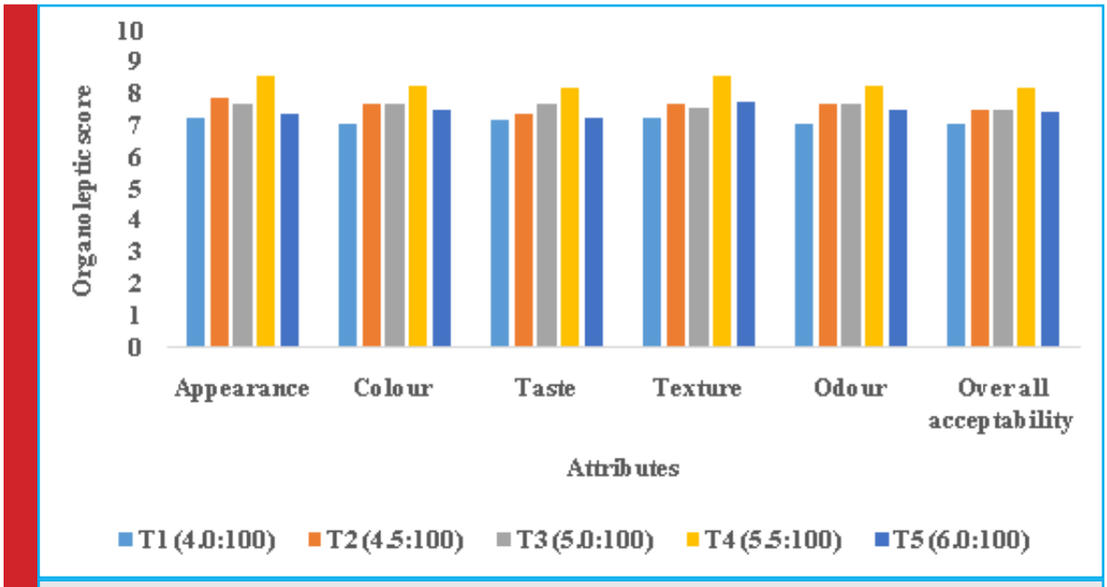

FIGURE 3. Organoleptic evaluation of Acetes cutlet prepared with different ratios of green chilly

tion of the cutlets indicated the highest score this standardised recipe as shown in Table 2 and Fig.6.

Standardization of different ratios of potato to Acetes mince in Acetes cutlet

Acetes cutlet prepared with 75:100 (w/w) ratio of potato to Acetes meat was superior as compared to the other ratios of potato to Acetes mince used for preparation of cutlets. Joseph and Perigreen (1989) used 50:100 ratio of potato in the cutlet prepared from horse mackerel, ribbon fish, pola, vatta and mackerel tuna. Kamat (1999) used 50:100 ratio of potato in cutlet prepared from mackerel (*Rastrelliger kanagurta*). Pawar *et al.* (2012) used 70:100 ratio of potato in the cutlet prepared from catla. Rathod *et al.* (2012) used 70:100 ratio of potato in the cutlet prepared from Pangassius (*Pangasianodon hypothalamus*). Species differentiation and higher ratio of potato to mince increased more starch and taste in the cutlet

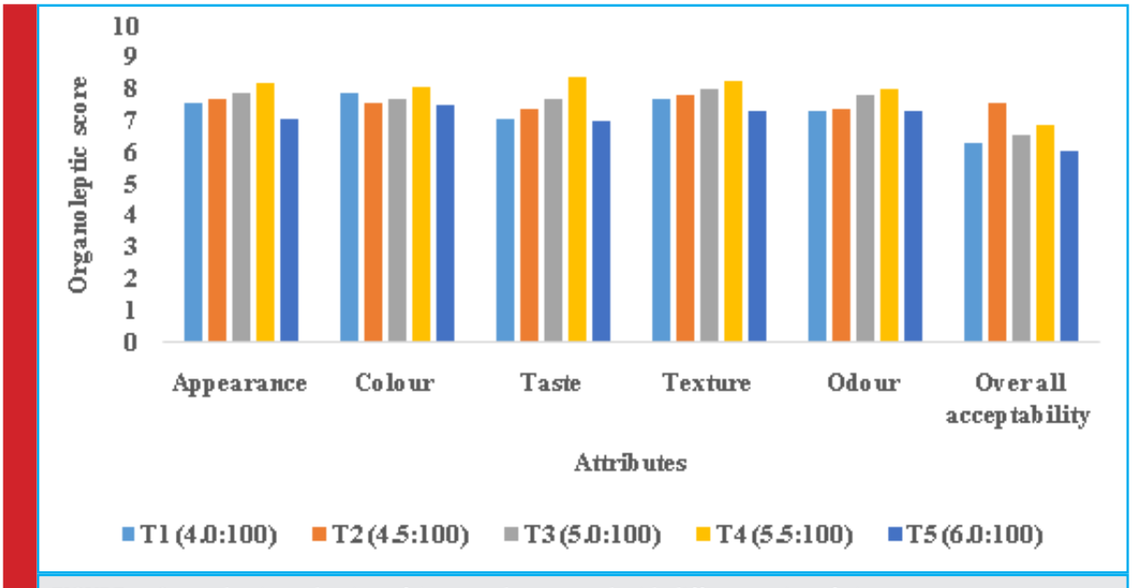

FIGURE 4. Organoleptic evaluation of Acetescutlet prepared with different ratios of ginger

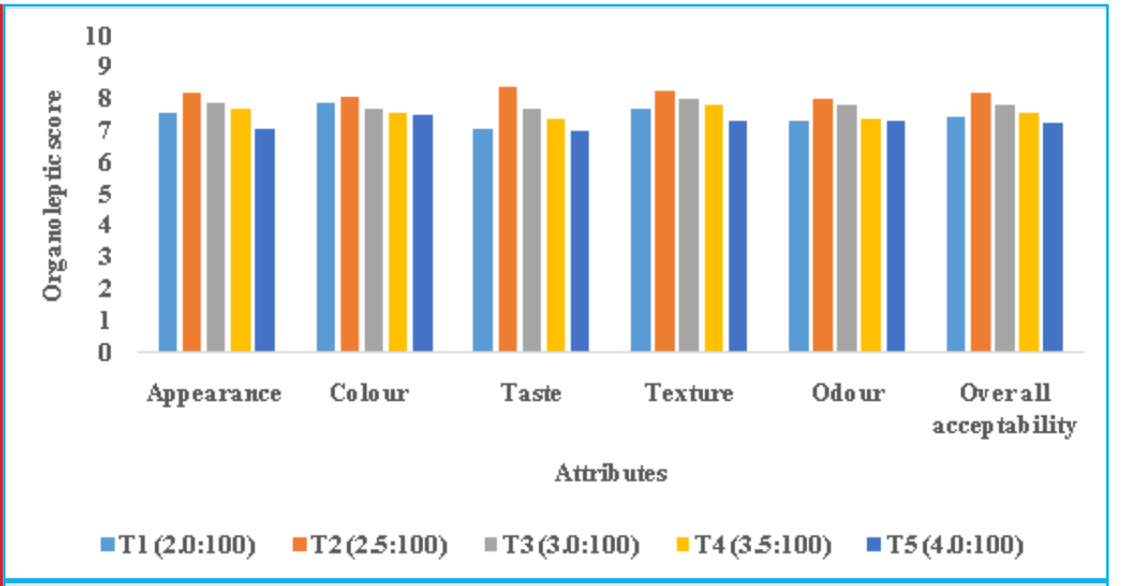

FIGURE 5. Organoleptic evaluation of Acetes cutlet prepared with different ratios of table salt

and bind all ingredients properly. Higher potato ratio reduces strong odour of Acetes as well as cost of the cutlets. Similar results with slight variation were found by Joseph, Perigreen (1989), Kamat (1999), Pawar *et al.* (2012) and Rathod *et al.* (2012).

Standardization of different ratios of onion to Acetes mince in Acetes cutlet

Acetes cutlets were prepared with 20:100 (w/w) ratio of onion to Acetes meat was superior as compared to the other ratios of onion to Acetes meat used for preparation of cutlet. Kamat (1999) used 25:100 ratio of onion in cutlet prepared from mackerel (*R. kanagurta*). Pawar *et al.* (2012) used 25:100 (w/w) ratio of onion to catla (*catla*) meat. Rathod *et al.* (2012) used 25:100 ratio of onion in the cutlet prepared from Pangassius (*P. hypothalamus*). Species differentiation may cause difference in the ratio of onion to meat. In the present, study as per the opinion of panellist, the higher ratio of onion gave a very excellent taste to the cutlets. Therefore, the

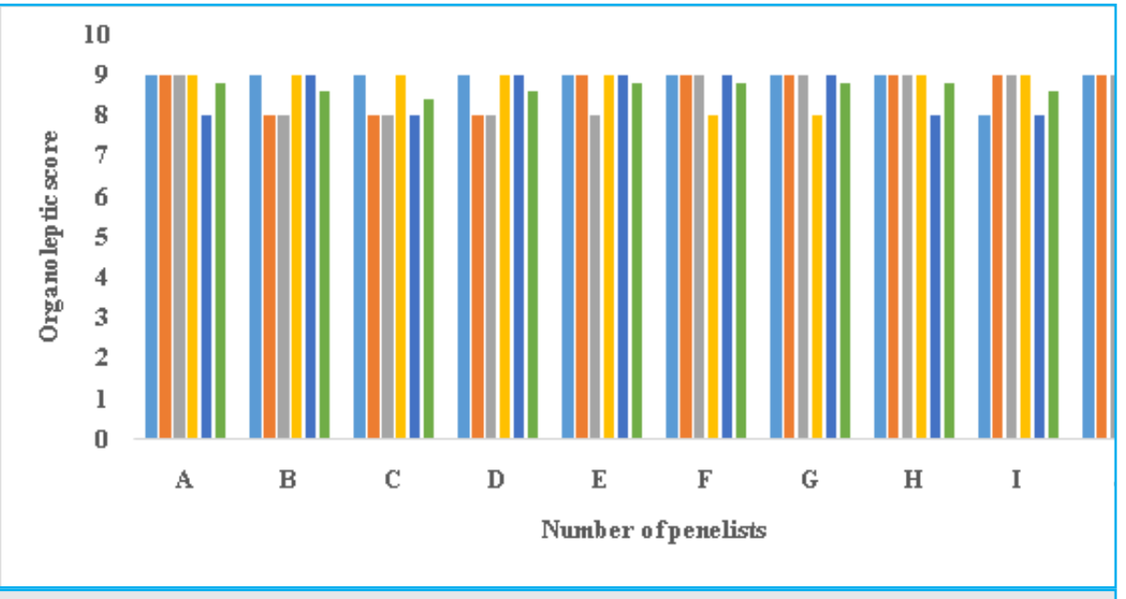

FIGURE 6. Organoleptic evaluation of Acetes cutlet prepared using standardized recipe

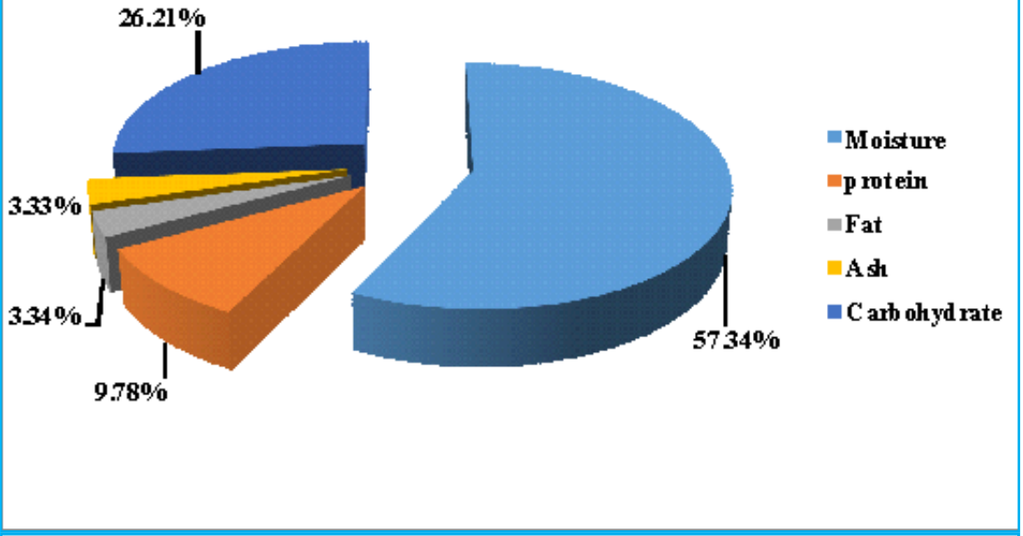

FIGURE 7. Proximate composition of Acetes cutlet

ratio of 20:100 (w/w) onion to meat was used for better taste.

Standardization of different ratios of green chilly to Acetes mince in Acetes cutlet

Acetes cutlet prepared with 5.5:100 (w/w) ratio of green chilly to Acetes meat was superior as compared to the other ratios of green chilly to Acetes mince used for preparation of cutlet. Kamat (1999) used 6.5:100 ratio of green chilly in cutlet prepared from mackerel (*R. kanagurta*). Pawar *et al.* (2012) used 5:100 ratio of green chilly in cutlet prepared from catla and similar Rathod *et al.* (2012) used 5:100 ratio of green chilly in cutlet prepared from Pangassius (*P. hypothalamus*) fish. In the present study as per the opinion of panellist, the higher ratio of green chilly was giving a very spicy taste of cutlet. Therefore, the ratio of 5.5:100 (w/w) green chilly to meat was used for better taste.

Table 1. Basic and standardized recipe of batter and breaded Acetes cutlet

Ingredients	Basic recipe (Pawar *et al.*, 2012) Quantity (g)	Standardized recipe Quantity (g)
Acetes mince	100	100
Table salt	3	2.5
Green chilly	5	5.5
Coriander leaves	5	5
Ginger	5	5.5
Garlic	5	5
Onion	25	20
Potato cooked	70	75
Pepper powder	0.3	0.3
Clove powder	0.3	0.3
Cinnamon powder	0.2	0.2
Turmeric powder	0.2	0.2
Bread powder	20	20
Note: Oil was used 10 ml for heating purpose		

Standardization of different ratios of ginger to Acetes mince in Acetes cutlet

Acetes cutlets were prepared with 5.5:100 (w/w) ratio of ginger to Acetes meat was superior as compared to the other ratios of ginger to Acetes meat was used for prepa-

Table 2. Average organoleptic score of Acetes cutlet prepared using standardized recipe

Attribute	Organoleptic Score
Appearance	8.90±0.32
Colour	8.70±0.48
Taste	8.60±0.52
Texture	8.70±0.48
Odour	8.60±0.52
Overall Acceptability	8.70±0.14

[1]Where number of panellist is 10 (n=10)
[2]Values expressed are means + S.D. of triplicate measurements

ration of the cutlet. Kamat (1999) used 7.5:100 ratio of ginger in cutlet prepared from mackerel (*R. kanagurta*). Pawar (2011) used 5:100 (w/w) ratio of ginger to catla (*catla*) meat. Rathod *et al.* (2012) used 6:100 ratio of ginger in cutlet prepared from Pangassius (*P. hypothalamus*). Species segregation may cause difference in the ratio of ginger to meat. In the present study as per the opinion of panellist, the higher ratio of ginger made the cutlets little spicy. Therefore, the ratio of 6:100 (w/w) ginger to meat were used for better taste.

Standardization of different ratios of table salt to Acetes mince in Acetes cutlet

Acetes cutlets were prepared with 2.5:100 (w/w) ratio of table salt to Acetes meat was superior as compared to the other ratios of salt to Acetes meat used for preparation of cutlet.

Similar results were observed by Joseph *et al.* (1984) they used 3:100 ratio of salt in the cutlet prepared from lizard fish, threadfin bream, jew fish and miscellaneous fish (comprising mainly soles, caranx, jew fish, threadfin bream and glassy perch). Ninan *et al.* (2008) used 3:100 ratio of salt in cutlet prepared from tilapia (*O. mossambicus*). Pawar *et al.* (2012) used 3:100 ratio of salt in the cutlet prepared from catla (*Catla catla*). Rathod *et al.* (2012) used 2.5:100 ratio of salt in cutlet prepared from Pangassius (*P. hypothalamus*). Species differentiation may cause difference in the ratio of salt to meat. In the present study as per the opinion of panellist, the higher ratio of salt was giving a more salty taste to the cutlet. Therefore, the ratio of 2.5:100 (w/w) salt to meat was used for better taste.

The proximate composition of Acetes cutlet showed moisture 57.34 %, crude protein 9.78 %, fat 3.34 % and ash 3.33 % and carbohydrate 26.21% (Fig 7). The increase in fat and reduction in moisture content in cutlet is due to deep frying with dehydration during chilled storage (Ninan *et al.* 2008 and Pawar *et al.* 2012). The reduction of protein is due to denaturation fish muscle during chilled and frozen storage (Gopakumar, 2002; Pawar *et. al.,* 2013; Rathod *et al.,* 2014). Joseph *et al.* (1984) reported moisture, protein, fat and ash content in flash fried cutlet was 62.65, 15.41, 5.92 and 1.88% respectively. Crab cutlet prepared by Raju *et al.* (1997) content and may found of moisture, protein, fat and ash were 67.72, 17.07, 8.36 and 4.00% respectively. Kamat (1999) reported fish cutlet prepared from bleached and unbleached fish meat content of moisture, protein, fat and ash were 65.01, 12.06, 6.31 and 1.39 %. Ninan *et al.* (2008) reported tilapia fish cutlet content of moisture, protein, carbohydrate, fat and ash were 65.10, 17.51, 13.47, 2.14 %. Pawar *et al.* (2012) reported initial moisture, protein, fat and ash content in flash fried cutlets was 65.71, 16.57, 14.50 and 3.22% respectively. Rathod *et al.* (2014) reported moisture, protein, fat, ash and carbohydrate content in fresh flash fried cutlets was 53.34, 18.43, 21.02, 2.78 and 4.43 % respectively. Praneetha *et al.* (2017) reported the moisture, protein, fat and ash content in fresh flash fried cutlets was 57.03, 21.74, 7.61, 3.41 % respectively.

CONCLUSION

Acetes can be utilized by preparing Acetes batter and breaded product like Acetes cutlet using standardized recipe. This standardized recipe showed excellent organoleptic characteristics. This developed technology will be useful to prepare nutritive products from Acetes as well as helpful to increase the income of fisherman, as well as SHG'S etc.

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Characterization and continuous production of ethanol using immobilized yeast cells

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ABSTRACT

In this study, characterization of the immobilized yeast cells for ethanol production by varying the initial sugar and yeast cell concentration was performed. The results have indicated that the effective fermentation can be achieved using 1% of the immobilized yeast cells with 10-12% of initial total reducing sugar concentration. The role of plant and fungal based chemicals as activators were studied and the studies indicated that *Chitin and Rhizopus Oryza* biomass has shown significant effects of increase in fermentation rate (47% and 23.94%) in free and immobilized cell –activator systems respectively. Continuous ethanol production studies with immobilized yeast cells suggested that the productivity can be improved up to 100% by reducing retention time. Also, these studies confirmed the reusability of beads for up to 16 days without losing activity.

KEY WORDS: ETHANOL FERMENTATION, MOLASSES, IMMOBILIZATION, ACTIVATORS, REDUCING SUGAR

INTRODUCTION

The gradual depletion and the environmental deterioration resulting from the over consumption of petroleum derived transportation fuels have gained great attention across the world. Hence, it is necessarily important to develop alternatives that are both renewable and environmentally friendly. Ethanol is one of the most exotic synthetic oxygen-containing organic chemicals. It has unique properties as a fuel, a solvent, a beverage, a germicide, an antifreeze that reduces greenhouse gas (GHG) emission, lessens the dependence on fossil fuels, and improves vehicle performance. It is generally manufactured by three types of agricultural raw materials viz., saccharine materials, starchy materials and lignocellulosic biomass using bacteria and yeast. Fermentation using yeast and sugarcane molasses is one of the widely used and ancient fermentation systems.

Despite the great advantages and importance, fuel ethanol is not competing to cheaper oil derivatives

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because of its high production costs due to low productivity. Hence, attempts cells, immobilization of whole cells, vacuum fermentation and optimization of operating parameters to reduce production costs. Several studies show that ethanol productivity can be increased by the addition of ergosterol (Patil, 1985, Kanellaki 1989, Patil 1990, Jozef 2003, Giuliano, 2004, Kara. 2005) chitin and polysaccharides (Neetu 2006), mineral kissiris skim powder and fungal mycelium

Different working parameter condition shows the optimal condition of initial sugar concentration, pH, temperature, dilution rate and bead size on maximum production of ethanol (Bardi,1994). Using continuous immobilized yeast fermentation, microalgal hydrolysate was converted into ethanol at a yield of 89% (Kyoung 2014) conversion of biomass into ethanol varies considerably depending on the nature of feedstock primarily due to the variation in biochemical composition, and so, only a few feedstocks have been exploited commercially (Zabed 2017).

In the present study, the effect of initial sugar concentration, yeast cell concentration on an immobilized cell system and the effect of different activators (saw-dust, chitin and *Rhizopus-Oryzae*) on free and immobilized cell system is studied. Continuous production of Ethanol was done to study the effect of flow rate and the reusability study. The reduction of bead size increased mass transfer of substrates from the liquid to the immobilized cells accelerating sugar consumption and ethanol production (2018). Fermentation of sugar for production of ethanol was carried out using *Saccharomyces cerevisiae* cells immobilized in calcium alginate films, (Leal 2018).

MATERIALS AND METHODS

MICROORGANISM

Commercially available fresh baker's yeast was used for experimentation. The culture was obtained as a cake. The same was stored under 0-4^{0}C temperature till it was further used.

PREPARATION OF IMMOBILIZED YEAST BEADS

About 4g of sodium alginate was weighed and dissolved in 100mL of boiling distilled water and made slurry. In another beaker 8g of fresh baker's yeast was dissolved in distilled water. The two solutions were mixed and made slurry. The final volume was adjusted to 200mL with distilled water. The slurry was extruded drop wise into 0.2M $CaCl_2$ solution with the help of a micro-tip connected to silicone tubing, which passes through peristaltic pump. The beads were left in calcium chloride solution over night for curing. The beads were then taken out, washed and stored till they were further used.

PREPARATION OF MOLASSES SOLUTIONS

Pre-activated yeast beads of 1% concentration were taken in four conical flasks (250mL), each containing different concentrations of molasses solution (i.e., 6%, 10%, 12%, 16% and 20%). The activity of immobilized yeast was observed for different time interval like 24, 48, 72, 96 and120 hours. The amount of total reducing sugars was measured at each time interval.

PREPARATION OF YEAST CELL CONCENTRATION

A series of batch experiments was conducted to test the effect of yeast bead concentration on alcohol yield. Yeast beads of various concentrations were taken (i.e., 0.5%, 1%, 2%, 3%, 4%, and 5%) and activated for overnight in the medium separately. Each of the beads was then transferred to 12 percent molasses solution and the reduction of total reducing sugars was estimated.

FERMENTATIONS WITH THE USE OF ACTIVATORS

Experiment on external activator compounds were conducted by using 1% of activators (i.e., chitin, sawdust and *Rhizopus-Oryzae* biomass) with 1% free and immobilized yeast cells in conical flasks (250mL). The fermentation was allowed for 10 hours. The amounts of total reducing sugars were measured after every two hours.

EXPERIMENTAL SETUP FOR REACTOR

Initially, the fermentation was carried out batch wise using immobilized yeast cells until the substrate was depleted and was then switched to a continuous mode with the feeding of molasses. Fresh molasses was continuously supplied to the fermenter with a peristaltic pump and at the same time cell free liquid was removed from the bio-reactor through the filter module. As a whole, the volume of broth in the fermenter was controlled at 5 liters by making the total filtrate flow (outlet) rate counter balance the total feed flow rate. The fermentation was carried out at a room temperature.

ANALYTICAL METHODS FOR SUGAR AND ETHANOL ESTIMATION

Amount of total reducing sugar was estimated by the DNS method (Milller,1985). The ethanol concentration was measured by gas chromatography (Thermofisher, 8610) using a chromosorb 101 column (80 to 100 mesh of packing material, 1/8 inch outer diameter and 6 ft long stainless steel tube) with flame ionization detector. Temperatures of the detector, injector and column oven

were 300°C, 200°C, and 150°C respectively. Nitrogen was used as a carrier gas. The data reported for sugar concentration and percent alcohol were the mean value of at least two independent samples analyzed in duplicate.

RESULTS AND DISCUSSION

EFFECT OF SUGAR CONCENTRATION OF ETHANOL FERMENTATION RATE

It was evident from the Fig.1 that total reducing sugar level in the fermentation broth has influence on the fermentation rate. The maximum reduction in the amount of total reducing sugar was observed after 24 hours of the fermentation in the broth with 12 % TRS. As this total reducing sugar level increased beyond 12 percent, the efficiency of the yeast fermentation has reduced, indicating the high sugar concentration decreased the fermentation rate.

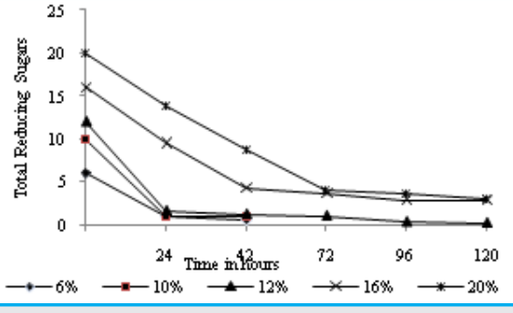

FIGURE 1. Effect of initial sugar concentration on ethanol fermentation rate.

The reduced sugar conversion with the higher concentration of molasses is probably due to osmotic effects (Roukas 1994), decreased water availability and plasmolysis of cells that leads to the growth inhibition of cells. Though the initial fermentation rate was less in 16 and 20% broth than in 12 percent broth, it increased with increase in fermentation time. The amount of TRS left after 120 hours of fermentation was higher at 16% and 20% broth than in 12% broth. This suggests that the fermentation rate decreased with increase in alcohol concentration.

EFFECT OF IMMOBILIZED YEAST CONCENTRATION ON ETHANOL FERMENTATION RATE

As evident from the Fig.2, the rate of fermentation increased with increase in immobilized yeast concentration. However, the left-over sugar concentration was

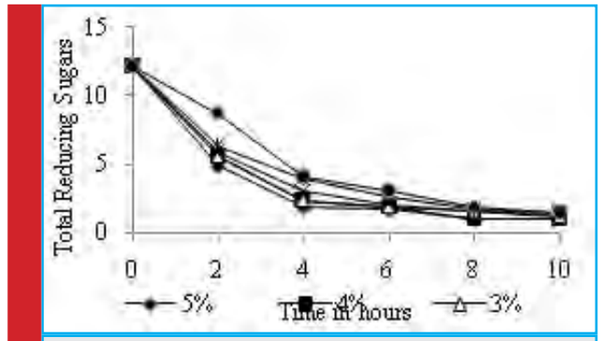

FIGURE 2. Effect of immobilized yeast concentration on ethanol fermentation rate.

observed to be increasing with decrease in amount of immobilized yeast crystal concentration. In case of fermentation with the 5 % immobilized yeast, the fermentable sugars were completely utilized within 4 h of fermentation time.

The data also indicated that the effective fermentation can be achieved using 1% of immobilized yeast crystals with 12% TRS level in the fermentation broth. However, the economic feasibility in ethanol fermentation can be achieved with 5 percent yeast crystals. The data acknowledge that 12% concentration broth is more productive for ethanol production.

EFFECT OF ACTIVATORS ON THE FERMENTATION RATE

As evidenced from the (figure 3), in the free cell system it was observed that control has shown more effect than activators for the initial 2 hours of fermentation time however , the activator effect was observed starting from 4 hours which was pronounced only at the end of 6 hours of fermentation time where chitin supplemented broth showed 47% increase in fermentation rate followed by Sawdust (20.86%) and *Rhizopus Oryzae* (12.5%) over control where maximum sugars were degraded.

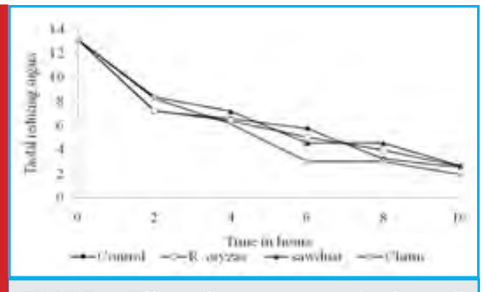

FIGURE 3. Effect of activators on the free cell fermentation rate

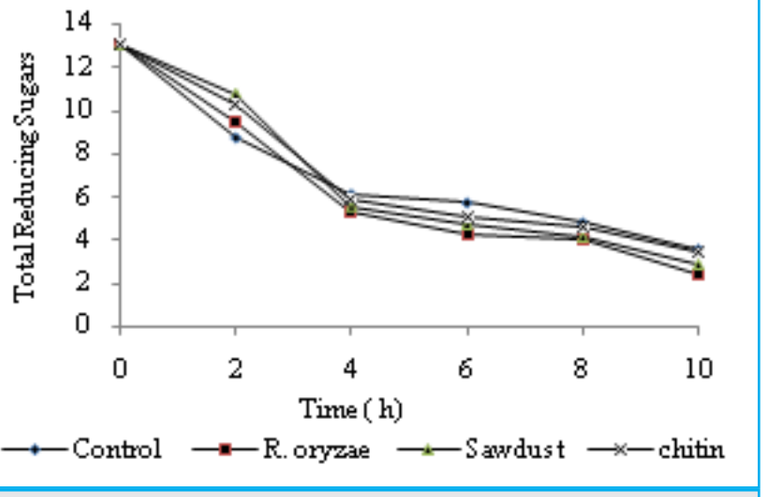

FIGURE 4. Effect of activators on immobilized cell fermentation rate

The effect of activators (Fig.4) on immbobilized cell system shows a similar trend of free cell system was repeated where control showed more effect than activators for initial 2 hours while the effect of activators was observed starting after 4hours , however, unlike the free cell system where chitin showed much effect here in this case *Rhizopus oryzae* has shown significant effect, i.e., a 23.94 % increase in fermentation rate followed by saw dust (16.37%) and Chitin (12.32%) with respect to control after 6h where maximum sugars have been degraded

Chitin, a cell wall component of yeast increased the ethanol production, which is probably not contributing as a nutritional factor needed by yeast cells, but may have released some nutritional factors that lead to the partial hydrolysis of complex substances present in the molasses or may be due to the removal of inhibitory compounds. The increased in ethanol productivity due to Sawdust is due to cells immobilized on dilignified cellulosic material that lead to an active bio-catalyst reducing the activation energy (Bardi, 1994). Besides, Sawdust may have enhanced the catalytic action of some enzymes involved in the fermentation pathways or any undesirable substances in molasses may be excluded by their adsorption on the dilignified materials.

Patil and Patil found the acceleration of ethanol production activity of yeast in cane molasses fermentation by the addition of fungal mycelium (Patil 1990). Besides, brewery yield of ethanol was increased by the addition of Proteo-lipid from *Aspergillus* and *Oryzae* promote yeast growth and yeast durability against ethanol and salt concentration.In the present work we hypothesize that the activators in common may have released some nutritional factors from other complex polysaccharides (as molasses contain different polysaccharide beside sugars) which degraded the sugar. This activator effect is seen more pronounced only at lower, reducing sugar concentrations (after 50% of initial sugar degradation) which is clearly reflected in our work, where the increase in fermentation activity has started at 4 hours of fermentation time both in free and immobilized activator system. This may probably due to less mass transfer effect of activators in high sugar concentration.

CONTINUOUS PRODUCTION OF ETHANOL USING IMMOBILIZED YEAST

To evaluate the sodium alginate- yeast beads for continuous production of ethanol, the experiments were planned using 5-L reactor with an inlet and outlet. The reactor was equipped with an overhead stirrer so as to provide uniform mixing.

Initially the reactor was run in batch mode for 24h there after the inlet feeding was initiated at a flow rate of 190mL h-1 having residence time of 26.3 hours. The productivity was observed to be 2.49g L-1 h-1. Once the stabilization was achieved at this flow rate it was increased so as to achieve the residence time of 21.73 h (flow rate-230mL h-1). It was noticed that at this residence time the productivity was increased in the outlet at the tune of 50%. The same was reflected by the decrease in sugar concentration in the outlet. To evaluate further, experiments was conducted at flow rates of 270mL h^{-1} and 280mL h^{-1} with residence time of 18.51 h and 17.85 h respectively.

As evidenced from the figure 5, the productivity increased with increasing flow rate up to a certain level (270mL h^{-1}) beyond which the increased flow-rate resulted in the decrease of productivity.

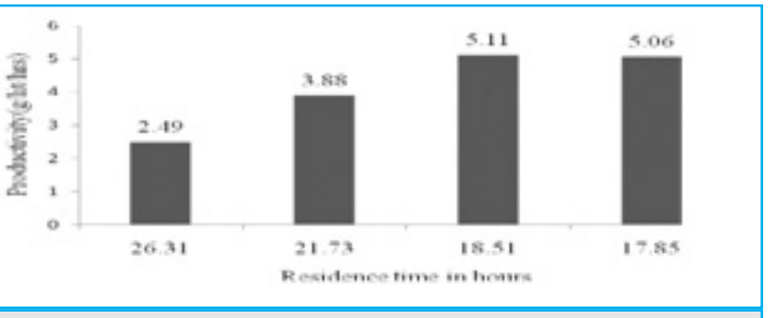

FIGURE 5. Continuous production of ethanol using immobilized yeast cells at different flow rates

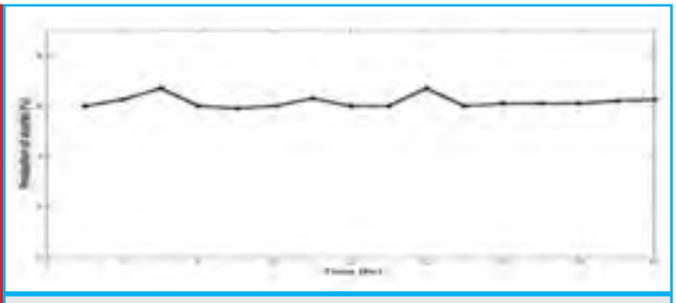

FIGURE 6. Continuous production of ethanol using immobilized yeast cells at a fixed flow rate

Data indicated that critical dilution was achieved at around (270mL h^{-1}) having maximum specific growth, increase in flow rate might have led to washing out of cells resulting in decreased productivity.

Based on the above data, the continuous production of alcohol using immobilized yeast beads in a CSTR was continued using at a flow rate of 190mL h^{-1} (residence time of 26.31h) to access the reusability of immobilized yeast breads. The data were presented in the graph (figure 6). The results indicated that production of ethanol ranged from 6-6.8%, depending on the initial TRS loading.On an average, consistent ethanol production was observed for more than 16 cycles (maximum cycles studied). It was observed that the productivity levels were varied as the fermentation time increased. A visual observation indicated that a slower rate of fermentation was observed in the molasses vessel because the medium is not sterilized.

CONCLUSION

Effective Fermentation can be made with 1% of Yeast cell concentration and 12% of initial TRS loading. Plant and Fungal based Activators were shown to significantly increase the fermentation Rate. Continuous production of Ethanol showed an increase in productivity up to 270mL h-1 beyond which showed reversed trend. Continuous production of ethanol at a fixed flow rate showed the beads can be reused up to 16 cycles.

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Differentiation of human fat mesenchymal stem cells using electromagnetic waves

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ABSTRACT

In orthopedic sciences, cartilage regeneration and repairing is an underlying issue investigated by many studies in tissue engineering. Bioactive growth factors, cell implantation and biocompatible scaffolding are the new developments in this field. Keeping the proliferation and differentiation ability of cultured cells is essential in producing extracellular matrix and cartilage. Adult stem cells can be used due to their high ability to be differentiated in several cell types in tissue engineering. Till recently hematopoietic stem cells and fat tissue stem cells have been used to repair the tissues. Studies indicates that using fat tissue stems cells is preferred because of easy access to adipose tissue and maintaining the ability of proliferation and differentiation to cartilage in high passages compared to hematopoietic stem cells. Therefore, this study has been conducted to analyze the differentiation of human fat mesenchymal stem cells using electromagnetic waves. In this work simultaneous effect of platelet-rich plasma and electromagnetic waves have been studied on efficiency of differentiation of fat mesenchymal stem cells to cartilage. Mesenchymal stem cells have been extracted from human fat tissue and then, the cells have been differentiated to fat, bone and cartilage tissue cells using different treatments. Cartilage differentiation was done in 2-D form and single-layer cell in bottom of 6-bed and 3-D cells in cell falcons. Required tests to estimate efficiency of cartilage differentiation were done in two general examinations for analysis of evaluation of specific and pathologic markers such as osteogenicity, angiogenesis and inflammation parameters by measuring the amount of TNF and VEGF with ELISA technique. The results obtained from this study showed reasonable use of platelet-rich plasma (PRP) and electromagnetic waves in medicine and tissue engineering. Although PRP enables cartilage differentiation, it cannot reduce pathologic symptoms.

KEY WORDS: DIFFERENTIATION OF HUMAN FAT MESENCHYMAL STEM CELLS, ELECTROMAGNETIC WAVES, PRP

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INTRODUCTION

The aim of tissue engineering is normalizing tissue performance through transferring living factors in body of patients, (Kocan *et al.* 2017, Bondarava *et al.* 2017). Continuous efforts of cell biologists, engineers, material engineers, mathematicians, geneticists and physicians should be in way of achievement and success in new tissue production, (Alonso-Goulart *et al.* 2017, Klar *et al.* 2017).

Nowadays, tissue engineering is being used as a technique for tissue or organ transplant. The lost and injured tissues and organs can be treated by engineered biological transplant (Zhai *et al.* 2016). Engineered products and components should have high performance and have the ability to form expected functional tissue (Zajdel *et al.* 2017). Mesenchymal stem cells (Skeletal Stem Cells or Bone marrow stromal stem cells) are plastic sticky non-hepatocytes cells placed in bone marrow stromal vessels (Seong *et al.* 2014 Ross *et al.* 2015 and Liu *et al.* 2017) and have multi-generation differentiation with self-renewal capacity (Heo *et al.* 2016 and Mobini *et al.* 2017). Deciding which type of cell should be applied to repair injured tissue is essential which can be adult brucellosis, mesenchymal stem cells or protozoal cells from hypothyroidism, pericardium or cells manipulated genetically, (Tan *et al.* 2017). Cartilage is non-vascular fibrous connective tissue having resistant extracellular matrix, which is stiffer than other connective tissues and includes intra-matrix chondrocytes, (Choi *et al.* 2018).

The first function of cartilage is protection of soft tissues and helping evolution and growth of long bones. Fat tissue stem cells are similar to bone marrow stromal cells in terms of physical form and can be appropriate replacement. These cells have common features compare with mesenchymal stem cells such as self-renewal ability, powerful cells, fibroblast appearance, ability to attach to plastic dishes for cell cultivation and ability to be changed into other mesenchymal types, (Dubois *et al.* 2008 and Tan *et al.* 2017).The differentiation of these cells from fat, bone, cartilage, skeletal muscular and cardiovascular cells has been reported in recent years by different researchers. Fat tissue stem cells, same as bone marrow stromal cells, can be converted to ectodermal types (like neural and glial cells) by the mediation of neural inducers, (Ivan *et al.* 2017).

Several studies have been conducted in fat tissue stem cells, especially for treatment of neural system lesions and revealed that direct injection of fat stem cells to brain ischemic models can convert these cells to quasi-neural cells. These cells can improve laboratory models in the place of lesion with (injured place by) secretion of neural growth factors (Gugjoo *et al.* 2016).In order to prepare mesenchymal cell, fat tissue is a considerable source, since it is available in large amount (liposuctioned) fat tissue or in fat tissue section and contains large amount of stem cells called fat-derived stem cells. The property has made it be considered as a good candidate for 1-step treatment of cartilage defects (Bruder *et al.* 1997).

In the past decade, PRP or platelet-rich plasma has gained many attentions, especially in regenerative medicine, (Liu *et al.* 2017). PRP can be considered as a part of blood plasma with platelet density higher than base level (Xu et al. 2017 Qian *et al.* 2017). Importance of PRP with appropriate density of platelet is the abundant growth factors and proteins used in tissue engineering, (Anitua, *et al.* 2007). In 2012, a study was done in China for regenerating cartilage, which evaluated and compared mesenchymal stem cells, extracted from bone marrow and fat tissue placed on the PRP-derived scaffolding. Findings showed that rich plasma is appropriate bioactive scaffolding with ability of secreting growth factor, bone marrow stem cells and fat mesenchymal stem cells -placed on PRP were differentiated from cartilage and efficient to repair cell-based cartilage, (Lee *et al.* 2012).

In a study, the effects of platelet-rich plasma on histological, biochemical and biomechanical properties of cartilage tissue engineering were studied. Cultured cells in PRP created 20% thicker cartilage tissue compared with other cultured cells. Hence, platelet-rich plasma in culture medium can cause creation of cartilage in vitro cartilage formation with increased content of glycosaminoglycan and more mechanical compressive properties at the same time with maintaining Phenotype Features of Hyaline cartilage (Petrera, *et al.* 2013). In 2014, a research team from Spain studied effects of PRP on human mesenchymal stem cells, which can increase or limit clinical uses of these cells. PRP can preserve differentiated immune of mesenchymal stem cells and can apparently postpone ageing phenomenon. They also provide data on exact molecular relation and its mechanisms, (Rubio-Azpeitia *et al.* 2014).

In 2014, an economic method was proposed to prepare PRP. PRP-derived growth factor-BB was measured under conditions of using Anticoagulant dextrose solution A (ACD-A), along with or in absence of Prostaglandin E1 (PGE1) (as Platelet aggregation inhibitor). The new method was successful in analysis of PRP with growth factor and high BB under all growth conditions and high volume of PRP is obtained using ACD-A and PGE1(Fukaya *et al.* 2014).

In 2014, the effect of PRP on cartilage cells differentiated from rabbit fat-derived stem cells was studied in vitro. Stem nature of rabbit cells were studied with differentiation of fat, bone and cartilage types in vitro. Collagen type 2 expression and agrican expression in PRP-treated cells was increased to 10% compared to

control group. Hence, the findings showed that PRP of rabbit can take cartilage differentiation in mesenchymal stem cells extracted from rabbit fat (Elder *et al.* 2014).

Analysis of the simultaneous effect of PRP and electromagnetic waves on cartilage differentiation efficiency in fat mesenchymal cells has been studied. Mesenchymal stem cells have been extracted from human fat tissue and then, the cells were differentiated to types of fat tissues, bone and cartilage using different treatments Therefore, these experiment have been conducted to analyze differentiation of mesenchymal human fat stem cells using electromagnetic waves.

MATERIALS AND METHODS

Extraction of mesenchymal stem cells from fact tissue: To transfer fat tissue, fat pieces were placed in DMEM medium containing Penicillin and streptomycin 3X , amphotericin 1x , without FBS and were transferred to laboratory in ice. After each time of adding buffer to 2-3 times of tissue volume, centrifuge was taken with speed of 1200rpm for 20min and buffer solution was disposed. A thin layer of fat was formed on the solution, which had to be removed, since it could cause problems such as choking on cells due to lack of oxygen while cultivation. In this step, suspended cells were transferred to flasks 75 containing 10ml culture medium with FBS and were then heated in incubator.

To make cells face electromagnetic waves, after counting cells using Neubauer lam method, a part of culture medium containing 200.000 cells was removed from culture flasks and was transferred to sterile falcons. Then, 1ml of culture medium without differentiation factors and containing Penicillin - Streptomycin and FBS was added and maintained for 1 day. After the heating in incubator, cells were accumulated in bottom of the falcon in rounded form and were prepared to continue the examination and to expose to electromagnetic radiation. To apply electromagnetic waves on cell culture, wave winding machine was used. The winding machine was placed inside the incubator and culture falcon was placed there to be exposed to electromagnetic waves for 6 hours per day. Frequency of the machine was set in range 171-173Hz and according to power about 30%, ultimate and real time power about 50Hz was obtained. Cells were exposed to such conditions in 14-day period and the culture medium was changed every 3 days. 5 falcons containing culture medium with 5% PRP were exposed to electromagnetic waves and other 4 falcons were not. Measurement of VEGF distributed in differentiated cells was done using supernatant of cultures in days 7, 14 and 21. The phosphorylation of this factor was measured by kit based on Sandwich ELISA method. The Sandwich ELISA method was used to measure the amount of TNFα propagated from differentiating cells. To this end, the supernatant collected from cell culture was examined in the days 7, 14 and 21. The final results were analyzed statistically at the confidence level of 95% to test significance of the difference of different groups using Prism software and using one-way ANOVA and T-test. Moreover, the diagrams were drawn using Excel and SPSS software. The results obtained from analysis of cartilage differentiation gene expression were analyzed using REST software by normalization with reference beta-actin gene. Each test was replicated 3 times and the significance was considered lower than 0.05.

RESULTS

Analysis of differentiation of stem cells extracted from fat tissue to cartilage : In this study, differentiation to cartilage was analyzed using different staining methods and Immunocytochemistry test during 14 days of cell differentiation. Expression of some genes relevant to cartilage was also studied at the end of the differentiation period.

Proof of cartilage differentiation: The Immunocytochemistry method was used to analyze expression and production of collagens types 2 and 10. Collagen type 2 was expressed during cartilage differentiation and collagen type 10 was one of the bone and hypertrophy markers expressed by chondrocytes. Figure 1 has illustrated expression of these collagens in treated cells in the day 14. The blue color in the figure is DAPI staining to stain cell core and red color shows presence of studied collagens in differentiating cells.

Analysis of bone markers during differentiation of stem cells to cartilage: At the time of applying differentiation factors such as using PRP as a bioactive substance or electromagnetic radiation to stimulate cartilage differentiation in mesenchymal stem cells of fat tissue, non-specified differentiation to bone is also possible. With taking tests such as measurement of Alkaline Phosphatase Activity and Calcium sedimentation measurements, the non-specified differentiation was also examined.

Measurement of Alkaline Phosphatase Activity: To determine non-specified differentiation towards bones, measurement of alkaline phosphatase enzyme was activity was done. As it is clear in diagram 1, the amount of alkaline phosphatase in treatment PRP.W is the highest level measured equal to 2 units of optical absorption per mg protein. Then, treatment PRP a with value of 1.44 unit absorption per mg protein shows highest alkaline phosphatase activity. The lowest level of phosphatase

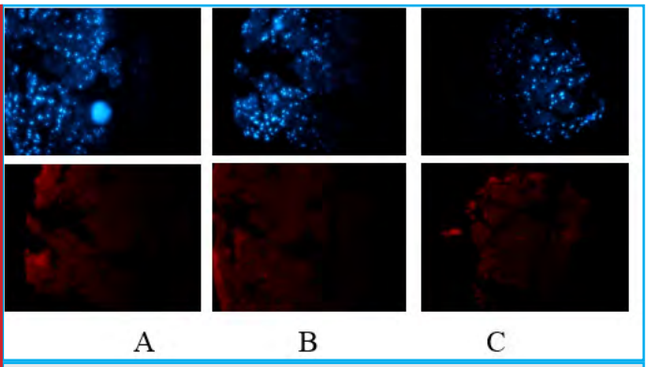


FIGURE 1. Immunocytochemistry staining of collagens type 2 and 10 in treated cells; A) collagen type 2 in treatment Dif.a; B) treatment Dif.W and c) collagen 10 in treatment PRP.a (the upper part is staining core by DAPI and the lower part is collagen staining); blue color shows cell core and red color refers to surface place of collagens; the line illustrates size of 0.26mm and magnification of image ×4.

alkaline was observed in treatment base.w at 0.32 unit absorption per mg protein. According to obtained results, treatments PRO.W and PRP.a motivate differentiation towards bones more than others and this can be inferred based on activity of alkaline phosphatase in these treatments. Despite to them, treatment base.w has caused lowest stimulation towards boning.

VEGF factor secretion : According to diagram 2, highest secretion level of VEGF was observed in treatments with base environment with PRP. In the base treatment with PRP.a, higher level of Endothelial Angiogenesis Growth Factor was observed compared to base environment in combination with PRP.w. The lowest secretion level was detected in base.w and it seems that the effect of electromagnetic wave treatments and TGF factor varies due to medium culture used. For example, in environment with Dif base, secretion in wave treatment is higher than others; although the results differ in base environment and secretion has been in higher level using TGF treatment. The effects of PRP have been also conflicting. This factor has shown different effects in combination with different culture media exposed to wave and TGF treatments. However, it seems that it has led to increased secretion of VEGF by itself, since secretion in treatments with PRP has been higher than others.

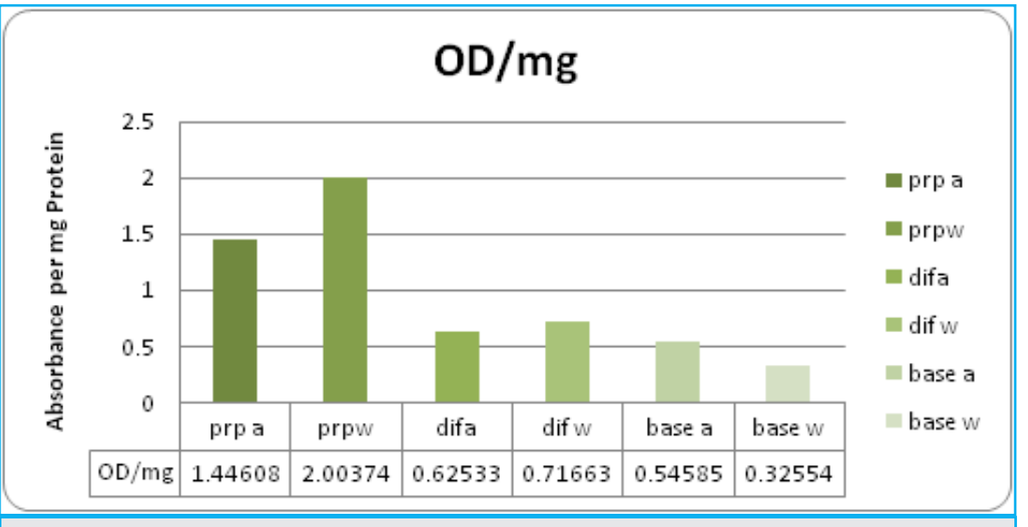


DIAGRAM 1. Phosphatase alkaline activity per unit optical absorption per mg protein in different treatments

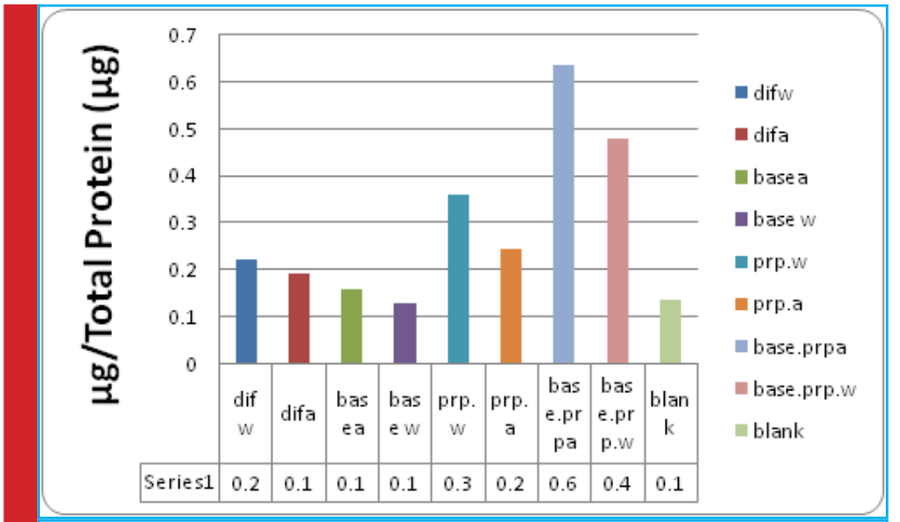


DIAGRAM 2. Secretion of VEGF in different treatments

Analysis of inflammation with TGFα measurement: Another symptom of cartilage-related articular diseases is inflammation. According to existing reports, PRP can cause inflammation in some cases and may intensify that. Moreover, it can inhibit and reduce inflammatory reactions. The conflict is because of wide range of inflammatory factors in PRP. Inflammatory potential of engineered tissue has been examined by measurement of TNFα. The results have been presented in diagram 3. According to these results, treatments Dif.w and PRPb.a have shown highest level of inflammatory activity. On the contrary, treatments Base.w and Base.A and PRP.W have shown low level of TNFα even lower than ELISA control level. Treatment Dif showed highest level of inflammation; although same factor showed lowest level of inflammatory activity in combination with Base medium. PRP.b factor has also shown different behavior in different combinations of treatment with TGF and electromagnetic radiation, so that it has shown high inflammatory activity in combination with TGF and inflammatory activity lower than control level in combination with electromagnetic radiation. The conflicting responses can be attributed to complexity of compounds in culture media and PRP compounds, since controlling

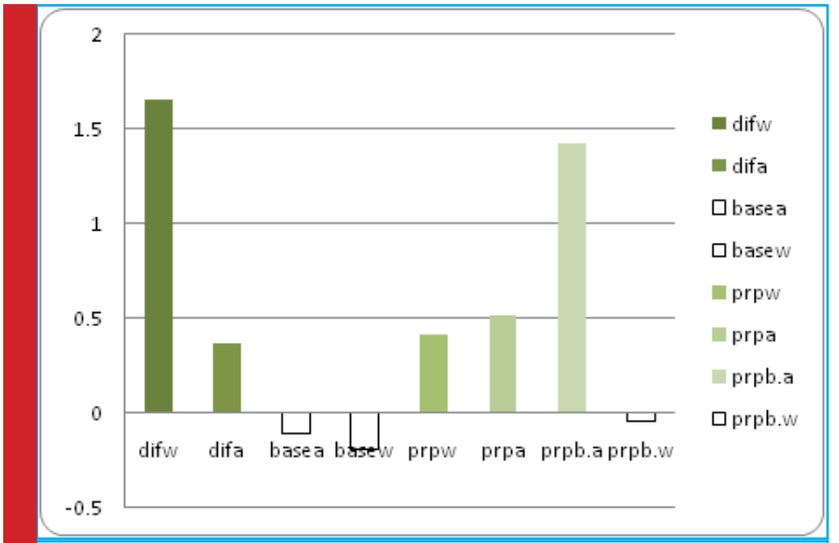


DIAGRAM 3. TNFα level in different treatments of induction of differentiation to cartilage

FIGURE 2. Agarose gel produced by PCR reaction for ALK5 M 1--bp marker gene

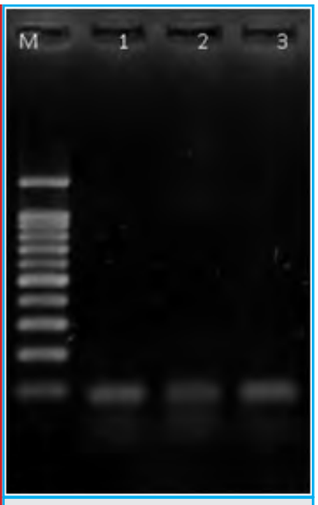


FIGURE 3. Agarose gel produced by PCR reaction for Pleotrophin M gene 100bp marker

these complex compounds and inhibition of interfering effects of factors in the combinations is difficult.

Measurement of cartilage and bone gene expression : Analysis of cartilage differentiation by gene expression using RT-PCR

Expression of Pleotrophin and ALK5 genes as markers in cartilage differentiation samples and collagen status type 2 gene were analyzed. Collagen type 2 includes 2 isoforms called CollIA and CollIB created as a result of alternative array of exon 2. Expression of these markers during Chondrogenic differentiation caused differentiation efficiency detection. The image of gels produced by reaction of these genes is presented in figures 2 and 3.

Emergence of 250bp band showed expression of ALK5 gene in samples, which can show cartilage differentiation in these samples. 111bp band emergence also showed expression of Pleotrophin in cells and confirms cartilage formation processes. However, no band was observed in proliferation of isoforms in collagen type 2 and the result of this gene proliferation was negative, which shows lack expression of this gene in this differentiation steps.

Analysis of specific cartilage genes expression using Real Time PCR

In order to analyze expression of specific cartilage genes such as sox9, colll and colX using b2m reference gene, Real Time PCR method was used.

Sox9 cartilage marker: Sox9 marker is the transcription factor required for expression of cartilage matrix genes

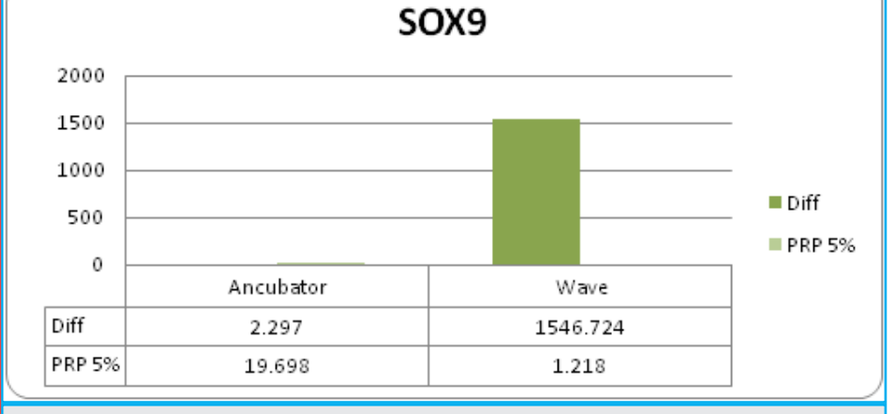
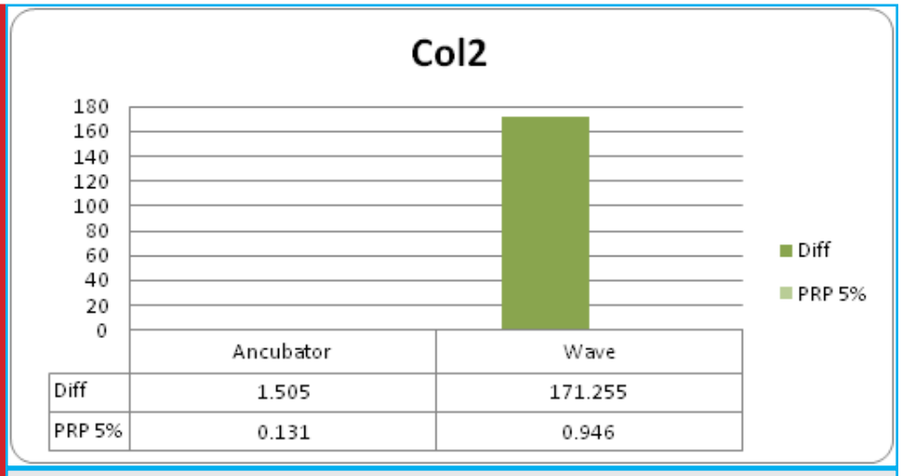


DIAGRAM 4. Sox9 gene expression during cartilage differentiation



	Ancubator	Wave
Diff	1.505	171.255
PRP 5%	0.131	0.946

DIAGRAM 5. Colll gene expression during differentiation process

such as collagen type 2. Diagram 4 illustrates sox9 gene expression during cartilage differentiation process. In the day 14 of treatment, expression of this gene in Dif medium exposed to electromagnetic waves was significantly high; although its expression in medium containing 5% PRP was not tracked. Moreover, expression of this gene was not observed or was insignificant in treatments without wave.

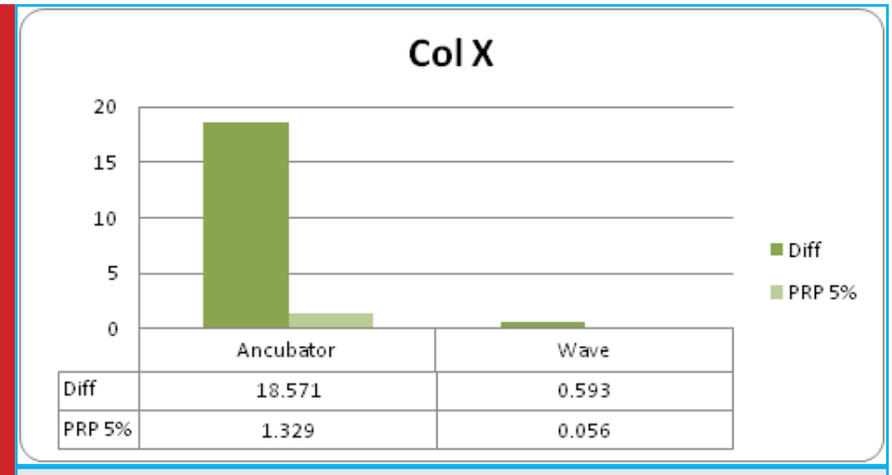
Collagen type 2: The products of this gene were observed in hyaline cartilage in form of fibril. The proteins can't form fiber. In this study, proalpha-1 chain has been detected, which has interfered in partial production of collagen type 2. According to diagram 5, colll gene expression has been significantly increased under effect of electromagnetic waves. Moreover, the results show that medium containing 5% PRP has been unable to induce expression of this gene and has also had inhibitory effects on wave induction capacity on the other hand.

Collagen type 10: Alpha-1 collagen chain was coded by COL10A1 gene. This gene can encode collagen type-10 alpha chain, which is expressed by hypertrophic chondrocytes during Endocardal bone formation.

Diagram 6 has illustrated adjustment of existing data of electromagnetic wave and 55 PRP on both inhibitory effects on expression of this gene. When the two factors are applied in same treatment on cells, no expression can be observed; although colX gene expression is in high level in presence of the two factors.

DISCUSSION

Mature stem cells can be used for tissue engineering purposes due to high differentiation ability to several



	Ancubator	Wave
Diff	18.571	0.593
PRP 5%	1.329	0.056

DIAGRAM 6. ColX gene expression during differentiation of stem cells to cartilage

cell types. Recently, fat tissue stem cells have been used to repair tissues. The study conducted by Zul et al. (2001) using stem cell extraction methods based on using collagenase could extract these cells from fat tissue of adult people, (Hunziker *et al.* 2002). Moreover, Estes Research Team used this method to extract stem cells to obtain fat tissue stem cells to create cartilage phenotype, (Jonstone *et al.* 1998).

According to findings of Pena et al. (2011), spindle cells with ability of adherence to bottom of culture dish and the ability of differentiation to mesodermic cell lines can be considered as mesenchymal stem cells (Yoo *et al.* 1998). Adherence ability of cells to plastic surfaces has been used for early separation of stem cell populations. Similar results were also observed in study of Tappe et al. (2009) and Aust et al. (2004), in which nature of fat tissue was analyzed using this type of staining, (Solchaga *et al.* 2006, Diduch *et al.* 2000).

In this study, to analyze the differentiation of stem cells extracted from fat tissue to cartilage, expression and production of collagens type 2 and 10 has been applied using Immunocytochemical method. Similar results were also obtained by Ogawa, (Williams *et al.* 2003). In this study, expression of collagen type 2 was observed during cartilage differentiation and type 10 as bone and hypertrophy markers in chondrocytes. It seems that Dif medium and electromagnetic wave treatment have induced cartilage differentiation and bone markers have been observed in PRP-contained treatment and it seems that the treatment has the ability of inducing hypertrophy and boning. Mishra et al. (2009) showed the effect of PRP on cartilage differentiation. Further, conflicting effects of PRP on cartilage and bone differentiation were revealed (Bosnakovski *et al.* 2006, Wakitani *et al.* 2002).

Alkaline phosphatase enzyme activity can be considered as one of the most underlying factors of hypertrophy and bone formation and when the cartilage cells are differentiated, they gain hypertrophic mode and start production of alkaline phosphatase enzyme, (Bruder *et al.* 1997). According to the results of this study, treatments PRP.W and PRP.a have stimulated differentiation to bone more than others and lowest alkaline phosphatase activity has been observed in treatment Base. W on the other hand. Measurements showed that highest secretion of VEGF was observed in treatments with base medium containing PRP. Lowest secretion level was also detected in Base. W medium. It is expected that PRP can induce such reaction because of nature rich of growth factors. However, there are some reports on inhibitory effect depended on PRP dose in VEGF secretion (Clin Pediatr Endocrinol 2014). The conflicts can be because of difference in type of treatments, PRP preparation method and its source and also type of cells under treatment.

According to results obtained from this study, treatments Dif.W and PRP.a have shown highest level of inflammatory activity. On the contrary, treatments Base.W, Base.A and PRPb.W have even shown TNFα level even lower than ELISA control level. It seems that combination of base medium lays key role in reduction of inflammatory activities regardless of other inductive factors.

The effects of electromagnetic radiation or using PRP showed different results. For example, radiation in combination with Dif medium showed highest inflammation level; although same factor showed lowest inflammation activity in combination with base medium. In 3 out of 4 treatments containing electromagnetic wave, TNFα level was low and insignificant and it could be inferred that electromagnetic wave can probably reduce inflammatory activity regardless of culture medium compounds.

Controlling expression of cartilage matrix gene like collagen type 2 was taken by transcription factor called sox9. During cartilage differentiation process, the transcription factor played key role and its expression was increased in chondrocytes and Chondroprogenitor cells. According to obtained results, expression of this gene in Dif medium under electromagnetic radiation was significantly increased; although its expression was not detected in medium containing 5% PRP. Moreover, the expression of this gene was not observed or was insignificant in treatments without wave. As a result of increased expression of sox9 gene, its downstream gene (colll) was also increased in terms of expression. Moreover, the results showed that medium containing 5% PRP has not the ability to induce expression of this gene and has had also inhibitory effects on wave induction capacity on the other hand. Colll expression pattern under treatment has been significantly similar to expression pattern of control gene (sox9).

Adjustment of data of electromagnetic wave and 5% PRP has shown that both of them have inhibitory effects on colX gene expression.

CONCLUSION

The results of the present study showed the efficient and logical use of PRP and electromagnetic waves in medicine and tissue engineering. However PRP allow cartilage differentiation in the right format, this method need further study and cannot decrease pathologic symptoms.

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Genetic characterization of native *Bacillus thuringiensis* strains isolated from Tamil Nadu, India

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ABSTRACT

B. thuringiensis is a crystalliferous bacteria used as a biocontrol agent against lepidopteran, dipteran, and coleopteran pests. Seventy eight *Bacillus thuringiensis* strains were isolated from 108 soil samples collected from Tamil Nadu, India. Phylogenetic relationship of *B. thuringiensis* isolates were evaluated based on PCR amplified fragment polymorphisms of flagellin genes (PCR-AFPF). The isolated *B. thuringiensis* strains comprised of 51.3% of known biochemical types and 48.7% of undescribed *B. thuringiensis* types. PCR-AFPF UPGMA dendrogram generated using Jaccard coefficient values showed two phylogenetic groups, group A and B comprised of I-XIII and XIV-XV clusters respectively. The present study concluded that *B. thuringiensis* isolates from Tamil Nadu have a high degree of genetic diversity and high rate of genetic exchange.

KEY WORDS: *BACILLUS THURINGIENSIS*, PCR-AFPF, GENETIC DIVERSITY, UPGMA DENDROGRAM

INTRODUCTION

Chemical insecticides that are currently used to control insect pests are extremely toxic to non-target organisms and many insects have developed resistance to different chemical pesticides, resulting in ineffective insect control programs. They are deleterious to the health of humans and animals, lead to cancer and immune system disorders. In addition, chemical insecticides are recalcitrant, it accumulates in the environment and result in soil and water pollution (Devine and Furlong, 2007). The use of microbial insecticides is an alternative to chemical pesticides for insect control. Biological insecticides are mainly based on entomatopathogenic bacteria, *Bacillus thuringiensis*.

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B. thuringiensis is a member of a group of crystalliferous spore-forming gram-positive bacteria of the family Bacillaceae (Schnepf et al., 1998). This bacterium is able to produce proteinaceous parasporal crystals that exhibit specific insecticidal and nematicidal activities (Khyami-Horani et al., 1996; Carneiro et al., 1998; Al-Banna and Khyami-Horani, 2004). *B. thuringiensis* is commonly used as an organic biopesticide against lepidopteran, dipteran, and coleopteran insect pests (Schnepf et al., 1998). *B. thuringiensis* was first isolated from diseased silkworm (*Bombyx mori*) larvae (Ishiwata, 1901). Over the last five decades, *B. thuringiensis* has been developed as a microbial agent against lepidopteran pests (Carlton, 1990). In 1977, Goldberg and Margalit (1977) discovered a novel *B. thuringiensis* strain that expressed specific insecticidal properties against Diptera and another *B. thuringiensis* strain that targeted against Coleoptera was explored by Krieg et al. (1983). The success of various *B. thuringiensis* strains in controlling various insect pests has driven the establishment of several screening programmes for more novel *B. thuringiensis* strains. As a result, it is estimated that today, more than 50,000 *B. thuringiensis* strains are kept in various private and public bacterial collection centres (Sanchis et al. 1996).

There are many methods have been proposed to classify *B. thuringiensis* into sub-species level. The main classification of *B. thuringiensis* isolates was developed on the basis of H-flagellar antigens by de Barjac and Bonnefoi (1962). *B. thuringiensis* strains are classified into more than 82 serovars using H-antigen method. However, there are two limitations with the H-classification for strains lacking parasporal inclusions and auto-agglutinated strains. Some *B. cereus* strains have antigens that cross-react with sera specific for *B. thuringiensis* H-serotypes (14) and such isolates may originate from older *B. thuringiensis* strains that have lost plasmid encoded crystals (Lecadet et al., 1999). These auto-agglutinated strains make up almost 3% of the *B. thuringiensis* in the International Entomopathogenic *Bacillus* Center (IEBC) collection (Burges et al., 1982) at the Institute of Pasteur, Paris, France, but the H-classification is completely useless on these strains. In addition, a few *B. thuringiensis* strains, called non-motile strains such as *B. thuringiensis* var. *wuhanensis*, also escape H-serotyping (de Barjac and Frachon, 1990).

With the development of molecular biology, the classification and identification of bacteria has changed from traditional phenotypic to genotypic methods in recent decades. Pulsed field gel electrophoresis (PFGE), used for genotyping different bacterial strains of a specific species as in the case of *B. thuringiensis* strains (Gaviria and Priest, 2003). However, as PFGE requires special equipment and chemicals, it is not easy to perform in many laboratories. Currently, molecular typing methods including Arbitrary Primer PCR technology (Brousseau et al., 1993), DNA reassociation measurements (Nakamura, 1994), ribosomal RNA gene restriction fragment length polymorphism (Priest et al., 1994; Akhurst et al., 1997), ribosomal RNA gene intergenic spacer sequences comparison (Bourque et al., 1995), DNA-colony hybridization and random amplified polymorphic DNA (RAPD) analysis (Hansen et al., 1998), have also been applied to a limited numbers of *B. thuringiensis* strains. To date, molecular techniques such as RAPD, RFLP, 16S rRNA probe, specific DNA probe, and ISR methods have not provided any great improvement over the H-classification method. Hence, in the present research, a faster, convenient and accurate method was followed to classify all subspecies of *B. thuringiensis* using PCR amplified fragment polymorphism of flagellin genes (PCR-AFPF).

MATERIALS AND METHODS

Soil collection and isolation of *B. thuringiensis*: Soil samples were collected from 108 locations in Tamil Nadu, India, that are very diverse in nature. The samples were collected from agricultural fields, high-altitude mountains, forests, grasslands and sewage. Soil samples were collected by scraping off surface material with a sterile spatula and then obtaining a 10g sample from 1 or 2 cm below the surface. These samples were stored in sterile plastic bags at ambient temperature. One gram of soil was added to 10ml of Luria Broth, which was buffered with 0.25M sodium acetate (Travers et al., 1987). The mixture was shaken for 4h at 250 rpm in 30°C, after incubation 1.5ml of sample mixture was taken and heat shocked at 80°C for 3min. 100µl of the suspension was plated on HiCrome™ Bacillus Agar supplemented with 10µg/ml of polymyxin B. Colonies formed after overnight growth at 30°C were selected based on colour and colony morphology and the colony was transferred onto TCHA medium supplemented with 0.3% glucose. Cultures were allowed to grow and sporulate for 40h at 30°C and the sporulated cultures were then checked for the presence of crystals, which was the criterion used to confirm the isolates as *B. thuringiensis* (Braun, 2000).

Biochemical identification

Fourteen biochemical tests such as acid production from glucose, arabinose, xylose, mannitol, mannose, salicin and sucrose; utilization of citrate and esculin; and production of protease, amylase, phospholipase C or lecithinase, and hemolysin were performed as described by Parry et al. (1983) to identify *B. thuringiensis* strains. For this study, only the results of the following four (the

most relevant) biochemical tests are presented: esculin utilization, acid formation from salicin and sucrose, and lecithinase production (Martin and Travers, 1989). Based on these four biochemical tests the *B. thuringiensis* isolates classified into 16 biochemical types. The classification of *B. thuringiensis* strains in these groups was corresponding to the distribution obtained by additional tests.

PCR-AFPF

DNA C1000 thermal cycler (Bio-Rad) was used to carry out PCR amplification. Cells of different *B. thuringiensis* strains were inoculated on Luria-Bertani (LB) agar plates and incubated at 30°C for 12 h. A loopful of cells was suspended in 100 µl of nuclease free water in a 1.5-ml Eppendorf tube. The cell suspension was frozen at -70°C for 20 min and then boiled in water bath for 10 min. The resultant lysate was centrifuged at 10,000 rpm for 10 min and five microliters of the supernatant was used as a source of DNA template. The AFPF primers used in this study was previously described by Yu et al. (2002) and are listed (Table 1). Primer was obtained from Eurofins MWG Operon, Germany. Each 50 µl of PCR mixture contained 200 µM deoxynucleotide triphosphates, 2 µM $MgCl_2$, 12.5 pmol per primer, and 2 U of DreamTaq™ DNA polymerase (Fermentas). Amplification was performed using a single denaturation of 3 min at 94°C followed by a 35 cycle program, with each cycle consisting of denaturation at 94°C for 30 s, annealing at 45°C for 30 s, and extension at 72°C for 2 min; the final extension step was 72°C for 10 min. The PCR amplified products were detected by 1% agarose gel electrophoresis. The gels were scanned using AlphaImager gel documentation system.

Data analysis

Cluster analysis was used to examine genotypic relationships among the environmental *B. thuringiensis* isolates and it was performed using the AlphaView software, version 4.2 (Proteinsimple, CA). For data analysis, the profiles were converted into binary matrix. The computer cluster analysis was performed on the basis of calculation of the Jaccard Coefficient using unweighted pair group method with arithmetic mean (UPGMA) (Kumar et al., 1993).

RESULTS AND DISCUSSION

Isolation of *B. thuringiensis* from soil: Totally 108 soil samples were collected from different locations of 26 districts in Tamil Nadu, India. Soil samples were collected from cultivated fields (rice, sugarcane, coffee, tea, mango, papaya, cabbage, onion, tomato, coconut, and potato), natural vegetation (pine forests, shola forest, reserved forest, tropical evergreen forest, and grasslands) and sewage soils. The elevations of the places from which the samples collected were highly variable, ranging from sea level to 7,200 m above sea level. Among 82 soil samples, twenty six samples were collected from hilly regions (Ooty, Yercaud, Kolli hills, Palani, Kodaikanal, Sirumalai).

Table 1. Nucleotide sequences of primers used for PCR-AFPF and multiplex PCR

Primer Name	*Sequence*
Fla5	F – GGCGTCGACATGAGAATTAATACAAACATT
Fla3	R – CGCCTGCAGTTATTGTAATAATTTAGAAGCC

After acetate and polymyxin B selection, 97 of 108 soil sample yield colonies. Of these 97 samples, 70 (72%) contained at least one crystal protein forming *B. thuringiensis* strains. From the 70 soil samples, 78 *B. thuringiensis* strains were isolated and separated from 173 other spore forming organisms. Overall, this suggests that Tamil Nadu soils were enriched with *B. thuringiensis*.With the advent of a very selective procedure for separating *B. thuringiensis* spores from the spores of other soil microbes, 78 *B. thuringiensis* strains have been isolated through acetate and polymyxin B selection. The distribution of *B. thuringiensis* in Tamil Nadu, is summarized (Table 2). In Tamil Nadu, the soil samples collected from districts such as, Vellore, Thanjavur and Theni were extremely rich in *B. thuringiensis*. Hundred percent of the soil sample contained *B. thuringiensis*. The soil sample collected from Krishnagiri, Nilgiris, Coimbatore, Trichy, Tirunelvelli and Kanyakumari had 75 – 80% of *B. thuringiensis* strains. On the other hand, soil samples collected from Ariyalur, Nagapatinam, Madurai, Virudhunagar and Tuticorin, only 25 – 33% of the soil samples contained *B. thuringiensis* strains.

Biochemical typing: The *B. thuringiensis* strains isolated from Tamil Nadu were identified and classified based on Martin and Travers (1989). In order to discriminate the isolated *B. thuringiensis* strains in to 16 biochemical types, four biochemical tests such as esculin utilization, acid formation from salicin and sucrose and lecithinase production were carried out and these were the most variable among *B. thuringiensis* isolates. *B. thuringiensis* isolates were given the biochemical type number based on the Martin and Travers (1989) and their occurrence in Tamil Nadu is also recorded (Table 3). Eventhough some of these types were differed by a single biochemical tests, these difference were more important. In this study *B. thuringiensis* subsp. *kurstaki* is widely available in the environment differed from leastly available *B. thuringiensis* subsp. *galleriae* by a single biochemical test, lecithinase production. Among

Table 2. Distribution of *B. thuringiensis* in soil

Districts	*No. of samples examined*	*% of samples with B. thuringiensis isolate (no. of samples)*	*B. thuringiensis indexa (no. of B. thuringiensis isolates)*
Chennai	4	50.0 (2)	0.22 (2)
Kanchipuram	4	50.0 (2)	0.33 (2)
Vellore	4	100.0 (4)	0.37 (3)
Krishnagiri	4	75.0 (3)	0.28 (4)
Villupuram	4	50.0 (2)	0.18 (2)
Nilgiris	4	75.0 (3)	0.40 (4)
Erode	4	50.0 (2)	0.22 (2)
Salem	4	75.0 (3)	0.38 (5)
Ariyalur	4	25.0 (1)	0.16 (1)
Perambulur	4	50.0 (2)	0.25 (2)
Namakkal	4	75.0 (3)	0.46 (6)
Coimbatore	4	75.0 (3)	0.36 (4)
Tiruppur	4	50.0 (2)	0.28 (2)
Karur	4	75.0 (3)	0.25 (2)
Trichy	4	75.0 (3)	0.27 (3)
Nagapattinam	4	33.3 (1)	0.16 (1)
Tiruvarur	4	66.6 (2)	0.40 (2)
Thanjavur	4	100.0 (4)	0.30 (4)
Pudukottai	4	75.0 (3)	0.33 (2)
Dindigul	4	100.0 (4)	0.30 (4)
Theni	4	100.0 (4)	0.33 (4)
Madurai	4	75.0 (2)	0.44 (4)
Virudhunagar	4	50.0 (2)	0.12 (1)
Tuticorin	4	50.0 (2)	0.28 (2)
Tirunelveli	4	75.0 (3)	0.36 (4)
Kanyakumari	4	75.0 (3)	0.60 (3)
Ramanathapuram	4	50.0(2)	0.28 (2)
Total	108	64.8 (70)	0.31 (78)

[a]*B. thuringiensis* index was calculated as a number of *B. thuringiensis* strains isolated divided by the number of colonies of all bacteria examined.

78 *B. thuringiensis* isolates, a maximum of 12 (15.4%) *B. thuringiensis* subsp. *kurstaki* strains were isolated from different soil samples. *B. thuringiensis* subsp. *israelensis* was not present in any of the samples. The biochemical types 9, 10, 11, 13, 15 and 16 made up a cluster of strains which accounted for 48.7% of all environmental isolates. The biochemical type of each isolates is recorded (Table 3). The results are similar to the study reported by Martin and Travers (1989), but they have got more isolates of six undescribed *B. thuringiensis* biochemical types (52%) than the known biochemical types (48%).

The widely accepted and well established typing method for *B. thuringiensis* strains is serotyping. But the current serotyping system is not suitable for auto agglutinated strains, non-motile strains and strains lacking a parasporal inclusion body. From the point of bacterial systematic classification, serotyping is a phenotypic system which cannot reveal phylogenetic relationships among the strains (Joung and Côté, 2001).

Analysis of AFPF-PCR amplified products: PCR was performed using cell lysate as DNA template with Fla5 and Fla3 primers. AFPF-PCR yielded multiple distinct DNA products of sizes ranging from approximately 100 to 2000bp of more than 300 fragments from 78 *B. thuringiensis* strains (Fig. 1). All *B. thuringiensis* isolates differed from one another in the specific amplified patterns of the PCR products, which correspond to the presence of flagellin gene sequence. In these isolates the major number of bands was observed at the size of 100, 225 and 425bp. Most of the amplified products were observed

Table 3. Biochemical types of *B. thuringiensis* and its occurrence in Tamil Nadu, India[a]

Biochemical Type	*Biochemical test result*[b]				*B. thuringiensis isolates in Tamil Nadu, India (%)*
	Esculin	*Salicin*	*Lecithinase*	*Sucrose*	
1 (*thuringiensis*)	+	+	+	+	4 (5.1)
2 (*kurstaki*)	+	+	+	-	12 (15.4)
3 (*indiana*)	+	+	-	+	3 (3.8)
4 (*galleriae*)	+	+	-	-	1 (1.3)
5 (*sotto*)	+	-	+	+	3 (3.9)
6 (*dendrolimus*)	+	-	+	-	11 (14.1)
7 (*morrisoni*)	+	-	-	+	3 (3.8)
8 (*darmstadiensis*)	+	-	-	-	2 (2.6)
9 (Biochemical type 9)	-	+	+	+	4 (5.1)
10 (Biochemical type 10)	-	+	+	-	6 (7.7)
11 (Biochemical type 11)	-	+	-	+	1 (1.3)
12 (ostriniae)	-	+	-	-	1 (1.3)
13 (Biochemical type 13)	-	-	+	+	9 (11.5)
14 (*israelensis*)	-	-	+	-	0 (0)
15 (Biochemical type 15)	-	-	-	+	10 (12.8)
16 (Biochemical type 16)	-	-	-	-	8 (10.3)
Total					78

[a]Martin and Travers, 1989
[b]The + sign indicates a positive reaction; – sign indicates a negative reaction

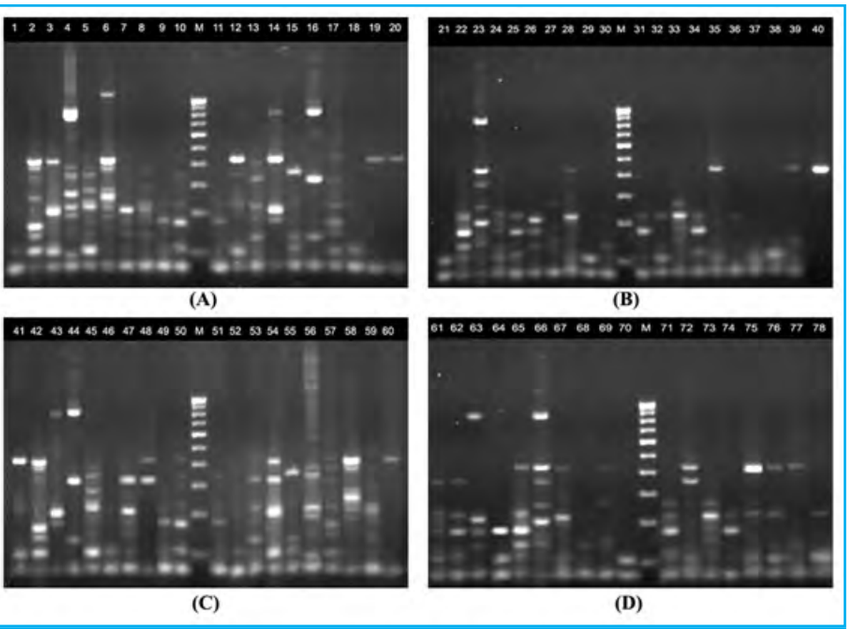

FIGURE 1. PCR-AFPR fingerprint patterns of environmental isolates of *Bacillus thuringiensis*. (A) *Bacillus thuringiensis* Tamil Nadu (BTTN) isolates BTTN01 – BTTN20; (B) BTTN20 – BTTN40; (C) BTTN41 – BTTN60; (D) BTTN61 – BTTN78. Lane M, 100bp ladder.

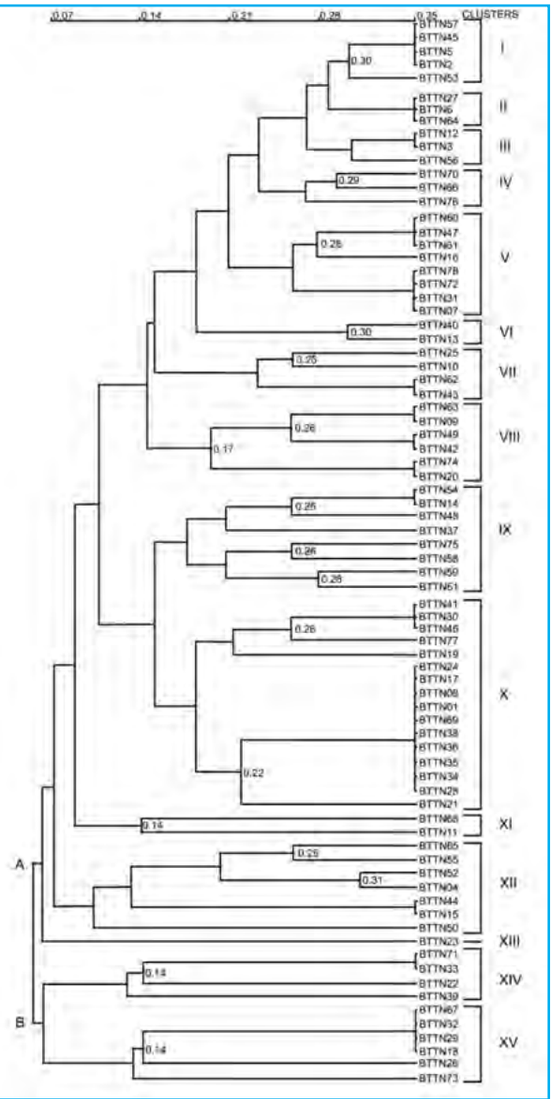

FIGURE 2. UPGMA dendrogram derived from similarity coefficients calculated by the Jaccard method based on the amplified bands obtained during PCR-AFPF analysis. The dendrogram shows the relationships among environmental isolates of *B. thunringiensis*.

between 100 to 1000bp and only few of the isolates (BTTN4, 6, 16, 23, 44, 56 and 66) contained fragments more than 1000bp. Only a distinct single product with a size of about 100 or 425bp was obtained in the strains such as, BTTN19, 20, 21, 29, 38, 40, 60 and 70. The maximum of 12 to 14 bands ranged from sizes 100 – 2000bp were observed in BTTN4, 23, 56 and 66.

PCR-AFPF is a preliminary attempt of Yu et al. (2002) to classify the subspecies of *B. thuringiensis* obtained from the Institute of Pasteur, France and in the present study this tool is used to discriminate between the *B. thuringiensis* serovars isolated from the environment. The chromosome of all *B. thuringiensis* strains contain flagellin gene and it was present even in their closely related species *B. cereus* and *B. anthracis*. Its PCR amplification pattern analysis is not subjected to any limitation associated with the *B. thuringiensis* serotyping system. The phyolgenetic analysis of *B. thuringiensis* using PCR-AFPF would be more accurate, than previously described molecular classification methods such as RAPD, RFLP, 16s rRNA probe, specific DNA probe, and ISR (Yu et al., 2002). The AFPF primers recognises differences in the prevalence and positions of annealing sites in the genome producing sets of fragments that are considered to reflect the genomic composition of the strain, therefore it gives a good opportunity to detect biodiversity of a group of isolates.

Phylogenetic analysis using UPGMA method: The genetic distance, Jaccard coefficient values were calculated from amplified bands and the values were ranged from 0.07 to 0.035 (Fig. 2). An unweighed pair group method with average (UPGMA) dendrogram was constructed using the Jaccard coefficient. In UPGMA dendrogram, the *B. thuringiensis* isolates were divided into 2 groups, group A and B. Group A was further classified into 13 clusters (I - XIII), which comprised of 68 *B. thuringiensis* isolates and remaining 10 strains, BTTN71, 33, 22, 39, 67, 32, 29, 18, 26 and 73 were categorized in cluster XIV and XV and these two clusters were grouped under group B. Among I to XV clusters, cluster I, X, XII and XIV contains the mixture of different *B. thuringiensis* biochemical types and rest of the 11 clusters contains any one of the biochemical types. The maximum of 20.5% of *B. thuringiensis* isolates were classified under cluster X and it comprised 6.25% of biochemical type 9, 25% of *B. thuringiensis* subsp. kurstaki and 68.75% of *B. thuringiensis* subsp. dendrolimus. At the least of cluster XIII contained only one biochemical type 9 strain and this type showed more degree of variations in their band pattern among their biochemical type and these four biochemical type 9 strains were characterized under 3 clusters (X, XI and XIII). This confirmation of variation or intermixing of biochemical type 9 within the clusters suggests that the specific phenotypes were acquired after the ancestors to each of the clusters were formed. The study supports the idea that horizontal gene transfer of plasmid is an important factor in defining the phenotypes of biochemical type 9 isolates evolved along perceptible large evolutionary distances to give rise to different clusters.

The study of Katara et al. (2012) demonstrated that molecular typing and diversity analysis of *B. thuringiensis* has enormous importance for discrimination of strains isolated from different sources. They distinguished 113 native *B. thuringiensis* strains isolated from various

locations in India using REP-PCR and ERIC-PCR. They explored that the *B. thuringiensis* isolates collected from diverse habitats in India had a high degree of genetic diversity. Similar to them, the *B. thuringiensis* strains which were isolated from Tamil Nadu showed diverse range of patterns and high level of genetic diversity.

CONCLUSION

Through the present study, it is suggested that the association between *B. thuringiensis* and insects is not obligative. *B. thuringiensis* was omnipresent when compared to other bacterial strains and based on the nutrient requirements the growth turnover is possible. There is no need that the population of *B. thuringiensis* has to be more in the soil samples with high levels of insect activity because several soil samples had been collected from the mosquito breeding regions but no *B. thuringiensis* isolates had shown any degree of mosquito larvicidal activity. Finally, the genetic heterogeneity were analysed in the *B. thuringiensis* strains isolated from Tamil Nadu using AFPF-PCR. This technique could be used for the separation of novel *B. thuringiensis* isolates from the environment.

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Ergonomic effects on workers of selected healthcare areas of King Abdulaziz Medical City, National Guard Hospital, Riyadh Saudi Arabia

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ABSTRACT

Ergonomics' is composed of two words which are 'ergo' a Greek word meaning work, and 'nomics' which means study. Ergonomics factors that contribute to the health are inappropriate lighting, tools design, chair design, heavy lifting and repetitive motion and others. These factors can cause musculoskeletal disorders (MSDs). The aim of this study was to assess ergonomic effect on workers of ultrasound and microbiology areas in King Abdulaziz Medical City, (KAMC)-Riyadh. The second objective of this study was to identify the ergonomic factors and the presence of work related injuries, also to compare awareness of ergonomics of work sites of health workers. This study was a cross sectional quantitative study conducted at KAMC,National Guard Hospital (NGH), the sample size for workers in radiology department was 27, and the sample size for workers in the laboratory was 27. Two questionnaires distributed among laboratory and radiology workers constructed of questions and demographic data were adapted for our study to determine the effect of ergonomics, the most common physical problems that workers experience in their workstation, and the awareness of ergonomic among the health care workers 18 participants (40.0% of the total) from the Microbiology-technicians were completed the questionnaire and were 27 participants (60.0%) form the Ultrasound-sonographers completed the questionnaire. There was an ergonomic effect on gender for microbiology technicians (p-value was 0.043). Moreover, the ultrasound-sonographers had a significant association between gender and pain

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related to work (p value < 0.001). Awareness however, in microbiology technicians, was found to be 83% who knew the meaning of ergonomics and 85% in ultrasound sonographers. In conclusion, there was a significant effect of ergonomic on sonographers and microbiology technician. The study showed a good level of knowledge and awareness about ergonomics, however, still there is a quiet high percentage of them who did not receive health education on ergonomics and also a high percentage who aren't implementing ergonomics. There is a need for educational and implementation empowerment programs in this regard.

KEY WORDS: ERGONOMIC, ULTRASOUND, MICROBIOLOGY, OCCUPATIONAL THERAPY, MUSCULOSKELETAL DISORDERS

INTRODUCTION

'Ergonomics' is composed of two words which are 'ergo' a Greek word meaning work, and 'nomics' which means study, Tayya et al. (1997) Pao et al. (2001) have defined ergonomics as a branch of science which analyses the optimal relationship between workers and their environment. It is also described as a system that the workers interact with the work environment, the tasks and the workplace, (Brooks 1998). Ergonomics significantly developed during World War II, and now includes design, medicine, and computer science, (Goyal et al., 2009) as it includes a variety of conditions that can affect workers in different aspects such as health and comfort. Besides, it also has factors that are contributing to the health such as lighting, tools design, chair design, heavy lifting and repetitive motion and others. These factors can cause injuries and problems related to muscles, tendons or nerves which can lead to musculoskeletal disorders (MSDs), (Jaffar et al., 2011, Mcatamney et al., 2017).

Musculoskeletal disorders MSDs are injuries and disorders that affect the nerves and soft tissues which are muscles, tendons, ligaments and joints. According to Yelin et al. (1999) the majority of the old workers were disabled due to MSDs, which were also observed among female care givers. Therefore, poor ergonomics have an impact on the worker's health and can lead to occupational health injuries. According to Lee, ergonomics is promoting compatibility between humans and systems, (Lee 2017).

Considering the workers' limitations and capabilities by fitting environments, and tasks to improve the productivity and safe work performance reduce costs due to work injuries. There are many types of jobs which require moderate to heavy physical work such as health care workers, engineers, food industry workers, manual workers, and office workers and other service staff. Inappropriate workplace design, tools and equipment machine lead to fatigue, frustrate, and hurt the workers. The most extreme risk factors which affect the workers on the worksite are the uncomfortable static position, repetitive motion, vibration, heavy lifting, temperature, and lighting. Many studies have shown that work injuries and pain caused by risk factors of ergonomics result from frequent bending, twisting, heavy physical activities, heavy (manual) lifting and whole-body vibration, (Estryn-Behar et al. 1990, Kuiper et al. 1999 and Lee 2017).

One of the most arduous professionals that require overload on the body, forced position, and long working hours are working in health care. Doctors, Nurses, Radiologists, Dentists and other groups in the healthcare professions show a high incidence of work-related injuries and pain result from a singular (acute) event to gradual events of repetitive movement which lead to handling patients and equipment. Several studies are showing high-risk factors of ergonomics among healthcare workers. The primary factors which can increase musculoskeletal injuries and induce pain among health care workers are load (weight and size of materials, the force needed to push or pull, a position of handholds, and a shape of handles) and posture (disadvantageous positions of the arms and legs, forward bend and twist of the trunk) and environment (inappropriate floor conditions, insufficient equipment, inadequate lighting and thermal conditions, and time pressure). Ergonomics have a high impact on the worker, and poor ergonomics may lead to MSDs. The job of health care workers and other professionals demand a tremendous physical load to improve the productivity of healthcare and hence adequate quality; the ergonomics prevents the risk factors and work-related injuries. Proper office ergonomics contribute to increase workers' effectiveness and reduce musculoskeletal injuries that associated with office working. Recent studies pointed out that poor ergonomics of the above areas lead to MSDs and pain on the workers.

Work in radiology area demands physical tasks such as patient's transfer and using imaging equipment and computer-related task. Accordingly, improved ergonomics of the radiology department will contribute to reduce the risk of work-related injuries and provide the safety when dealing with patients, (Siegal et al. 2010, Ruess et al. 2003). In regards to laboratory work, it needs a prolonged standing position. Because of that laboratory healthcare workers are more exposed to MSDs and poor ergonomics can lead to pain in the different area of the body, (Agrawal et al. 2015).

There is a paucity of data on the ergonomics of places involving patient care, sites of diagnosis of diseases such as radiology and laboratories. Hence the present study was planned and proposed so that the evidence created from the study will give light to the ergonomic effects

on workers of selected healthcare areas. This study was aimed to assess the ergonomic effect on workers of ultrasound and microbiology technicians of areas in KAMC-Riyadh, and to identify the ergonomic factors and the presence of work-related injuries, it also compared the ergonomical awareness of the workers.

MATERIAL AND METHODS

This was a cross-sectional quantitative study conducted at National Guard Hospital (NGH), in Riyadh over 6-months period from Aug to Nov 2017. The study was approved by the Institutional Review Board at King Abdullah International Medical Research Center (KAIMRC). The study included technicians from two different areas. The first area is radiology; the inclusion criteria of radiologist workers were a technician working at Ultrasound areas (which include sonographers works in general Ultrasound, Mammogram areas, Echo areas, and OB-GYN areas), including all ages and both genders. The exclusion criteria were the other areas such as Magnetic Resonance Imaging (MRI) and Computed Tomography (CT). The other area is a laboratory, the inclusion criteria were microbiology technician, including all ages and both gender. The exclusion criteria were workers in other areas of laboratory such as hematology. With a population of 60 technicians, 50% margin of error of 95% of confidence level, the required sample size was calculated as 53. Selection of samples based on the departments. The sample size for workers in the radiology department is 18, and the sample size for workers in the laboratory is 27.

A self-developed questionnaire was used to collect the data from the workers. The questionnaires constructed of yes or no questions (microbiology department 37 questions and radiography department 30 questions) and demographic data was adapted for our study to determine the most common physical problems that workers experience in their workstation, the effect of ergonomics, and the awareness of ergonomic among the healthcare workers. The procedure of the study done after identification of the subjects and the consent form was obtained from the participants before enrolling in the study. The questionnaires were distributed randomly among laboratory and radiology workers. All the data collected were analyzed by SPSS version 21 software, and descriptive statistics were used to summarize the data.

RESULTS AND DISCUSSION

STUDY POPULATION

We identified 45 microbiology-technicians and Ultrasound-sonographers. The Microbiology-technicians were 18 participants (40.0%) completed the questionnaire, with a mean age of 26 years (SD = 3.97). The Ultrasound-sonographers were 27 participants (60.0%) completed the questionnaire, with a mean age of 35 years (SD = 7.17) (see Table 1 for further information).

Table 1. Participant characteristics

	Male	Female
Microbiology-technicians (N)	4	14
Percentage (%)	22.2	77.8
Years of experience (mean)	2.67	
Ultrasound-sonographers (N)	4	23
Percentage (%)	14.8	85.2
Years of experience (mean)	10.22	

Ergonomic Effects on Health Care Workers

Ergonomic Effects on Microbiology-technicians

A significant association between gender and pain related to work (p-value was 0.043). We found that 50% of male and 92% of female have pain or discomfort related to work. We reveals that the most common pain positions among both genders were lower back (16%), followed by Feet heel (14%), Neck and shoulder blade (12%), Shoulders and headache (10%), dry eyes (9%), eye strain (7%), depression and wrist (5%). We found that (60%) of both genders reported that the pain started at the end time of the workday (p-value was 0.017). Table 2 & figure 1 shows that most common activities of daily living that affected by work-related pain were social activities and sleep (33%), family demands (27%), work productivity and health maintenance (16%). The results show that, the most common factor the participants were complaining of was chair type (55%), followed by chair width (44%), smell (38%), narrow space (33%), excessive noise (27%), table (16%), high light and computer monitor and contact pressure (11%). Regarding the techniques and changes that technician used to decrease the pain related to work, we found that most of them applying stretching exercise (50%), massage (44%), and the rest either take painkillers, smoking or

Table 2. Activities that affected by work injuries on microbiology technicians

	n	Total	Pearson Chi-Square	p-value
Social activity	6	18	.643[a]	0.432
Sleep	6	18	.643[a]	0.423
Family demands	5	18	.020[a]	0.888
Work productivity	3	18	.257[a]	0.612
Health maintenance	3	18	4.114[a]	0.043

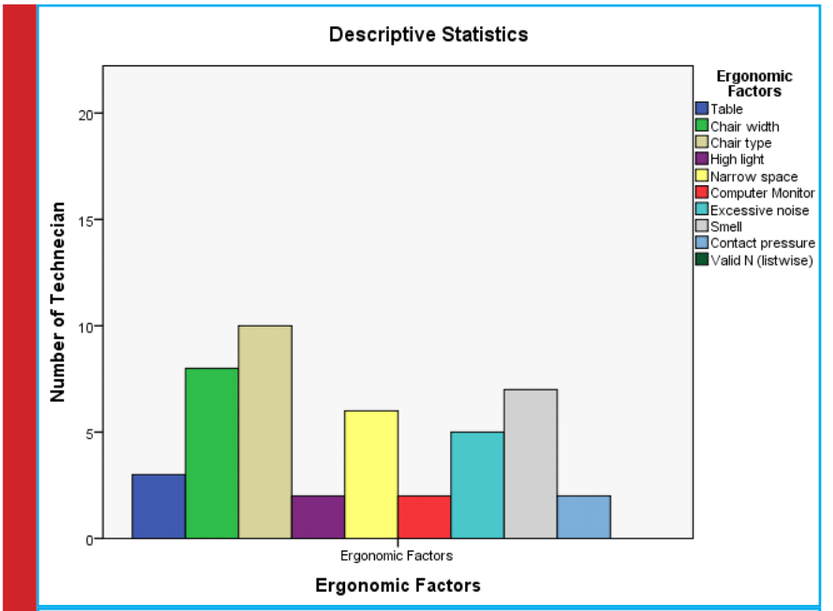

FIGURE 1. The most ergonomic factors that microbiology complaining

nothing. None of them try to visit Occupational therapy clinics to get help and education about some techniques and changes that help to decrease and eliminate pain and injuries related to work. As a solution to reduce and eliminate pain related to work, we found that most of technician suggest to add regular short breaks (50%), decrease the work hours (33%), increase the number of technicians (33%), add regular stretching exercise (33%), made changes in the workplace (22%), and training and education about proper ergonomics (22%).

Ergonomic Effects on Ultrasound-sonographers.

A significant association between gender and pain related to work (p-value < 0.001). We found that 75% of the male and 86% of the female have pain or discomfort related to work. Fig 2 reveals that the most common pain positions among both genders were shoulders (17%), followed by upper back (12%), Neck (11%), shoulder blade and lower back (10%), foot heel and dry eyes (8%), fingers (7%), wrist (6%), and elbow-forearm (5%). We found that (77%) of both genders reported that the pain started at the end time of the workday. Table 3 shows that most common activities of daily living that affected by work-related pain were family demands (48%), work productivity (40%), social activities (37%), sleep (33%), and health maintenance (18%). The results show that, the most common factor the participants were complaining of was chair type (44%), followed by heavy tools (40%), computer monitor (29%), repetitive motion (25%), narrow space and poor light and chair width (14%), excessive noise and high light (7%), and table (3%) (Figure 2). Regarding the techniques and changes that sonographers used to decrease the pain related to work, we found that most of them doing massage (62%), stretching exercise (55%), ask for sick leave and go to physician (14%), and go to physiotherapy clinics (7%). None of them try to visit Occupational therapy clinics to get help and education about some techniques and changes that help to decrease and eliminate pain and injuries related to work. As a solution to minimize and eliminate pain related to work, we found that most of technicians suggest having training and education

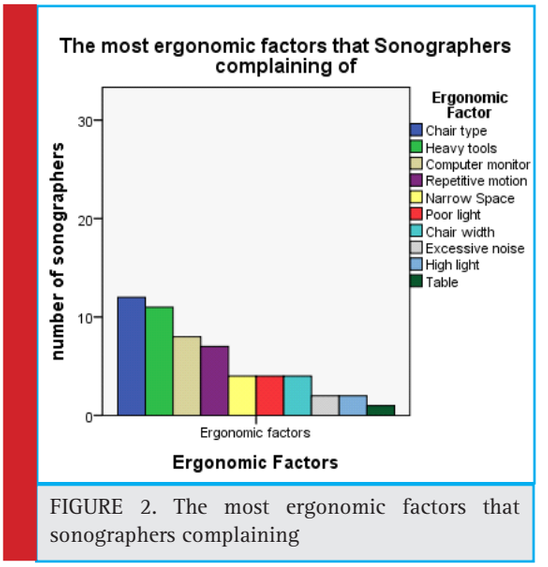

FIGURE 2. The most ergonomic factors that sonographers complaining

Table 3. Activities that affected by work injuries on sonographers

	N	Total	Pearson Chi-Square	p-value
Family demands	13	27	.006[a]	0.088
Work productivity	11	27	.167[a]	0.683
Social Activities	10	27	2.902[a]	0.936
Sleep	9	27	.147[a]	0.720
Health maintenance	5	27	1.067[a]	0.302

about proper ergonomics (62%), increasing the number of sonographers and adding regular stretching exercise (59%), adding regular short breaks (40%), decreasing the work hours (25%), and made changes in the workplace (18%).

3.3. Awareness

Regarding the awareness in Microbiology technician, we found that 83% who know the meaning of ergonomics, 88% who think poor ergonomics lead to health risk, 50% who get an education about proper posture and body mechanics, and 44% who implemented the appropriate posture and body mechanics. In Ultrasound sonographers, we found that 85% who know the meaning of ergonomics, 81% who think poor ergonomics lead to health risk, 66% who get an education about proper posture and body mechanics, and 55% who implemented the proper posture and body mechanics. (see Table 4 for further information).

The current study findings showed that there are significantly higher feelings of pain and discomfort in female compared to male participants. This finding is

Table 4. Awareness of Ergonomics

		Gender		Total	Pearson Chi-Square	p-value
		Male	Female			
Microbiology-technicians Know the meaning of ergonomics	No	1	2	3	.257[a]	.612
	Yes	3	12	15		
Total		4	14	18		
Poor ergonomics lead to health risk	No	1	1	2		
	Yes	3	13	16	1.004[a]	.316
Total		4	14	18		
Educated about proper posture	No	1	8	9		
	Yes	3	6	9	1.286[a]	.257
Total		4	14	18		
Implement proper body mechanics	No	2	5	7		
	Yes	1	7	8		
	not applicable	1	2	3	.815[a]	.665
Total		4	14	18		
Ultrasound-sonographers Know the meaning of ergonomics	No	1	3	4		
	Yes	3	20	23	.386[a]	.534
Total		4	23	27		
Poor ergonomic lead to health risk	No	1	4	5		
	Yes	3	19	22	.131[a]	.718
Total		4	23	27		
Education about proper posture Yes	No	2	7	9		
	2	16	18	.587[a]	.444	
Total		4	23	27		
Implement proper body mechanics	No	1	5	6		
	Yes	1	14	15		
	Not applicable	2	4	6	2.436[a]	.296
Total		4	23	27		

by what had been previously reported in the literature, where the current human results concerning sex differences in experimental pain indicate greater pain sensitivity among females compared with males for most pain modalities. Additionally, many previous studies confirmed differences between genders and documented that women are more likely to experience musculoskeletal pain, (Cook et al., 2000, Ranasinghe et al., 2011).

In line with the study of Kaliniene et al. (2016), the current study showed that low back pain was the highest prevalent type of pain among the studied cohort. Additionally, a similar study conducted in Nigeria, and the authors found a high preponderance of upper and lower back pain Kayoed et al. (2013). The predominant involvement of the back, as observed in the current study, also is in agreement with reports of studies carried out in Roma, British Columbia, and United States, (Pike, et al. 1997, Mirk,et al 1999). Additionally, shoulder pain was among the most reported pain positions in the current study and in that of Kaliniene et al. (2016).

The current study finding highlighted the point that the lack of attention to ergonomics could lead to damage to the healthcare workers mainly in the form of musculoskeletal pain, stress injuries and eye strain which can lead to increase the workers' fatigue and decrease their productivity, and this is in accordance with what has been reported in the literature, (Hills et al., 2012). It has been demonstrated that MSDs are highly relevant in the context of work and that the current economic and social implications of these conditions are sizeable and often underestimated. There are some types of jobs and specific sectors including home care and nursing, represent a heightened risk of developing or aggravating MSDs. The converse of work environments that aggravate MSDs is that work context can also contribute to improvements in MSD outcomes mainly through ergonomic design and job duty adjustment, (Summers, et al., 2015).

In sonography, surveys done among American and Canadian sonographers in 1997 showed that the incidence of MSDs was 84%; however, this incidence had increased to 90% (Evans et al., 2009). An ergonomic workstation in sonography according to Baker and workers should include an ergonomic task chair, the chair should be easy to operate and be adjustable from a settled position, has a different lift, vinyl upholstery that is antimicrobial, a foot ring, special casters, and detailed instructions on its use for different types of studies, however, then they reported that the ergonomic features of the examination room equipment are only as good as the workers willingness to use them, (Baker et al., 2015) .

The effectiveness key of these features is changing the worker work postures so that they maintain neutral postures for the majority of each examination. Comfortable work postures can make any ultrasound workstation ergonomic, increase worker comfort, reduce injury risk, and impact the quality of patient care Baker et al. (2015). In the Nigerian study published in 2013, the most significant proportion of the participants reported that the use of chairs of low height and scanning chair both precipitated and aggravated their symptoms and feeling with pain, and they recommended the use of chairs and scanning tables of ideal heights that can decrease risks to musculoskeletal disorders associated with Ultrasonography, (Kayoed et al., 2013). The current study participants reported that their primary complaint and width. For ultrasound practitioners, to empower safe working practices the room should was from the chairs type be of an adequate size, (Tayyari et al., 1997) with lighting that does not cause glare on the monitor and heating that is suitable for the working conditions, (Baker et al., 2015 and Harrison et al., 2015).

This is in line with what the current respondents reported that narrow space is one of the common factors of their complaint. Massage and stretching exercises were the most common techniques and changes that sonographers in the current study used to decrease the pain related to work. Harrison and Harris (2015) in their review concluded that there are many factors involved in the prevention or the reduction of work-related MSDs for ultrasound practitioners. These factors include ergonomic issues, management of workload, psychosocial factors, physical factors and general fitness levels. Sunley et al. (2006) highlighted that ergonomics education for staff is essential to ensure that they are aware of best practice guidelines, ways of risk reduction to themselves and others and how to report and monitored pain and injury to ensure a long and healthy career, (Sunley et al., 2006). In the current study, results indicated that there is a shortage in ergonomics education; additionally, a high percentage of the respondents show their wish to receive education and training about ergonomics.

As per the results of several studies, the effects of occupational MSD interventions have been particularly strong when using a multi-branched intervention approach, combining physical exercise with another component, like worksite ergonomic changes, (Dawson et al., 2007, Holtermann et al., 2010). Unfortunately, none of the participants in the current study tried to visit occupational therapy clinics to get help and education about some techniques and changes that help to decrease and eliminate pain and injuries related to work. For healthcare workers, the previous study has shown that social, environmental factors such as work demands and social support are related to report MSDs, (Sorensen et al., 2011), which is almost the same with our study findings. Taken together with the evidence from previous interventions, these findings support intervention

strategies that incorporate targeted changes to the physical and social work environment along with worker education.

For tackling MSDs at work, several preventive strategies can be taken. These prevention strategies primarily include risk assessment, and technical/ergonomic, organizational and person-oriented intervention. The secondary prevention strategy involves the identification and health monitoring of workers at risks, while the tertiary prevention strategy comprises a return to work actions, Mirk et al., 1999, EU-OSHA 2008. Suggestions of the current study respondents regarding a solution to decrease and eliminate pain related to work were within the context of those prevention strategies with adding regular short breaks being the highest scored suggestion. In line with this finding, a previous study identified that lack of rest breaks and use of facilities that are not ergonomic were the main contributing factors to work-related MSDs Kayoed et al. (2013).

Some studies on sonographers have also linked regular breaks and reduced workload to reduced musculoskeletal symptoms, Schoenfeld et al. (1999). On the other hand, the work of Schoenfeld et al. (2013) did not find reduced scanning frequency to be associated with reduced symptoms among sonographers. Microbiology technicians participated in the current study showed a right awareness level with the term "ergonomics," a result which is better compared to the Nigerian research about awareness and knowledge of ergonomics among medical laboratory scientists, in which awareness of ergonomics and knowledge of gains of its right application was reduced, Oladeinde et al. (2015).

Additionally, it is better than what recorded elsewhere among computer users and manufacturing workers, (Loo et al., 2012, Shantakumari et al., 2012). The strengths of this study include that the data about ergonomics is scarce in Saudi Arabia, and to the best of our knowledge, this is the first study in Saudi Arabia that assesses the ergonomic effects on workers of selected healthcare areas. Second, healthcare workers especially those who participate in interventional procedures such as laboratory and radiology are well known and more prone to have musculoskeletal pain. Third, the data come from one of the biggest national hospitals in Riyadh. This study has some limitations including mainly the small sample size in microbiology technicians, and the participants were only from one healthcare institution. The results of this study may be further enhanced in the future by increasing the sample size.

CONCLUSION

In conclusion, there is a significant effect of ergonomic on sonographers and the laboratory technicians. The study showed a good level of knowledge and awareness about ergonomics; however, still, there is a quite high percentage of them who did not receive health education on ergonomics and also a high percentage who aren't implementing it. There is a need for educational and implementation empowerment programs in this regard.

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Isolation, biochemical characterization and antibiotic profiling of members of *Enterobacteriacea* isolated from animal fecal matter

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ABSTRACT

In the present study, fecal samples were collected from different localities of Balawala, Dehradun city. Out of 87 isolates, 50% were *E. coli*, 25% were *Klebsiella spp*., 15% were *Enterobacteriaceae spp*. and 10% were *Proteus spp*. The isolates were then checked for antibiotic sensitivity. 50% strains were resistant for novabiocin, 25% were resistant for cefixime, 15% were resistant for clotrimazole and 10% were resistant for amoxicillin and most of these showed sensitivity against the antibiotics- Amikacin, amoxicillin, cefixime, cephalexin, ciprofloxacin, clotrimazole, gentamicin, novabiocin, ofloxacin and trimethoprim. In the minimum inhibitory concentration test, 50% of the isolates showed resistance against the antibiotics amoxicillin, ampicillin, streptomycin at different concentrations (8µg/ml, 16µg/ml, 32µg/ml, 64µg/ml and 128µg/ml respectuvely) and 50% showed sensitivity against the antibiotics cefoparazone sulbactum, meropenem and piperacillin tazobactum. In conclusion, the data of the present study determine the resistance profile of enteric pathogens in animal fecal samples and is helpful from the community infection point of view. The study provides some insight on the prevalence dynamics of enteric pathogens from animal fecal which can be helpful to clinicians to formulate proper antimicrobial therapy.

KEY WORDS: AMIKACIN, RESISTANCE PROFILE, MINNIMUM INHIBITORY CONCENTRATION, MRSA, ENTEROBACTERIACEA

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INTRODUCTION

Bacterial resistance to antibiotics continues to curb our ability to treat, cure and control infectious diseases. Two organisms in particular that have become major public health threats are methicillin-resistant *Staphylococcus aureus* and penicillin-resistant *Streptococcus pneumoniae*. Resistance to aminocyclitol aminoglycosides is an important clinical problem since these antibiotics are widely used in the treatment of serious infections, **(Larson *et al.,* 1986; Garcia *et al.,* 1989).** Large quantities of enteric bacteria from animal fecal wastes can be released into rivers and lakes that serve as sources of water for drinking, recreation or irrigation. Fecal contamination is considered to be main contributor of enteric pathogens to natural water sources. Infection originating from such sources specially diarrhea and typhoid fever. The family of *Enetrobacteriaceae* is accountable for these illnesses. The important members of *Enterobacteriaceae* are *E. coli, Salmonella* and *Shigella*. Amikacin has been the drug of choice for treating nosocomial infections refractory to other aminoglycosides **(Gerding *et al*., 1990; Levine *et al*., 1985, Kalita *et al*., 2016).**

In recent years, resistance to amikacin due to production of 3'-aminoglycoside-phosphotransferases, 2" –adenyltransferases and aminoglycoside-6'- N-acetyltransferases has been reported **(Hopkins *et al.,* 1991; Shaw *et al*., 1993; Shimizu *et al.,* 1985).** Transmission of this microbe is usually through uncooked meats and eggs. The disease is spread via the fecal-oral route and requires very low cell numbers to initiate infection. In many cases, *Shigella* infection will lead to diarrhea accompanied by fever. Among the disease caused by poultry and other farms and their products some are often severe and sometimes lethal infection such as meningitis, endocarditis, urinary tract infections, septecimia, epidemic diarrhea of adults and children. Resistance are more commonly observed among isolates of animal fecal. The relatively intensive conditions under which animal are housed may be associated with greater disease potential and therefore a greater potential and therefore a greater tendency for antibiotic use of disease control **(Bywater *et al*., 2004).**

Resistance to antimicrobials and particularly multidrug resistance is an emerging problem in Enterobacteriaceae for developing and developed countries **(Schwarz and White, 2005).** Resistant microorganisms have emerged as a result of improper use of antibiotics in human health as well as in agricultural practices **(Khachatourians, 1998).** Investigators have reported evidence of some low-level resistance to antibiotics, but overall the bacteria studied were sensitive to most antibiotics prior to exposure **(Datta and Hughes, 1983; Dancer, 1997).**

MATERIALS AND METHODS

Isolation of Enteric Pathogens: Sample was diluted appropriately in sterile saline by serial dilution method and then an appropriate dilution (0.1ml) was plated on selective media and incubated at 37 ˚C for 24 to 48 h **(Pelcezar *et al.,* 1986)** and then observed for the growth.

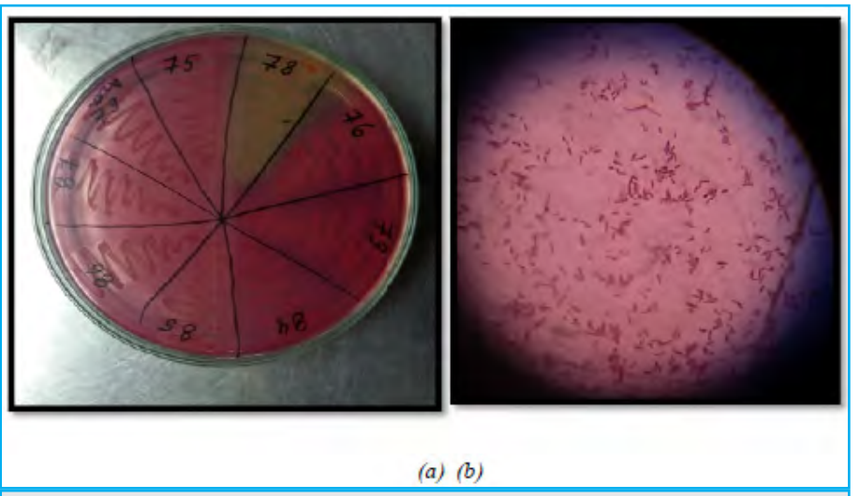
Identification and characterization of Enteric pathogens: All suspected colonies on respective selective media were presumptive forms identified using identification scheme of Bergey's manual (1997) that identifies bacteria on the basis of morphological, cultural and biochemical characteristics. The methods suggested in the microbiological methods were followed **(Borrego and Figueras, 1997)** for characterization of the bacterial isolates.

Antibiotic Susceptibility Test: Bacterial isolates viz., *E. coli, Enterobacteriaceae, Klebsiella sps., Proteus sps.* were screened for their sensitivity to antibiotics because the frequency of occurrence of these pathogens was very high. Multidrug resistant strains of these pathogens are emerging worldwide. Overnight growth of respective bacterial isolates was used for the sensitivity test. The Kirby Bauer modified disk diffusion technique was was used to determine the sensitivitity to antibiotics.The polydiscs (Micromaster Laboratories) were evenly distributed on sterile Mueller Hinton agar medium. Plates were then incubated at 37 ˚C for 24 h. The inhibition zone diameters were measured using meter scale. Inhibition zone diameters were compared with the standard inhibition zone for resistance, intermediate and susceptible character **(Kalita *et al*., 2016).**

Minimal Inhibitory Concentration (MIC): Minimum inhibitory concentration was determined according to the method described earlier by adding various concentrations of antibiotics (8-128 µg/ml) in Nutrient Broth. Further, 100 µl of inoculum was added to each tube and incubated the tubes at 37°C for 24 hours **(Sharma *et al*., 2011).**

RESULTS AND DISCUSSION

Isolation of Enteric pathogens from Animal excreta: Samples of animals were collected aseptically and transported to the laboratory immediately for isolation of enteric pathogens on Mac-Conkey agar, Eosine methylene agar, Cystine–lactose–electrolyte-deficient agar plates. The plates were incubated for 14- 16 hours *at* 37°C and after incubation observations were made there are appearances of isolated colonies. The isolated colonies were further pure cultured by sub-streaking on Mac-Conkey agar plates **(shown in Fig. 1).** The culture



(a) (b)

FIGURE 1. Isolation of Enteric pathogen(a) Selective Isolation of Enteric Pathogens on Ma Conkey Agar (b)Microscopic Observation- Gram Negative Rods

thus obtained and the details of healthy animals and humans are given in **Table 1**

In this study susceptibility pattern of pathogens liable for urinary tract infections in Poland to cogently used antimicrobial agents. A most entire study of 141 pathogens from hospital – acquired infections and 460 pathogens from community- acquired infections were isolated between July 1998 and May 1999. The most common ecological agent was *E. coli* (73.0 %), followed by *Proteus spp*. (8.9 %) and other species of *Enterobacteriaceae* (9.6 %). Few community infections were caused by Gram-positive cocci were isolated more frequently from a hospital setting (14.1 %) and the most common was *Enterococcus spp*. (8.5 %). *Pseudomonas aeruginosa* was found only among hospital isolates and was responsible for 10.7 % of infections. *E.coli* isolates from both community and hospital infections were highly affected to many antimicrobial agents with the explusion of those isolates generating elongated spectrum beta- lactamases (ESBLs). Of all *Enterobacteriaceae* tested, 38 strains (6.9 %) were able to generating ESBLs **(**Ahmed *et al*., 2011**)**.

ANTIBIOTIC SENSITIVITY TEST

Antibiotic sensitivity of all the 87 isolates was determined against 10 antibiotics belonging to β-lactam and non β-lactam group. The antibiotics included are Amikacin, Amoxycillin, Cefixime, Cephalexin, Ciprofloxacin, Clotrimazole, Gentamycin, Kanamycin, Novabiocin and Ofloxacin. There sensitivity to different antibiotics is represented in *Graph 1, 2 & 3*.

According to **Ergin & Mutlu,** 197 bacterial isolates from Sudanese patients with diarrhea or urinary tract infections. *Shigella dysenteriae* type 1 and enteropathogenic *E. coli* Showed high resistance rates against the commonly used antimicrobial agents: ampicillin, chloramphenocol, amoxycillin, co-trimoxazole, tetracycline, malidixic acid, sulfonamide and neomycin. The uropathogens wre completely sensitive to ciprofloxacin. Resistance to tetracycline, amoxicillin, ampicillin, cotrimoxazole and sulfonamide was the most frequent pattern. The common urinary tract pathogens *Klebsiella pneumonia, E. coli* and *Proteus mirabilis* showed high rates of resistance to ampicilin, co-trimoxazole, amoxicillin, tetracycline, trimethoprim, sulfonamide, streptomycin and carbenicillin.

MINIMUM INHIBITORY CONCENTRATION

Of all 87 samples 25 samples were selected for carrying out MIC of Amoxycillin, Ampicillin, Pipracillin tazobactum, Streptomycin, Meropenem and Cefoparazone sulbactum. The MIC was conducted at different concentrations like (8µg, 16µg, 32µg, 64µg and 128µg). Maximum isolates showed resistance against Amoxycillin and minimum against Meropenem and Cefoparazone sulbactum. In decresing order of resistance antibiotics can be placed as Amoxycillin>Ampicillin>Streptomycin >Pipracillintazobactum>Meropenem>Cefoparazone sulbactum. The MIC result of isolates is shown in *Graph. 4, 5, 6 & 7*.

In this study they determined the distribution rates of *Pseudomonas aeuroginosa* in clinics and its resistance to antibiotics. The antibiotic resistance rates were detected by minimal inhibitory concentration (MIC). The clinical and specimen distribution properties of *Pseudomonas* were evaluated based on their resistance pattern. *Pseudomonas* was the fourth common bacteria in all isolates.

Table 1. Cultures obtained from Animal Fecal Matter

S. No	Sample Number	Growth On MacConkey Agar	Morphology	Motility
1.	AH1	Small pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
2.	AH2	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
3.	AH3	Pink color colnies/ pink background	Small pink color colonies and mucoid	-ve
4.	AH4	Pink color colonies/ pink background	Small pink color colonies and mucoid	-ve
5.	AH5	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
6.	AH6	Pink color colonies/ pink background	Small pink color colonies and mucoid	-ve
7.	AH7	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
8.	AH8	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
9.	AH9	Pink color colonies/ pink background	Small pink color colonies and mucoid	-ve
10.	AH10	Yellow swarming colonies/ yellow background	Yellow color colony and show motility	+ve
11.	AH11	Small pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
12.	AH12	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
13.	AH13	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
14.	AH14	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
15.	AH15	Yellow color colonies/ yellow background	Gram –ve, non-motile and rod shaped	-ve
16.	AH16	Colorless colonies/ white background	Gram –ve, non-motile and rod shaped	+ve
17.	AH17	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
18.	AH18	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
19.	AH19	Translucent gummy colonies/ pink background	Gram –ve, non-motile and rod shaped	+ve
20.	AH20	Colorless colonies/ pink background	Gram –ve, non-motile and rod shaped	+ve
21.	AH21	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
22.	AH22	Pink color colonies/ pink background	Small pink color colonies and mucoid	-ve
23.	AH23	Translucent gummy colonies/ pink background	Gram –ve, non-motile and rod shaped	+ve
24.	AH24	Colorless colonies/ pink background	Gram –ve, non-motile and rod shaped	+ve
25.	AH25	Yellowish gummy colonies/ yellow background	Gram –ve, non-motile and rod shaped	-ve
26.	AH26	Colorless colonies/ white background	Gram –ve, non-motile and rod shaped	+ve
27.	AH27	Colorless colonies/ white background	Gram –ve, non-motile and rod shaped	+ve
28.	AH28	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
29.	AH29	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
30.	AH30	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
31.	AH31	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
32.	AH32	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
33.	AH33	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
34.	AH34	Pink color colonies/ pink background	Small pink color colonies and mucoid	-ve
35.	AH35	Colorless colonies/ pink background	Gram –ve, non-motile and rod shaped	+ve
36.	AH36	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
37.	AH37	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
38.	AH38	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
39.	AH39	Pink color colonies/ pink background	Small pink color colonies and mucoid	-ve
40.	AH40	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
41.	AH41	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
42.	AH42	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
43.	AH43	Colorless colonies/ pink background	Gram –ve, non-motile and rod shaped	+ve
44.	AH44	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve

44.	AH44	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
45.	AH45	Colorless colonies/ pink background	Gram –ve, non-motile and rod shaped	+ve
46.	AH46	Colorless colonies/ pink background	Gram –ve, non-motile and rod shaped	-ve
47.	AH47	Translucent gummy colonies/ pink background	Gram –ve, non-motile and rod shaped	+ve
48.	AH48	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
49.	AH49	Small orange color colonies/ pink background	Gram –ve, non-motile and rod shaped	+ve
50.	AH50	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
51.	AH51	Colorless colonies/ pink background	Gram –ve, non-motile and rod shaped	+ve
52.	AH52	Pink color colonies/ pink background	Small pink color colonies and mucoid	-ve
53.	AH53	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
54.	AH54	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
55.	AH55	Translucent gummy colonies/ pink background	Gram –ve, non-motile and rod shaped	+ve
56.	AH56	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
57.	AH57	Translucent gummy colonies/ pink background	Gram –ve, non-motile and rod shaped	+ve
58.	AH58	Colorless colonies/ pink background	Gram –ve, non-motile and rod shaped	+ve
59.	AH59	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
60.	AH60	Colorless colonies/ pink background	Gram –ve, non-motile and rod shaped	-ve
61.	AH61	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
62.	AH62	Yellow swarming colonies/ yellow background	Yellow color colony and show motility	+ve
63.	AH63	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
64.	AH64	Colorless colonies/ pink background	Gram –ve, non-motile and rod shaped	-ve
65.	AH65	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
66.	AH66	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
67.	AH67	Yellow swarming colonies/ yellow background	Yellow color colony and show motility	+ve
68.	AH68	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
69.	AH69	Colorless colonies/ pink background	Gram –ve, non-motile and rod shaped	+ve
70.	AH70	Translucent gummy colonies/ white background	Gram –ve, non-motile and rod shaped	+ve
71.	AH71	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
72.	AH72	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
73.	AH73	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
74.	AH74	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
75.	AH75	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
76.	AH76	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
77.	AH77	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
78.	AH78	Translucent gummy colonies/ yellow background	Gram –ve, non-motile and rod shaped	+ve
79.	AH79	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
80.	AH80	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
81.	AH81	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
82.	AH82	Pink color colonies/ white background	Small pink color colonies and mucoid	+ve
83.	AH83	Colorless colonies/ white background	Gram –ve, non-motile and rod shaped	+ve
84.	AH84	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
85.	AH85	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
86.	AH86	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve
87.	AH87	Pink color colonies/ pink background	Small pink color colonies and mucoid	+ve

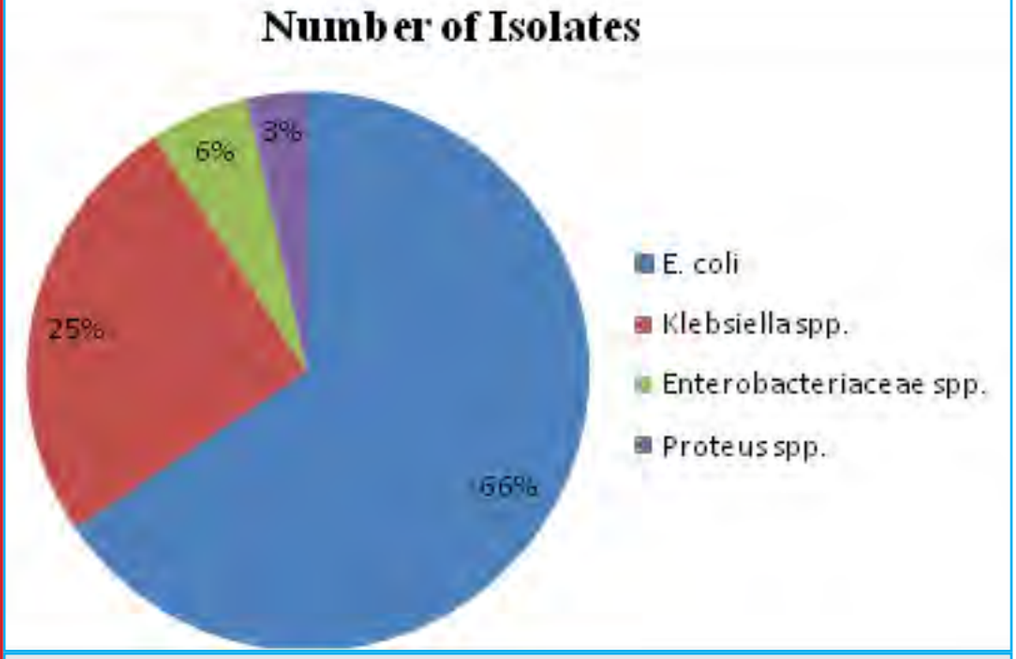
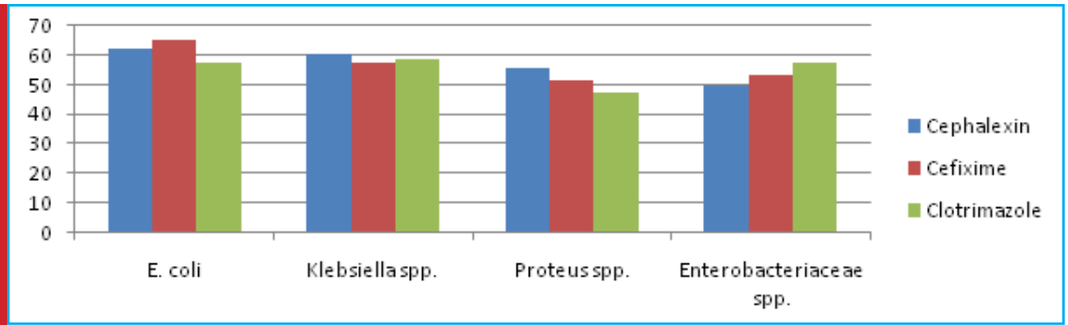
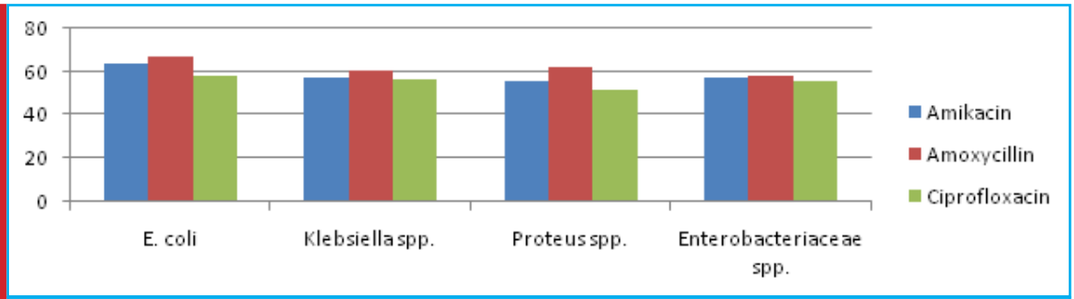


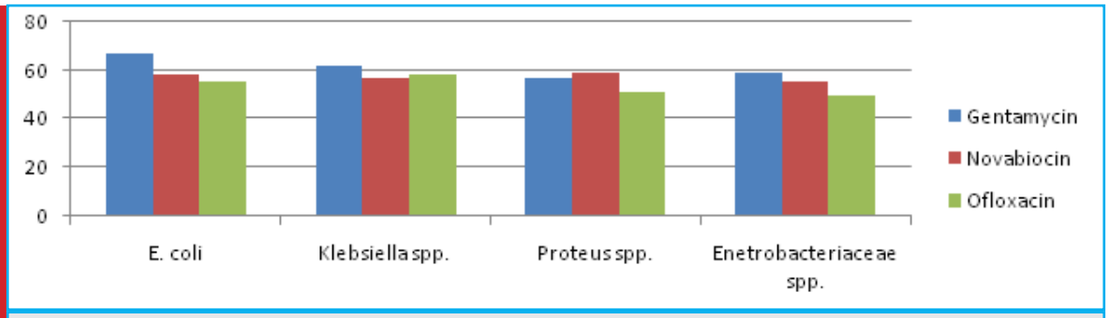
FIGURE 2. Cultures obtained from Animal Fecal Matter



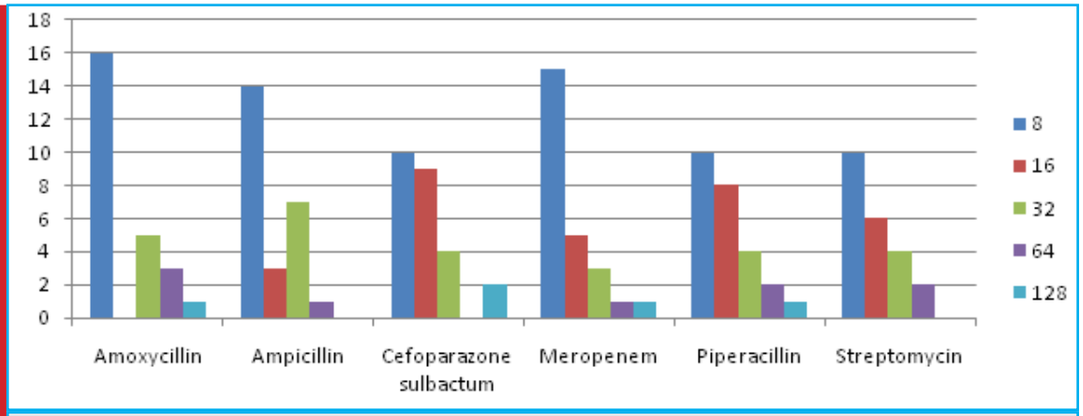
GRAPH 1. Antibiotic Sensitivity Of Selected Strain To β-Lactam Antibiotics



GRAPH 2. Antibiotic Sensitivity Of Selected Strain To Non β-Lactam Antibiotics



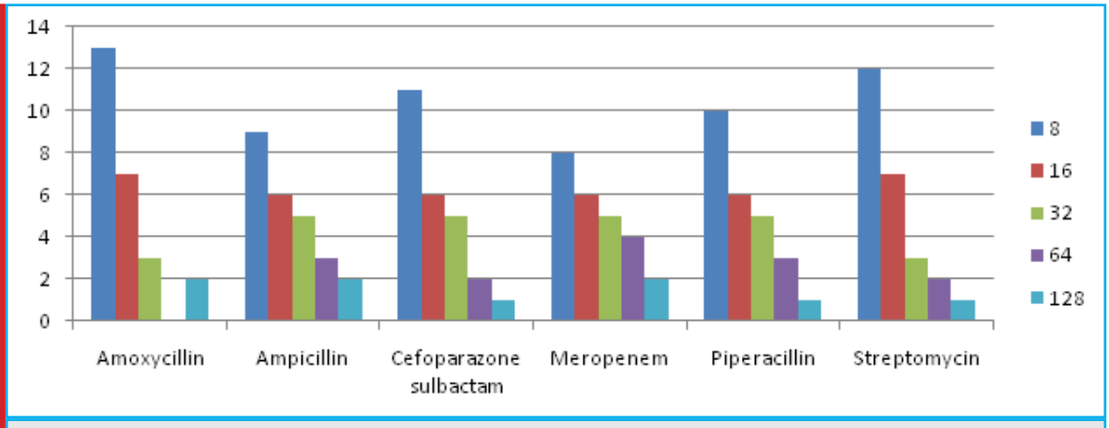
GRAPH 3. Antibiotic Sensitivity Of Selected Strain To Non β-Lactam Antibiotics



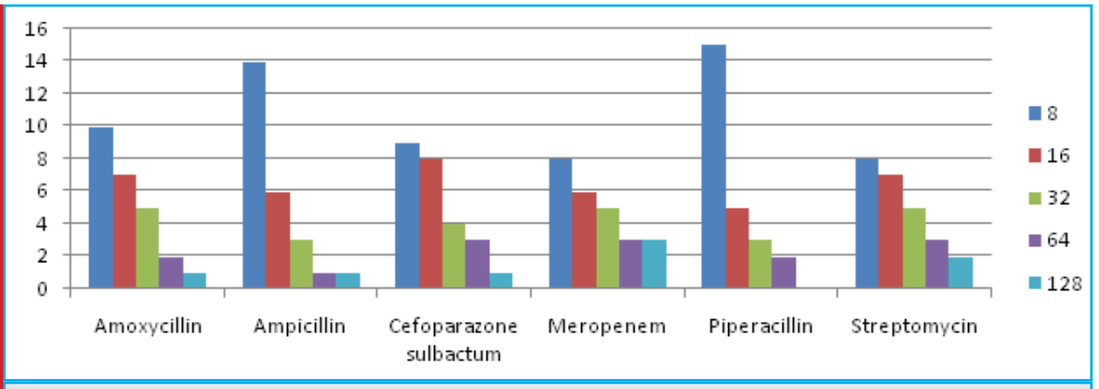
GRAPH 4. MIC Concentration Against Different Antibiotics for E.Coli

Tracheal aspirates, sputum and wound, pus were important sources for *Pseudomonas aeruginosa* isolation in intensive and nonintensive care units of surgery wards (SW-ICU, SW-nonICU) ($p<0.05$). on the basis of MIC criteria, the resistance ratios of the isolates to cefriaxone, cefotaxime, ceftazidime, imipenem, ofloxacin and ciprofloxacin were 8.4%, 15.0%, 13.3%, 0.0%, 11.6 % and 8.3% respectively **(Hryniewicz *et al.*, 2001)**.

A wide range of pathogenic microorganisms can be transmitted to humans via water contaminated with fecal matter. These include enteropathogenic agents such as *E. coli, Shigella, salmonella, enteroviruses* and multicellular parasites as well as opportunistic pathogens like *Pseudomonas aeruginosa, Klebsiella* etc. Applications of antibiotics bring about an increase in resistance to antibiotics not only in pathogenic bacterial



GRAPH 5. MIC Concentration Against Different Antibiotics for Enterobacteriaceae sps.



GRAPH 6. MIC Concentration Against Different Antibiotics for Klebsiella sps.

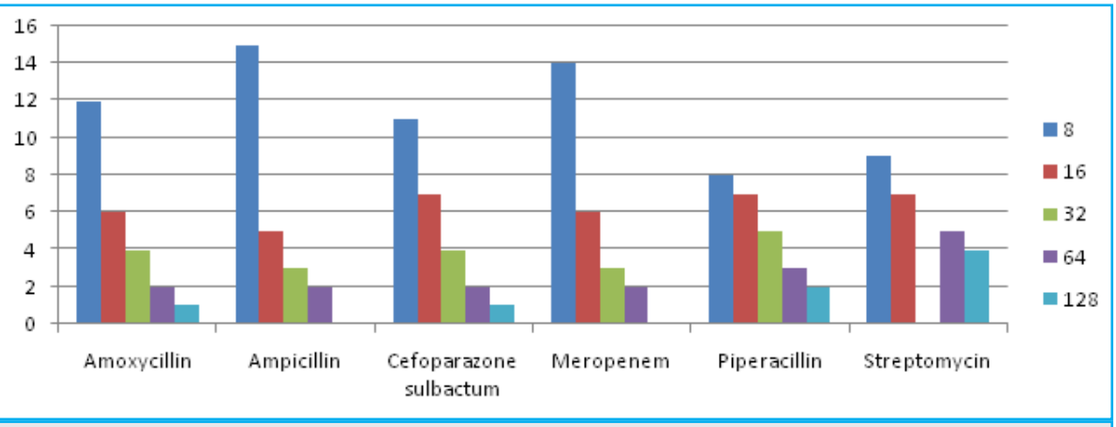
strains, but also in commensal bacteria **(Luzzaro *et al.*, 2001)**

In the present study, the samples from different localities of Balawala were collected and total of 87 isolates were obtained from them and among these 50% were *E. coli*, 25% were *Klebsiella* spp., 15% were *Enterobacteriaceae* spp., and 10% were *Proteus* spp. The isolates were then identified on the basis of biochemical characteristics and the *Klebsiella, E. coli, Proteus, Enterobacteria* and *Pseudomonas* were isolated from the excreta of animals. Antibiotic resistance among the isolates was also evaluated using for antibiotics- amikacin, gentamicin, novabiocin, ofloxacin, ciprofloxacin, cephalexin, cefixime, amoxicillin, clotrimazole, trimethoprim, kanamycin, ampicillin, streptomycin, meropenem, piperacillin tazobactam and cefoparazone sulbactum.

In our study it has been seen that resistance was seen for novabiocin (50%), cefixime (25%), clotrimazole (15%) and amoxicillin (10%). It was also found to be sensitive for gentamicin, amikacin, kanamicin, trimethoprim, ciprofloxacin and ofloxacin. The MIC test was also conducted during this study those isolates are chosen for the MIC that showed more resistance efficacy. The MIC has been performed by chosing the different isolates in which following antibiotics was used viz amoxicillin, ampicillin, cefoparazone sulbactum, meropenem, piperacillin tazobactum and streptomycin. 50% of the isolates showed resistance among the antibiotic amoxicillin, ampicillin, streptomycin at different concentrations (8µg/ml, 16µg/ml, 32µg/ml, 64µg/ml and 128µg/ml) and 50% showed sensitivity against the antibiotic cefoparazone sulbactum, meropenem and piperacillin tazobactum. The high density of enteric pathogen and prevalence of multidrug resistant *E. coli, Proteus* and *Kleibsiella* in the fecal matter may pose severe public health risk.

CONCLUSION

In this study, we analysed the susceptibility pattern of different aminoglycosides in different locality of Bala-



GRAPH 7. MIC Concentration Against Different Antibiotics for Proteus sps.

wala, strain collections of *E. coli*, *Klebsiella spp*., *Pseudomonas spp*., *Enterobacteriaceae spp*. and *Proteus spp*. Enteric pathogens, which are of great concern since they are the most common causes of infection among humans and animals. Aminoglycosides represent an important class of antimicrobial agents. The prevalence of aminoglycoside resistance among Gram-negative bacteria in Dehradun is low, but an increased prevalence among clinical isolates of *Escherichia coli* has been observed during the last years. The most prevalent resistance mechanism is aminoglycoside modifying enzymes.

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Heavy metal tolerance in association with plasmid mediated multiple antibiotic resistances among clinical bacterial isolates

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ABSTRACT

The heavy metal tolerance in association with plasmid mediated antibiotic resistance among bacteria has been reported around the globe. This communication conducted an experiment to explore the co-existence of antibiotic resistance and heavy metal tolerance in clinical bacteria and the involvement of R-plasmid in such phenomenon. By disc diffusion method, 6 clinical bacteria: *Escherichia coli* (n=3), *Pseudomonas aeruginosa* (n=2) and *Proteus mirabilis* (n=1), utilized in the study, displayed resistance to multiple antibiotics with MAR (multiple antibiotic resistance) indices 0.15 – 0.77; such bacterial isolates showed tolerance to Hg^{2+}, Cd^{2+}, Cr^{6+} and Cu^{2+} at 3 – 37.5 µg/ml, 75 – 800 µg/ml, 100 – 400 µg/ml and 600 – 900 µg/ml, respectively. The SDS treatment induced the test bacteria to mislay their resistance property (following susceptibility test) with a parallel loss of single plasmid (following agarose gel electrophoretic analysis) contained in them. This study confirms the antibiotic co-resistance with heavy metal tolerance among human pathogenic bacteria, and underlines the regular vigilance of bacterial R-plasmid in order to combat the multiple antibiotic resistances of such bacteria as well as the infection caused by them.

KEY WORDS: HUMAN PATHOGENIC BACTERIA, R-PLASMID, HEAVY METAL TOLERANCE, ANTIBIOTIC RESISTANCE, MAR INDEX

INTRODUCTION

The antibiotics, which are still the gold standard therapeutics against a large number of bacterial infections, and the heavy metals, which are in use in various anthropogenic activities, remain the two universal categories of environmental pollutants, and are unsafe to public health and biological safety (Zhu et al., 2013). Several anthropogenic processes cause contamination of environment with heavy metals leading to the selection and

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emergence of bacteria possessing the tolerance capacity to heavy metals in the niches (Nakahara et al., 1977), and, as such, the heavy metal accumulation in the environment accounts for the bacterial antibiotic co-resistance (Baker-Austin et al., 2006; Berg et al., 2010; Das et al., 2016). The imprudent use of antibiotics, on the other hand, results emergence of antibiotic resistant bacteria having the capacity to cause life-threatening infection to humans, around the world (Tenover, 2006; Mandal 2015). It has been reported that the exposure of heavy metals causes an effect in the co-selection of metal tolerant and antibiotic resistant bacteria (Filali et al., 2000), and such co-resistances are plasmid mediated (Smith, 1967; Das et al., 2018). Garhwal et al. (2014) observed a significant change in MAR (multiple antibiotic resistance) index in clinical bacterial isolates before and after lead (Pb^{2+}) exposure. Nakahara et al. (1977) studied the frequency of antibiotic and heavy metal resistance in clinical isolates of *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and reported a similar as well as different heavy-metal resistance frequency, when compared to the antibiotic resistance frequency, among the isolates, and such resistances were proved to be plasmid mediated. The occurrence of R-plasmid (antibiotic resistance plasmid) conferring heavy metal tolerance in river water isolates of *E. coli* and *Ps. aeruginosa* has been documented earlier (Das et al., 2016). The heavy metal induced antibiotic resistance in bacteria has also been reported (Chen et al., 2015). A conjugative plasmid, approximately of 56.4 kb, encoding resistance to heavy metals (Hg^{2+}, Cu^{2+}, Pb^{2+}, Cd^{2+}) as well as antibiotics was detected among nosocomial isolates of *E. coli* and *K. pneumoniae* (Karbasizaed et al., 2003). Thus, an emerging concern, predominantly in the developing countries, for the treatment of infectious disease is the acquisition and dissemination of bacterial plasmid mediated resistance to multiple antibiotics. Hence, in order to evade the bacterial antibiotic resistance, by fixing an appropriate treatment 'to-do-list', precise and prompt detection of resistance phenotype is an emergent and imperative issue (Doddaiah and Anjaneya, 2014), since bacterial antibiotic resistance has been marked as the global public health crisis (Martinez, 2008). Therefore, the current study has been undertaken to determine the association between antibiotic resistance and heavy metal tolerance among clinical bacterial isolates: *E. coli*, *Ps. aeruginosa* and *Pr. mirabilis*, West Bengal state, India.

MATERIAL AND METHODS

BACTERIAL STRAIN AND MEDIA

A total of 6 randomly selected clinical bacterial isolates: *Escherichia coli* (n=3), *Pseudomonas aeruginosa* (n=2) and *Proteus mirabilis* (n=1), were considered for the current study. The tests, in the current study, were carried out by the utilization of nutrient broth (for subculturing, bacterial inocula preparation and plasmid DNA isolation) and nutrient agar (for performing antibiotic susceptibility and heavy metal tolerance test) media (Hi-Media, India).

ANTIBIOTIC SUSCEPTIBILITY TEST

The antibiotic susceptibility test for the bacterial isolates were determined following Kirby-Bauer disc diffusion (Bauer and Kirby, 1966), using tetracycline (Tc; 30-µg), gentamicin (Gm; 10-µg), cefotaxime (Ct; 30-µg), cefpodoxime (Ce; 10-µg), ampicillin (Am; 10-µg), meropenem (Mp; 10-µg), chloramphenicol (Cm: 10-µg), ciprofloxacin (Cp; 10-µg), cefoxitin (Cx; 30-µg), piperacillin (Pc; 100-µg), piperacillin/tazobactam (PT; 100/10-µg), amikacin (Ak; 30-µg) and nalidixic acid (Nx; 30-µg). The results, in terms of ZDI (zone diameter of inhibition) obtained around each of the antibiotic discs, for the test isolates were interpreted according to the CLSI criteria (CLSI, 2011).

MAXIMUM TOLERANCE CONCENTRATION OF HEAVY METAL

The MTC (maximum tolerance concentration) values, for the bacterial isolates, of heavy metals: using 4 salts, such as $HgCl_2$ (Hg^{2+}), $CdCl_2$ (Cd^{2+}), $K_2Cr_2O_7$ (Cr^{6+}), and $CuSo_4$ (Cu^{2+}) were determined by agar dilution method, using $\approx 10^4$ CFU/spot inocula, as described earlier (Das et al., 2016). The concentrations of heavy metals utilized included: Hg^{2+} (3 – 50 µg/ml), Cd^{2+} (25 – 1000 µg/ml), Cr^{2+} (25 – 500 µg/ml), Cu^{2+} (200 – 1000 µg/ml). The obtained results were interpreted as described earlier (Das et al., 2016). The bacterial isolates grown in presence of each of the heavy metals, at concentrations ≥3 µg/ml, were considered as heavy metal tolerant.

PLASMID ANALYSIS

As mentioned earlier (Das et al., 2016), the plasmid DNA from the test bacteria were isolated following the protocol of Kado and Liu (1981), and the agarose gel electrophoresis of the isolated plasmids were done following Maniatis et al. (1982). The plasmid DNA bands, in the gel after ethidium bromide staining, were visualized and documented using gel-doc system.

In order to investigate the loss of plasmid, the randomly selected bacterial isolates (*Pr. mirabilis* CSD1, *Ps. aeruginosa* CSD3, and *E. coli* CSD5) were subjected to plasmid curing with SDS, following the protocol of Anjanappa et al. (1993), as described elsewhere (Mandal et al., 2008; Das et al., 2016). The loss of antibiotic

resistance and heavy metal tolerance, along with the loss of plasmid, was determined based on the resistance patterns of the cured bacterial strains, and absence of plasmid in the gel following agarose gel electrophoresis for the cured bacterial strains.

RESULTS AND DISCUSSION

The antibiotic susceptibility test results, in terms of ZDI, are depicted in Table 1. The mounting use of antibiotics, not only in health care but also in agriculture and animal husbandry contribute to an emergent problem of antibiotic resistant bacteria (Dhanorkar and Tambekar, 2004). Pokhrel et al. (2018) reported, among the isolated environmental bacteria, 3-antibiotic resistance, 4 to 10-antibiotic resistances and more than 10-antibiotic resistance in 6.1%, 44.89% and 48.97% isolates, respectively. The fecal as well as soil isolates of *Klebsiella*, *Citrobacter*, *Shigella* and *Staphylococcus*, showed resistance to 6 – 10 antibiotics tested, and the MAR indices for the isolates ranged 6 – 10 (Ayandele et al., 2018). The Mahananda river water bacterial isolates had resistance to multiple antibiotics, among Am, Cm, Ce, Cx and Tm, as per the report of the earlier study (Das et al., 2016). In the current study, *Pr. mirabilis* (n=1) had 2-drug resistance "Cx-Pc", *Ps. aeruginosa* isolates (n=2) had 8-drug resistance of two different patterns "Am-Ce-Cm-Ct-Cx-Nx-Pc-PT" and "Am-Ce-Cp-Ct-Cx-Nx-Pc-PT", and the *E. coli* isolates (n=3) showed 3 different patterns of resistance to antibiotics: 8-drug resistance "Am-Ce-Cm-Cp-Cx- Mp-Nx-Pc", 9-drug resistance "Am-Ce-Cp-Ct-Cx-Mp-Nx-Pc-PT" and 10-drug resistance "Am-Ce-Cp-Ct-Cx-Mp- Nx-Pc-PT-Tc" (Table 2). As per the report of Malema et al. (2018), among the 100 pathogenic *E. coli* test isolates, 52% had multiple antibiotic resistance, of which 10 showed to 9 antibiotics, and 24 different MAR phenotypes have been identified.

The MAR indices for the human pathogenic bacteria are depicted in Figure 1. As has been reported by Sandhu et al. (2016), the majority of the clinical isolates of *Acinetobacter* had resistance to cotrimoxazole, Cp, Gm, Ak, A/S, cefepime, Im, Mp, with overall MAR indices of 0.3 – 1.0 for the isolates. Subramani et al. (2012) reported high MAR indices (0.64 - 0.74) among *Staphylococcus aureus* isolates from clinical settings demonstrating the origin of the bacteria from niches with high antibiotic exposure/contamination. The MAR indices of potential pathogenic bacteria, *E. coli* (MAR index: 0.44) and *Ps. aeruginosa* (MAR index: 0.43-0.57), were all > 0.2, indicating their origin from high risk source of antibiotic contaminated region (Oko et al., 2016). In the previous communication, the MAR indices have been reported to be 0.47 in *Ps. aeruginosa* and zero to 0.2 in *E. coli* isolates from Mahananda river water, Malda (India) (Das et al., 2016). In the current study, the MAR indices for the clinical bacteria were: 0.15 for *Pr. mirabilis* and 0.62 for *Ps. aeruginosa*, while the values ranged 0.62 – 0.77

Table 1. Antibiotic susceptibility test results for clinical bacterial isolates (ZDI; zone diameter of inhibition)

Antibiotic	ZDI (mm)					
	CSD1	CSD2	CSD3	CSD4	CSD5	CSD6
Tc	40	10	18	17	12	13
Gm	27	15	30	26	20	20
Ct	30	6	12	6	6	25
Ce	20	10	6	6	6	13
Am	20	6	6	6	6	6
Mp	30	12	33	34	14	18
Cm	18	22	8	22	25	8
Cp	40	8	46	15	10	6
Cx	14	6	6	6	6	11
Pc	6	6	6	6	6	6
PT	30	15	17	6	6	20
Ak	30	20	18	30	26	15
Nx	25	6	12	10	6	6

Ak: amikacin, Am: ampicillin, Ce: cefpodoxime, Cm: chloramphenicol, Cp: ciprofloxacin, Ct: cefotaxime, Cx: cefoxitin, Gm: gentamycin, Mp: meropenem, Nx: nalidixic acid, Pc: piperacillin, PT: piperacillin/tazobactam, Tc: tetracycline, CSD1: *Pr. mirabilis*; CSD2: *E. coli*; CSD3: *Ps. aeruginosa*; CSD4: *Ps. aeruginosa*; CSD5: *E. coli*; CSD6: *E. coli*.

Table 2. Antibiotic resistance and heavy metal tolerance patterns of clinical bacterial isolates and their cured derivatives

Bacterial isolates*	Resistance/tolerance patterns		Resistance patterns of cured bacteria
	Antibiotic resistance	Heavy metal tolerance	
Pr. mirabilis CSD1	Cx-Pc	Hg^{2+}-Cd^{2+}-Cr^{6+}-Cu^{2+}	Pc
E. coli CSD2	Am-Ce-Cp-Ct-Cx-Mp-Nx-Pc-PT-Tc	Hg^{2+}-Cd^{2+}-Cr^{6+}-Cu^{2+}	ND
Ps. aeruginosa CSD3	Am-Ce-Cm-Ct-Cx-Nx-Pc-PT	Hg^{2+}-Cd^{2+}-Cr^{6+}-Cu^{2+}	Nx-Pc-PT
Ps. aeruginosa CSD4	Am-Ce-Cp-Ct-Cx-Nx-Pc-PT	Hg^{2+}-Cd^{2+}-Cr^{6+}-Cu^{2+}	ND
E. coli CSD5	Am-Ce-Cp-Ct- Cx-Mp-Nx-Pc-PT	Hg^{2+}-Cd^{2+}-Cr^{6+}-Cu^{2+}	Cp-Mp-Nx-Pc-PT
E. coli CSD6	Am-Ce-Cm- Cp-Cx- Mp-Nx-Pc	Hg^{2+}-Cd^{2+}-Cr^{6+}-Cu^{2+}	ND

*The clinical bacterial isolates possessed a single plasmid of ≈54 kb, and the cured bacterial strains were plasmid-less. ND: curing not done.
Am: ampicillin, Ce: cefpodoxime, Cm: chloramphenicol, Cp: ciprofloxacin, Ct: cefotaxime, Cx: cefoxitin, Gm: gentamycin, Mp: meropenem, Nx: nalidixic acid, Pc: piperacillin, PT: piperacillin/tazobactam, Tc: tetracycline.

for *E. coli* isolates. Thus, considering the fact of origin of bacterial contamination from human-fecal sources, based on the MAR indices of >0.4 (Tambekar et al., 2005; Kaneene et al., 2007), and from high risk zone of contamination with antibiotics, based on the MAR indices of >0.2 (Krumperman, 1983), the currently studied clinical bacteria (*Ps. aeruginosa* and *E. coli*) might have been originated from niches with human-fecal contamination, due to antibiotic selection pressure.

The bacterial heavy metal tolerance has been depicted in Table 2. As has been reported by Mustapha and Halimoon (2015), the bacterial isolates from industrial effluents had tolerance to Cd^{2+}, Cr^{6+}, Pb^{2+} and Cu^{2+}, at the concentration of 50 µg/ml, while one of the isolate showed resistance to high level of Cu^{2+} (200 µg/ml), and for one isolate the Cd^{2+} MIC (minimum inhibitory concentration) was recorded as high as 200 µg/ml. Zhu et al. (2013) determined the MICs of Pb^{2+}, Cu^{2+}, Zn^{2+}, Cr^{6+} and Hg^{2+} as 125, 100, 100, 100 and 25 µg/ml, respectively, for the livestock isolate of *Ps. fluorescens*, and recorded the occurrence of enhancement of bacterial resistance to antibiotics due to the presence of some heavy metals at certain concentrations. *Ps. aeruginosa*, *Ps. putida* and *Klebsiella pneumoniae* had Cd^{2+} MICs 300 – 950 µg/ml; such isolates had Zn^{2+} MICs of 1150, 1100 and 2000 µg/ml, respectively, and Hg^{2+} MICs of 20, 80 and 90 µg/ml, respectively (Yamina et al., 2014). *Ps. aeruginosa* and *E. coli*, isolated from Mahananda river water,

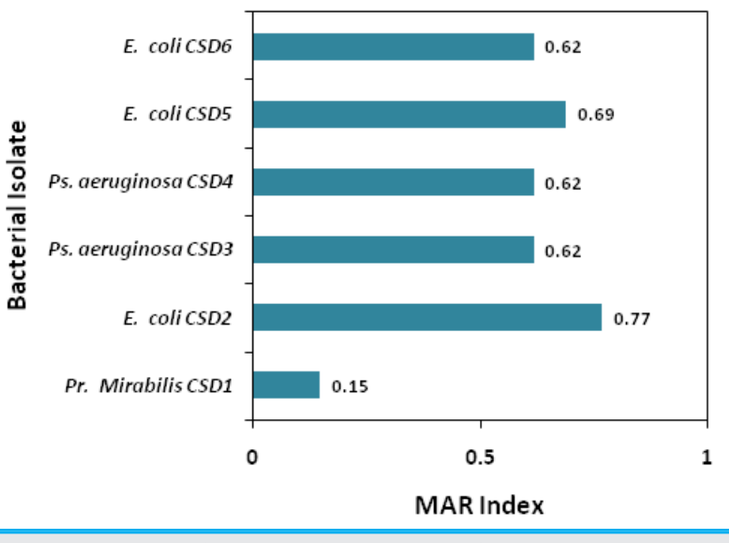

FIGURE 1. The MAR (multiple antibiotic resistance) index for clinical bacterial isolates (n=6).

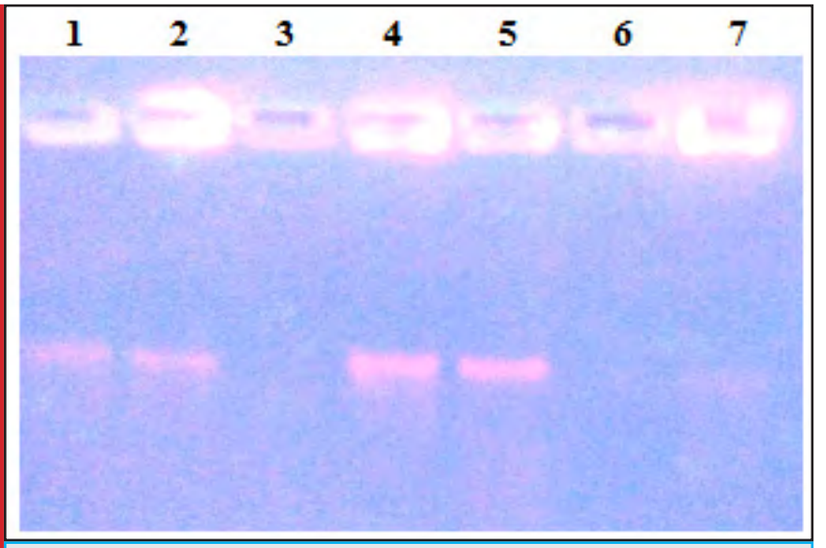

FIGURE 2. Plasmid profile of clinical bacterial isolates; lane 1: E. coli V517 (54 kb), lane 2: *Pr. mirabilis* CSD1, lane 3: *Pr. mirabilis* CSD1 (cured strain), lane 4: *Ps. aeruginosa* CSD3, lane 5: *E. coli* CSD5, lane 6: *Ps. aeruginosa* CSD3 (cured strain), lane 7: *E. coli* CSD5 (cured strain). Note the absence of plasmid in cured bacterial strains (lane 3, 6 and 7).

Malda (India) had resistance to Cd^{2+} and Hg^{2+} (Das et al., 2016). The continued usage of heavy metals since the ancient, in medicine and other anthropogenic purposes, select heavy metal resistant bacteria in polluted niches. For the bacteria utilized in the current study, the level of tolerance to Hg^{2+}, Cd^{2+}, Cr^{6+} and Cu^{2+} ranged 3 – 37.5 µg/ml, 75 – 800 µg/ml, 100 – 400 µg/ml and 600 – 900 µg/ml, respectively (Table 3). This communication is, for the first as we believe, to demonstrate the heavy metal tolerance among clinical bacterial isolates of *E. coli*, *Pr. mirabilis* and *Ps. aeruginosa* from our part of the globe (West Bengal state, India).

The plasmid profile of clinical bacterial isolates and the cured derivatives are represented in Figure 2. Apprehension grows in recent times in connection with the co-selection for antibiotic resistance among bacteria on exposure to heavy metals, in several ecological niches (Wales and Davies, 2015). Because the heavy metal (pollution) acts discriminatorily as a selective agent in the emergence and propagation of antibiotic resistance among bacteria, wherein, along with the genes conferring antibiotic resistance, metal tolerance genes are also encoded in the same plasmids (Foster, 1983; Fang *et al.*, 2016). The clinical isolates of *Pseudomonas* spp., as reported by Rajasekar and Mohankumar (2016), had resistance to multiple antibiotics and heavy metals, and the resistance properties were shown to be plasmid (10 kb) mediated. A conjugative plasmid (≈56.4 kb), carrying resistance to multiple heavy metals, such as, Hg^{2+}, Cu^{2+}, Pb^{2+}, Cd^{2+}, and also antibiotics was detected among the isolates of *E. coli* and *K. pneumoniae* causing nosocomial infections (Karbasizaed et al., 2003). Das et al.

Table 3. Heavy metal tolerance level for clinical bacterial isolates (n=6)

Bacterial isolates	MTC of heavy metals (µg/ml)			
	$HgCl_2$	$CdCl_2$	$K_2Cr_2O_7$	$CuSo_4$
Pr. mirabilis CSD1	12.5	75	250	700
E. coli CSD2	37.5	500	250	600
Ps. aeruginosa CSD3	3	800	400	800
Ps. aeruginosa CSD4	9	100	200	900
E. coli CSD5	25	500	250	600
E. coli CSD6	9	100	100	800

MTC: maximum tolerance concentration

(2016) reported the occurrence of R-plasmid (antibiotic resistance plasmid) encoding heavy metal tolerance among *E. coli* and *Ps. aeruginosa*, isolated from river water. Herein, we demonstrated the involvement of ≈54 kb plasmid (Figure 2) conferring heavy metal tolerance to Hg^{2+}, Cd^{2+}, Cr^{6+} and Cu^{2+}, with associated multiple antibiotic resistances among the clinical bacterial isolates (Table 2).

The co-resistance to antibiotics and heavy metals has been reported among bacterial food pathogens (Wales and Davies, 2015). Wright et al. (2006) reported highest occurrence of heavy metal tolerance and antibiotic resistance among bacteria isolated from the contaminated most location, indicating the direct selection of heavy metal tolerant bacteria due to the exposure of heavy metals, thereby co-selecting bacterial antibiotic resistances. The *E. coli* isolates from urinary tract infection cases harbored copper/silver resistance genes, '*pco/sil*' with MIC of 500-µg/ml, presenting resistance to extended spectrum β-lactam antibiotics, too (Sutterlin et al., 2018). Co-spread of antibiotic resistance (β-lactams: *bla*CTX-M; quinolones: *oqxAB*; aminoglycosides: *aac-Ib-cr*; amphenicols: *floR*; fosfomycin: *fosA3*) as well as the heavy metal resistance (Cu: *pco*; Ag: *sil*) genes, have been shown to be plasmid mediated (Zhu et al., 2013). The Cd^{2+} resistant isolates of *Ps. aerµginosa* and *Ps. putida* showed multidrug resistance to kanamycin (Km), oxacillin (Oc), Nx and sulfonamids, while *K. pneumoniae* had resistance to Ct in addition to Km, Oc, Nx and sulfonamids resistances (Yamina et al., 2014). In the earlier study, antibiotics (Am-Cm-Ce- Cx-Tm) and heavy metals (Cd^{2+}-Hg^{2+}) co-resistances have been reported among the river water bacteria (Das et al., 2016). The co-resistance to heavy metals and antibiotics is, thus, a global concern, and the phenomenon among clinical bacteria, in our part of the globe, is not uncommon. The plasmid mediated resistance to the test heavy metals and to a number of antibiotics, as has been supported by SDS curing, approved the fact of heavy metal-antibiotic co-resistance in *Ps. aerµginosa*, *E. coli*, and *Pr. mirabilis* clinical isolates. The bacterial isolates displaying MAR indices of >0.4 have been regarded to be derived from human-fecal contaminated niches (Tambekar et al., 2005; Kaneene et al., 2007) and the bacterial isolates, with MAR indices of >0.2, deemed to be originated from highly antibiotic polluted regions (Krumperman, 1983). Still, the heavy metal inducing phenomenon of bacterial antibiotic resistances suggests that the emergence of multiple antibiotic resistant bacteria might be due to either the heavy metal or the antibiotic selection pressure, or both, and hence the bacterial high MAR index does also mean their (bacteria) origin from a region with high metal pollution, too.

CONCLUSION

The human pathogenic bacteria (*Ps. aerµginosa*, *E. coli*, and *Pr. mirabilis*) had resistance to two or more antibiotics, which in association with heavy metal resistance were found to be plasmid linked. This study endorses the dissemination of bacterial antibiotic resistance under the heavy metal as well as antibiotic selective pressure. Therefore, regular inspection of antibiotic resistance plasmid among human pathogenic bacteria, from our part of the globe, is urgently needed, in order to combat the bacterial multiple antibiotic resistance as well as the bacterial infection to humans.

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Role of physical therapy in overcoming the barrier and adaptive behaviour for return to work in subjects with non-specific chronic low back pain: A qualitative study

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ABSTRACT

Clinical investigation has revealed that individuals face challenges in adapting to their gruesome employee-roles after undergoing a back intervention program. The aim of this research was to investigate the back-rehabilitated patient's perspectives and understandings of the difficulties faced while adapting as employees. Research aimed to extract the patient's perspectives and understandings of barriers, facilitators and adaptive procedures which influenced their capability to continue their employee-roles. Qualitative investigation method was used for investigating the study topic. Focus groups comprised of broad questions followed by probing were utilized to obtain detailed descriptions about the client's understandings and perspectives. Recommendations involved developing the insight of stakeholders regarding early, valuable on-job training, initiating health-promotion by teaching in the workplace and in the society, along with improving flexible job and health policy. Results of the investigation signify that the goal of physiotherapy and extent of service to back injured patients needs to rebuild.

KEY WORDS: BACK INJURED PERSON, BACK REHABILITATION, BARRIERS, FACILITATORS AND ADAPTIVE PROCEDURES

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INTRODUCTION

Industrialization has resulted in increasing number of back disabilities especially within industries which need frequent carrying of heavy substances. Back injury is quoted as the costliest health concerned problem within people aged between 30-50 years and the primary reason of disorder within population less than 45 years of age (Jones and Kumar, 2002). Back pain which persists for lesser than 3 months can be called 'acute' because a person can improve without therapeutic treatment (Drezner & Herring, 2001). The growth of a chronic condition can be associated with fear of recurrence of injury, excessively caring partners and being aware of sickness role (Joy et al., 2000). It can hence, be debated that such supporting aspects lead to an inadequate return to work rate especially if people are being financially compensated for their losses.

As per Friesen et al. (2001), employee attitudes and motivation to participate must be acknowledged and addressed if more injured employees are to be successful within return to permanent employment. Henceforth, theories based on the patient's motivation were viewed as being of value during investigating returning to work process. Cognitive theories of motivation have been recommended as suitable constructs to understand individual dissimilarities in motivation within return to work behaviour following work injury (Roessler, 1989). It signifies that an employee's personal viewpoint of himself within context of being re-injured in the work atmosphere plays an influential part within returning to work process. Research has revealed that money in form of worker's compensation or legal actions can also behave as a motivator for pain behaviour, (Reneman, 2001 Cancelliere et al., 2016 Wong et al., 2017).

It signifies that pain faced by some back injured subjects is worthwhile within itself due to the economical rewards, empathy, notice and concern it creates in favour of such subjects. As per Hildebrandt (1998), efficacy of present intervention strategies have not been proven considerably and it is apparent within yearly rise of patients having Chronic Low Back Pain (CLBP). This could be assumed that earlier back pain studies have strong efforts on rehabilitation from a health-expert's viewpoint, failing to comprehensively realise the back injured client's perceptions or views during planning treatment approaches, (Gatchel 2018).

Therefore this study is aimed to extract patient's perspectives and understandings of barriers, facilitators and adaptive procedures which influenced their capability to continue their employee-roles.

Methods: Qualitative investigation method was used for investigating the study topic. Subjects were chosen through random sampling from S.M.S (Sawai Man Singh) Hospital, Jaipur, Rajasthan, India -Physiotherapy Department. Focus groups comprising of broad questions followed by probing were utilized in order to obtain detailed descriptions about the client's understandings and perspectives. A pilot testing group and 3 one-hour focus groups were conducted consisting of 5 subjects in each group on an average. Data which was produced was qualitatively examined by manual coding system to achieve codes, categories and themes. Aspects which were viewed as facilitators involved enhanced communication and precision between stakeholders, injury prevention approaches and understanding among stakeholders which facilitated team-work. Aspects which obstructed subjects to resume as employees were considered to be barriers. These involved delays and all kinds of inefficacy for instance, inadequate management processes, bad quality medical treatments and uncaring work atmosphere.

Study-design: As explained by Bailey (1997), a qualitative investigation illustrates multiple realities and interpretations that aimed to enhance an in-depth understanding of the perceptions and knowledge of the participants. Because of this, a qualitative investigation concept was selected to conduct this research. Objective of this research asked for a qualitative-methodological-analysis to be performed because the highlight was on illustrating the perspectives and understandings of back-rehabilitated subjects on their return to workplace.

DATA COLLECTING PROCEDURE

Focus group Interview: Focus group interview was chosen for collecting data. Because of its interacting nature they are an ideal method to explore participant's own meaning and to understand issues like health and illness. This method of investigations is mainly helpful to assess the viewpoints of those who have been inadequately served by a traditional research Wilkinson (1998). Pilot-focus group interviewa were conducted, to provide a direction towards establishing a range of approaches that were essential in order to investigate aims of the research. When no issues were established within questions of pilot focus group, minor modifications were done to the questions of the focus groups which followed. Data obtained from the pilot focus group was included in the study in order to contribute to aim of the research.

Participants: Participants were selected from statistical records of S.M.S Hospital-Physiotherapy Department. Since it is the largest and only government Hospital situated in Jaipur (capital of Rajasthan, the largest state of India), it was ideal for selecting subjects having work-related pain for this research. Because of the fact that a number of subjects with different kinds of injuries are assessed at the hospital, subjects having only back pain

were selected from statistical records of the hospital. Aim of the study was described to the participants on telephone by contacting them at their place of work, if employed or at their residence, if unemployed.

Sampling: Simple random sampling (Kerlinger 1986), was utilized as a procedure in order to give every patient on statistical records of the hospital, an equivalent opportunity to be selected in the research. A table of random digits were used to select the subjects which was followed by giving a number to each name. A random start-point had been selected on a random table and first 24 numbers and the names that corresponded them were selected. After that, subjects that were chosen were invited to take part within this project.

Sample size: According to Krueger (1994), an ideal number of participants in a focus group ought to be between 3 and 9, therefore for this study 24 participants (8 participants per group) were selected to be involved within the 3 focus groups. However, only 15 participants turned up for the interview.

Selection Criteria: The inclusion criteria for this study were; subjects with a medically diagnosed chronic back pain affecting their work, who had received medical and physical therapy treatment. Subjects were not included if they had any form of psychiatric diagnosis, or undergone any invasive or surgical treatment for chronic back pain because their quality of life and psychosocial adaptations as employees could have provided an unhelpful influence on the investigation.

Study Protocol: A pilot testing group and 3 one-hour focus groups were performed and an average of 5 participants attended each group. It was done to serve the purpose of discovering the perspectives and understandings of a range of participants, hence to ensure dissimilarity among the views of study participants. Broad questions were asked which was followed by probing for eliciting detailed and rich explanations. A kind of debriefing was included at the end of each session, in which subjects were facilitated to clear their queries and express their feelings of contributiion within a group.

Focus groups were performed within one of the consulting rooms of physiotherapy department as the venue was familiar to all the subjects. As per the convenience of the subjects, the group session were scheduled every afternoon between 2-3 pm, for three continuous days. Subjects were seated around a table ensuring that there was a proper eye contact. Name tags were given to build a rapport between subjects. An audiotape was put at a pre-meditated position so that all the data received could be free from unrelated external stimuli.

Focus group questions were offered to clients both in English and Hindi. Subjects were informed that they were free to withdraw their consent before participating or at any stage of discussions. Participants were introduced to each other so that they could unwind themselves and become familiar to each other and to the environmental setting. Investigator set goals for discussion and guaranteed the clients that their feelings and views will be valued. Questions were discussed with the tutor in advance and changes were made accordingly. This allowed the investigator to become conscious of several group-dynamics that could play an important part, for e.g. difference between traditions, attitude towards life and viewpoints.

Data-analysis: Within this investigation, 4 cognitive procedures allowed the investigator to examine the qualitative data through comprehending, synthesizing (de-contextualizing), theorizing and re-contextualizing (Morse & Field, 1996).

Comprehending: Transcriptions were done directly after interviewing, providing investigator the time to study as well as identify the interviewed data before arranging for the next interview. After that, information was coded through line-by-line analysis to discover essential meanings within the transcript. Then, the investigator became capable of identifying the experiences that are a part of the research investigation and the patterns that foresee possible outcomes.

De-contexualizing: This phase was achieved at the completion of third focus group. Transcripts were evaluated during analysing, where codes were built into categories through commonalities, consisting of segments of transcripts.

Theorizing: Different descriptions were investigated against the data until the data for the research was best described. Questions were asked on the data for establishing links to the theory. This organized procedure facilitated the investigator to inductively develop formal themes from data. This also allowed investigator to acknowledge the quality of data to contribute within a particular experience or perspective.

Re-contextualizing: Comparing the similarities and dissimilarities between the data and the published research of other investigators played an essential role to ensure credibility of the data. Investigator made an effort to put the outcomes of the investigation within the context of standard knowledge and appreciated the outcomes which supported the literature.

RESULTS

THEMES

The themes of the research directly associated to the research aim and investigate the difficulties that back-

Table 1. Demographic and Clinical Data of participants

Variable	Category	Number of subjects in pilot group(m=men/w=women)	Number of subjects in focus groups (m=men/w=women)
Gender	Men	3	7
	Women	0	5
Age in years		18-40 = 2 (m) 41-65 = 1 (m)	18-40=5 (m), 41-65=2 (m), 18-40=3 (w), 41-65=2 (w)
Employed	Yes	2	8 (m), 3 (w)
	No	1	2 (m), 2 (w)
Medical Intervention	Type of organizations in which rehabilitation was obtained	D.H = 1 (m) B.R.F = 2 (m)	D.H=4(m), 2(w) B.R.F=2(m),1(w) P.T= 1(m), 2 (w)
Data collection	No. of focus groups	1 session	3 sessions (different clients in each group)

*Key : D.H - (Day Hospital), B.R.F - (Back Rehabilitation Facility), P.T - (Physiotherapy consultant in a home visit)

rehabilitated individuals came across on their return to work. Theme one associates with the barriers which obstructed back injured individual's adaptation as employees, theme two associates with the facilitators which supported individuals to adapt to their employee-roles, theme three associates with the individual's perceptions and experiences of adapting to their role as employees. Table 2 present the themes which emerged after the data-analysis

Barriers: Fisher (1994) documented that psycho-social maladjustment and psychological health of an injured employee can be revealed within perspectives generated in reaction to depressing or destructive social aspects. By the information obtained, this was apparent that client's perspectives of themselves concerning their capability to execute job-associated tasks were either pessimistic or optimistic. Subjects frequently mentioned that unhelpful perceptions of employers as well as society forced them in losing self-confidence and build a feeling of uncertainty within their performance capabilities.

Doleys & Gochneaur (1989) describes that not everybody who has an injury develops a chronic condi-

Table 2. Themes categorizing the difficulties of back-rehabilitated individuals affecting their return to work.

Themes	Category	Subcategory
1. Barriers (Feeling of Uncertainty)	1. Feeling of uncertainty by stakeholders	Employer building an uncaring approach and uncertainty in the employee.
		Health-service building an uncaring approach and uncertainty in the client's condition
	2. Uncertainty in own potentials for accomplishment at work	Uncertainty in return to work approaches
		Uncertainty in discovering a meaning within the employee-role
2. Facilitators (Approaching an idealistic rehabilitation-program)	1. Informed stakeholders	Informed employers
		Informed consultants
	2. Work promoting approaches	Multi-trained health-experts
		Pain and disorder evaluation teams
3. Adaptors (Self-Responsible)	1. Managing yourself	Reorganising yourself
		Building an optimistic view and accepting your clinical condition
	2. Building competency in your employee-role	Awareness and training of the employee
		Asserting yourself

tion. Instead, a feeling of external sense of control while performance participation results in a sense of insufficiency and it depicts itself within the employee not being able to control the pain. By such a perception, it could be assumed that an external feeling of control in which client's certainty within himself in continuing his employee-role was affected by the perspectives of others in the environment. Such an uncertainty affected the person's feelings of self-efficacy during executing job-associated tasks.

Bandura (1989) debated that self-efficacy expectations powerfully influence motivation. Few subjects in present investigation were inadequately motivated in returning to work after injury. Such inadequate motivation can be seen as a consequence of barriers for instance, their uncertainty in opportunities after being injured. Subjects felt that they had not been capable enough in performing job-tasks which they did earlier. A sub-category 'uncertainty in return to work approaches' interprets their perspectives of fear in which they perceived that recurring injury might cause increase in pain and restrictions which can impact on their economical and psycho-social conditions. Such perceptions caused the subjects to avoid tasks in the workplace and lose confidence within their own potentials and capabilities.

Such results correspond to those of Fritz and George (2002) who examined fear-avoiding beliefs, and came to a conclusion that these are significant variables within foreseeing client's achievement within treatment programs. It henceforth, signified that subjects who feared re-injury may face problems to complete rehabilitation programs and can be at risk of long-lasting work limitations. Nevertheless, Forman & Murphy (1996) view the person as an aware agent that vigorously understands the environmental requirements and behaves accordingly with these perspectives. An implication of this would be that a person builds an internal sense of control whereby he obtains encouraging response from the environment and builds a constructive self-perception as well. Such a constructive self-perception reflected within approach of the subjects during completing intervention programs as well as during return to work. Subjects perceived that an optimistic approach along with internal motivation supported them to effectively complete intervention programs.

Facilitators: Subjects who performed productively in the work atmosphere repeatedly mentioned injury prevention as a facilitator. Injury prevention in terms of this research involved positive communication and confidence within stakeholders who were directly in touch with the worker. The participants described that the interpersonal-relations between the stakeholders confirmed that their treatment programs were frequently supervised and hence, promoted a quicker return to work rate.

DISCUSSION

This result is similar to a research by Friesen et al. (2001) in which interaction, team-effort, faith and sincerity were established to be supportive aspects during return to work-process. Such aspects henceforth, decided success by connecting the gaps for the patient from treatment processes to participating as employees. Conventional health-service has concentrated on a bio-medical model of rehabilitation in which a person's elements of performances have been remediated for participating in their work roles for instance, an employee, homemaker, sportsperson, etc. (Smithline & Dunlop, 2001, Cancelliere et al., 2016). Physiotherapy consultants working in private as well as for the government are frequently pushed for time due to excessive patient loads that they are bound to tackle with. It frequently leads to physiotherapists finding hard to perform holistic assessment processes like identifying the outcomes of work atmosphere and home atmosphere on the back injured client's performance. Physiotherapy consultants are in a favourable situation of being capable of incorporating holistic assessment and treatment in an individual's functioning as an environmental frame of reference (Christiansen, 1997, Gatchel 2018).

Nonetheless, consultants who desire to practice a career in comprehensive evaluation and intervention of an injured worker are required to focus on particular courses on ergonomic assessment of the injured employee's workplace as well as develop their focus on government laws for example, employment justice, code of high-quality service and workplace safety. Focus into such courses and government laws would enable physiotherapists in empowering their back injured patients within context of their legal rights at workplace and would facilitate them to adapt and retain their employee-roles. By a rise within workers taking legal action against companies concerning workplace injuries and unjustified removal, physiotherapists can be invited in future to give specialist legal authentication on basis of a person's work performance.

The results signified that the subjects which were successful to resume work after pain-process received a caring atmosphere at home and in workplace. This meant that barriers which impacted on the injured were mostly environmental in nature and such barriers were viewed as challenging when it influenced subjects personally. A majority of subjects within this research showed a powerful internal sense of control and it was visible within their capability to be responsible for their own treatment.

Nonetheless, they had to adjust regardless of working in an unsupportive working atmosphere. According to their perspective, they kept trying by searching meaning within the work that they were occupied with, by precise work-placement strategies, managing injury on their own and keeping a powerful value system which enabled them to resume their tasks. Physiotherapists, with their understanding about pathology and job-placement strategies have a high influence within the occupational treatment field. This will be their duty to facilitate an injured person in precisely identifying their difficulties and assets with regard to their capabilities and suitably match it to the job requirements. Subjects in the investigation presented with restrictions concerning the physical aspects of their performances such as to bend, to carry and to climb. It forced the back injured to observe increasing back pain. Physiotherapist will in turn support the back injured to modify the person's substantial and psycho-social job surroundings. Physical adaptation was viewed as modification of equipment and structural environment of the worker to equalise their performance capabilities. Psycho-social adaptation was viewed as informing the manager and the employee concerning the depressing outcomes of an unhelpful working atmosphere on the back injured person. Physiotherapist will henceforth, be capable of consulting with other rehabilitation-experts as well as employers within context of most appropriate job-placement and dealing with approaches of back injured people.

Health-promotion was considered to be a potential structure of recommendation for building a caring atmosphere for back injured people. Back injured client's workplace and home was considered to be examples of a caring atmosphere in which preventing back disorders and reinforcing intervention procedures can be initiated. Back injured people have presently been on a risk for not identifying their potentials because they do not use their complete mental/emotional as well as physical capability at work. Such capability in terms of this research can henceforth, only be remediated by enhancement of a caring society and a supportive work atmosphere.

CONCLUSION AND FUTURE RECOMMENDATIONS

The main aim of the research was to build a larger focus into the issues which challenge the back injured individuals. Results of the investigation signify that the goal of physiotherapy and extent of service to back injured patients needs to rebuild.

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Enviromental behavior of steel beams embedded in concrete under static loads

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ABSTRACT

In this article environmental behavior of steel beams embedded in concrete under static loads was investigated. The obtained experimental results were compared for precision of the sample configuration of a three-dimensional finite element model. In this study, for modeling concrete, Concrete Damaged Plasticity was used. The analytical method used in this study for explicit nonlinear dynamic analysis Dynamic Explicit is selected. In contrast, in samples with a ratio of 0.5 steel flange width, usually bending failure were observed.

KEY WORDS: MIXED BEAMS, FLEXURAL BEHAVIOR, STATIC LOADING, FINITE ELEMENT METHOD.

INTRODUCTION

Analytical studies were conducted, including the members of the Security, by Spacone and El-Tawilin 2004. In this study, nonlinear analysis steel-concrete composite structures were investigated. Another study by Camps et al., (2017) has analyzed flexural behavior of composite beams with deformable connection. How the tensions in the interface of concrete and steel sections and increase ductility and impact assessed concrete creep and shrinkage behavior of the composite was investigated. Another study evaluated the short beam strength composite columns by Mirza and Skranek which was conducted in 1991. Other research studies on prestressed beams have been combined in this research, which are based on analytically studied proposals for the design of this type of beam bending is presented, (Zhao and Li 2017).

The survey in 1989 was conducted by Saadatmanesh and colleagues. The use of prestressed steel beams for composite steel - concrete increases the bearing capacity of steel beams to the current stage and also increases the resistance of the composite section final. At the same

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time prestressing tension flange reduced the amplitude cyclic stress and improve fatigue resistance.In another study conducted in 2005 by Ellbody and Young, shear bond strength impact and efficiency in composite beams has been investigated. Another study under the title of ultimate strength of continuous composite beams under combined bending and cutting was done by Liang et al. Based on numerical results, a new design model based on vertical shear resistance and interaction Anchor - cutting the continuous composite beams have been proposed. The proposed design models were compared with experimental results and good agreement is shown (Goldston, et al. 2016 and Ongpenget al.2018).

Zhao and Li in 2008, conducted a numerical study in conjunction with a new way to create continuity in composite beams. The flow of steel beam to reduce the local rigidity and lead to brittle fracture the concrete. In 2005, Lam and his colleagues evaluated the behavior of shear connectors for composite beams was conducted stud.

In this study, a model of efficient numerical simulation using the finite element method for laying out tests have been proposed. The model was validated against experimental results and with common standards such as BS5950 and the information given in EC4 and AISC were compared. By a number of researchers based on laboratory analysis and numerical research has been done on shear connectors, length, maximum width of cracks in concrete was found to be reduced. Lam and colleagues in 2005 studied pull tests on 12 samples of pre-fabricated hollow concrete slab, the scale is complete. In another work by Lam and El-Lobody (2005) new work has been developed to FEM modeling computational which has been obtained from experimental results which have been discussed in terms of accuracy. Comparison has been made on the conducted data and the results show good correlation between the finite element model from environmental point of view. In this study, the flexural behavior of steel beams embedded in concrete under static loads is investigated for sustainable environmental development.

STATEMENT OF THE PROBLEM

Full of strength, using techniques to enhance the capacity of these members is very important. Capacity is up against loads of material to be used simultaneously and efficiently.

MODELINGALMAN LIMIT FOR CONSTITUENTS MERCURY COMPOUND

In this article for a sample configuration of a three-dimensional finite element model is used. For concrete modeling of three-dimensional elements (C3D8R) that an 8-node elements with reduced integration is used. Similar elements are considered for modeling steel sections. For longitudinal reinforcement and stirrups of the beam elements (B31) is used.

CONCRETE IN ABAQUS FINITE ELEMENT METHOD

In this study, the method used for modeling concrete, Concrete Damaged Plasticity is the model. This model is based on the assumption of isotropic damage survey and design for use in concrete under different environmentalloading conditions and the desired effect was accomplished. The embedded model is used for reinforcement.

LOAD AND BOUNDARY CONDITIONS

In the modeling study of rigid plates with a width of 10 cm was used as loading pages. Pages load and anchor tie in place by indicating that they have been fixed. Anchor plates on the boundary conditions with zero degrees of freedom U1 and U2 and UR2 and UR3 have taken place.

MESHED MODEL

Analysis of each of these models were calculated. By comparing the maximum load capacity for different meshing, percentage differences obtained for meshing

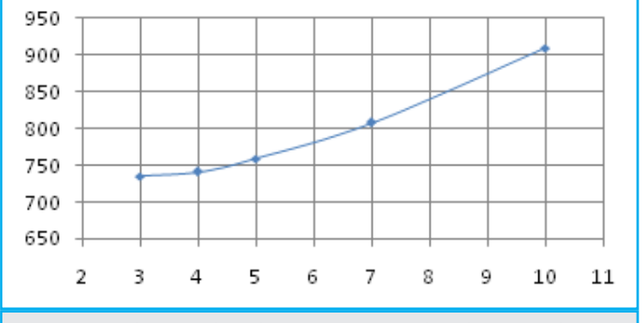

FIGURE 1. Change Chart bearing capacity by changing the size of the mesh

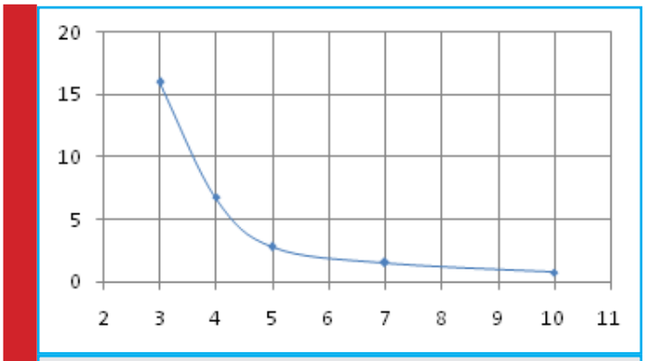

FIGURE 2. Change graph analysis time by changing the size of the mesh

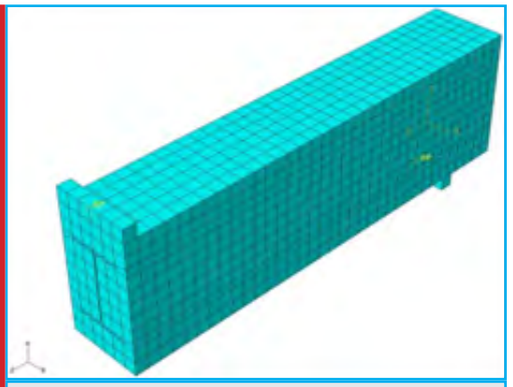

FIGURE 3. For an example of meshing in finite element software

with meshing elements smaller than 5 cm, less than 42/2 percent is obtained. That's why the next 5 inches as the maximum size for meshing elements have been selected.

Extrusion method used to create pieces of concrete and steel beam is therefore for the mesh generator parts Swept meshing techniques are used. Elements used in the mesh of hexagonal Hex been selected. Seeded maximum size of 5 cm is selected and used to control the curvature maximum deviation factor by default, the software is intended to 1/0. In Figure 3 meshed model for one of the samples shown.

ANALYSIS MODEL

Given the magnitude of the displacement and the need for non-linear analysis, dynamic methods for the analysis of selected models. The analytical method used in this study for explicit nonlinear dynamic analysis Dynamic Explicit is selected.

THE IMPACT OF CHANGING THE ENTIRE WIDTH OF THE BEAM FLANGE WIDTH STEEL TOOLS

One of the important parameters influencing the behavior of the composite beams of steel embedded in concrete, steel and concrete beam cross section Dimensions. the probability of failure is compounded in July.

Is. For this purpose, five different models, including models with wing width ratios C1, C2, C3, C4 and C5

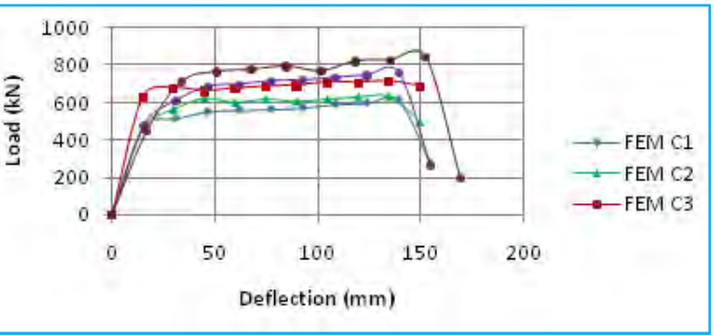

FIGURE 4. Bar chart - midspan displacement for models C1, C2, C3, C4 and C5

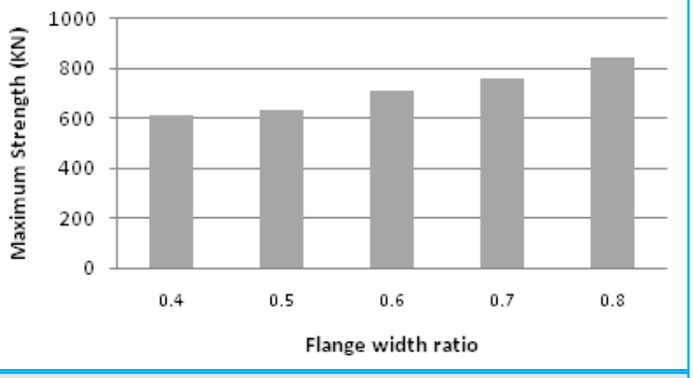

FIGURE 5. The graph ultimate bearing capacity for models C1, C2, C3, C4 and C5

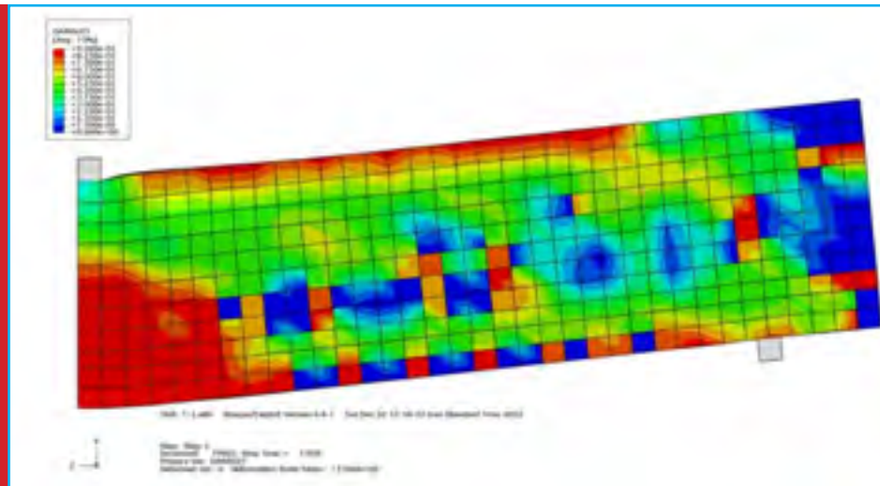

FIGURE 6. Contour stretch injury model C1

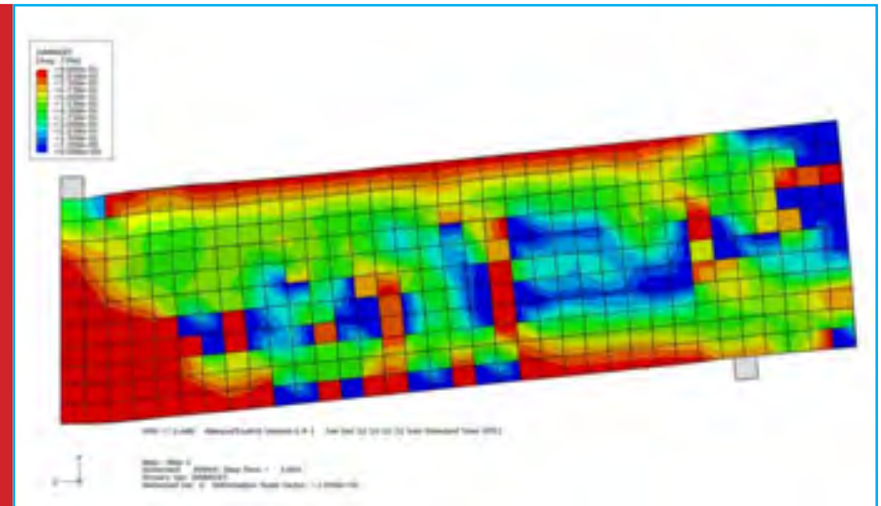

FIGURE 7. Contour stretch injury model C2

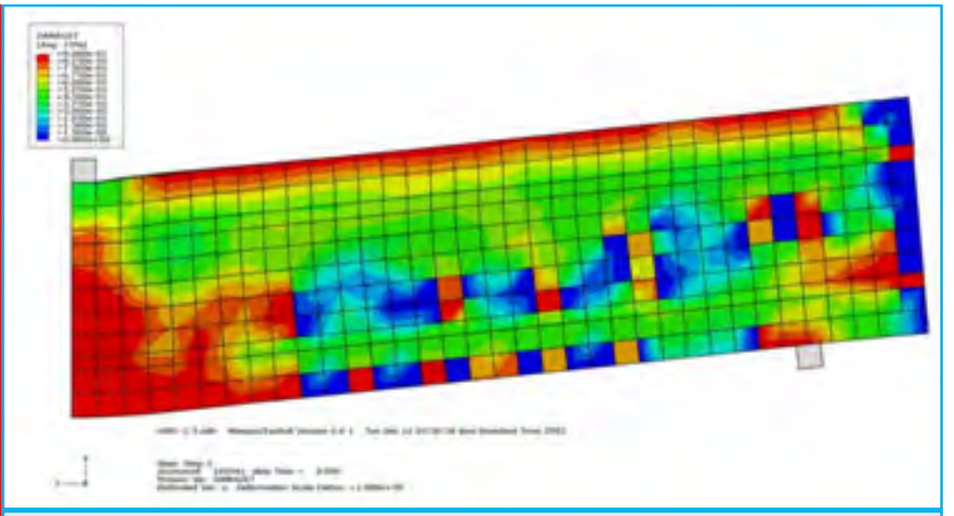

FIGURE 8. Injury contour drawing model C3

respectively steel with cross-sections of 120, 150, 180, 210 and 240 cm are selected.

All models have cross-sectional dimensions of 300 × 500 mm, respectively. Longitudinal bars diameter 19 mm and diameter of 10 mm are inadequate. The distance of each other 150 mm is considered inadequate and has not been used in any of these models is cutting.

GRAPHS TIME – DISPLACEMENT

Bar graphs - C1 to C5 models displacement is presented in Figure 4. As the charts once - movement can be observed with increasing steel flange width, capacity, as well as the final beam forming compound has increased. Figure 5 shows the curve of the ultimate capacity models. According to this chart, the increase in capacity of 22.3% for the C2 than C1 models, for models from C3 to C1 model 37/16%, 91/23% and times for the C4 to C1 model to model C5 to C1 models from 94/37% is obtained.

TENSILE CONTOURS INJURY

In the form of (6) to (10) C1 to C5 models tensile injury contours are provided. With a little care can be noted that in models with smaller steel flange width, propagation of cracks at midspan is more considerable.

RESHAPE THE CONTOURS OF PLASTIC

Stickers (11) to (15) of plastic deformation contours C1 to C5 models for the show. three models have been predicted failure for shear.

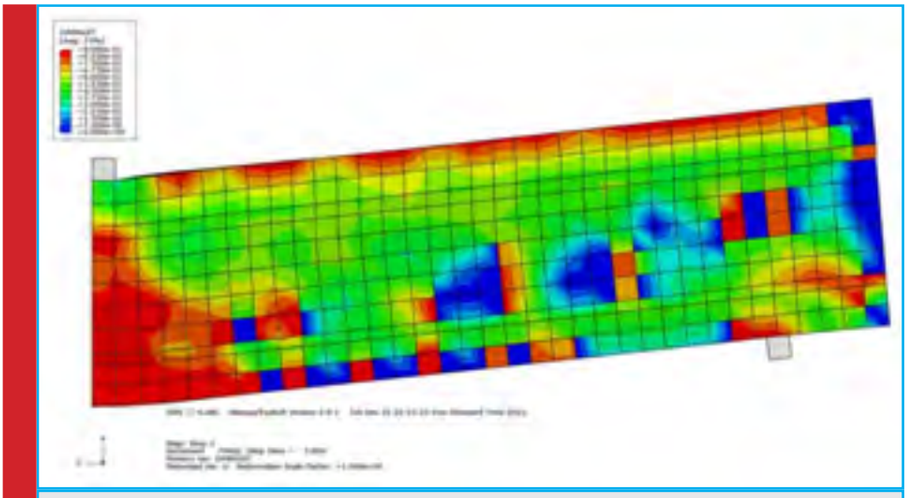

FIGURE 9. Injury contour drawing model C4

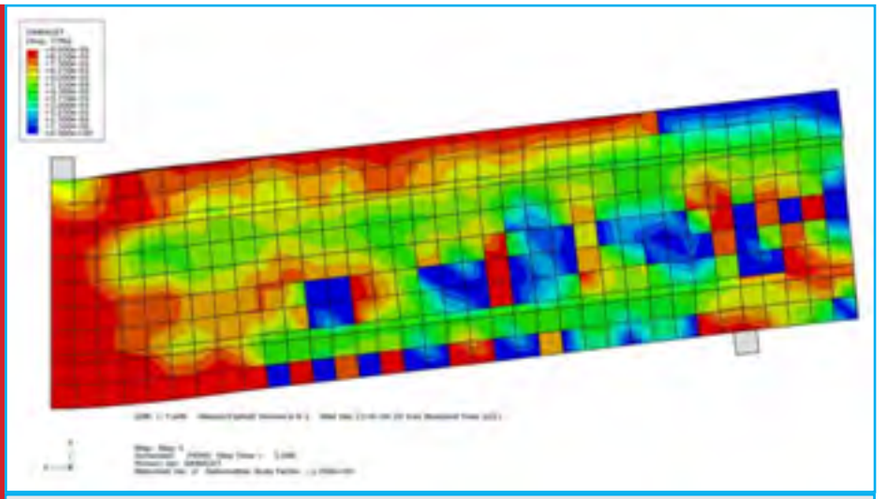

FIGURE 10. Contour injury tensile C5 model

In Table 1. The results of the analysis and prediction models include the strength of the type of failure is presented. The finite element analysis results in compliance with Regulation Iran relations have been evaluated. According to a survey made very good match between the results of finite element analysis of the samples and relations are provided in the bylaws. The ultimate shear capacity of 353.8 kN point in the relations of the Regulations is obtained. We see that in the C1 and C2 maximum shear force obtained from finite element analysis section is smaller than the shear capacity. Therefore, the models are broken due to reach its ultimate flexural capacity. Finite element analysis of bending failure for these models is the result. The results of finite element analysis also shows shear failure for these models.

CONCLUSION

In this article Environmental behavior of steel beams embedded in concrete under static loads were investigated. In contrast, in samples with a ratio of 0.5 steel flange width, usually bending failure have been observed. Results demonstrated that by optimizing the static load in the process of the production, the environmental side effects will be reduced and can be efficient in clean concrete production.

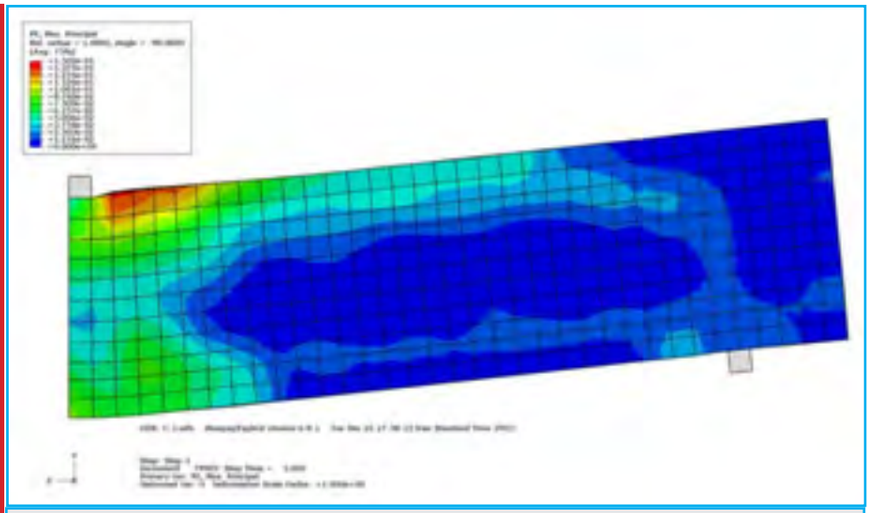

FIGURE 11. Contour relative deformation Plastic Model C1

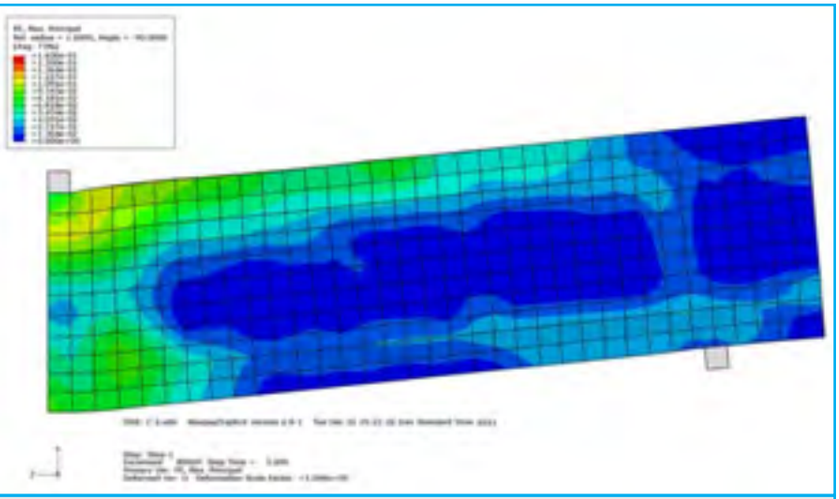

FIGURE 12. Contour relative deformation Plastic Model C2

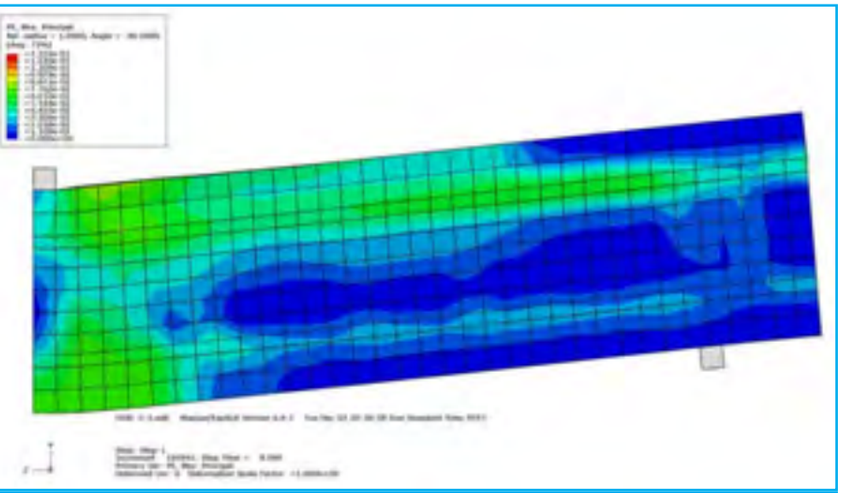

FIGURE 13. Contour relative deformation Plastic Model C3

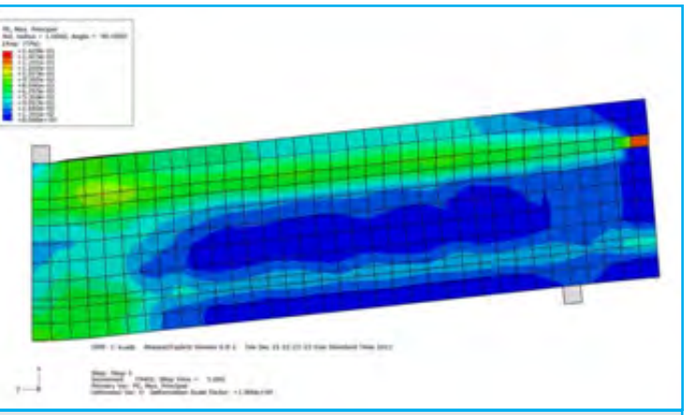

FIGURE 14. Contour relative deformation Plastic Model C4

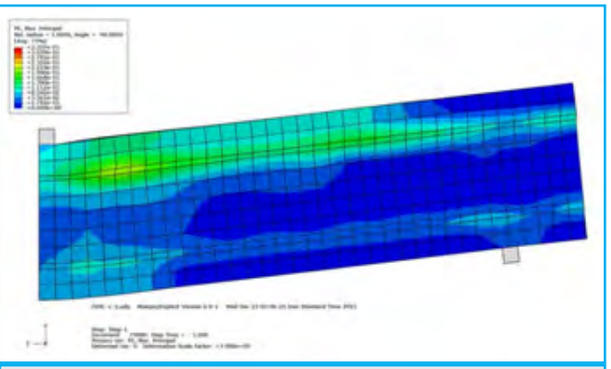

FIGURE 15. Contour relative deformation Plastic Model C5

Table 1. resistance and failure mode of composite beams with steel flange width ratios of

Model	Width steel	Width ratio	Resistance models		The type of fracture
			V (KN)	M (KN m)	
C1	120	0.4	306.5	459.8	Bending
C2	150	0.5	316.4	474.6	Bending
C3	180	0.6	356.7	535.1	Bending
C4	210	0.7	379.8	569.7	Bending
C5	240	0.8	422.8	634.2	Bending

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Lignocellulose biodegradation: An advance technology for sustainable environment

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ABSTRACT

The ever increasing energy load has attracted significant attention on the development and growth of renewable resources. Many workers have reported conversion of waste materials to useful compost. Lignocellulose consists of the three constituents indicated as cellulose, hemicelluloses and lignin. Lignocellulosic biomass can be reused in the production of chemicals and fuels. Cellulose and hemicellulose can be degraded into sugars, which are preliminary source for fermentation, biocatalytic and chemocatalytic processes to value-added products. A large number of microorganisms including bacteria and fungi are capable of producing cellulases and hemicellulases but only a limited number of these microorganisms are capable of producing lignin degrading enzymes. There are numerous methods available for the isolation of lignocellulose degrading microbial consortium. Lignocellulose compounds are most abundant agricultural residues present in the world. In this short review, updated account is presented on various aspects of lignocellulose compounds which can be utilized by fungi, actinomycetes and bacteria. The lignocellulytic utilizing microbial consortium can be used for the conversion of biomass feedstock to useful bio-based products. The use of farming crop wastes involves a separation of the polymeric compounds - cellulose and hemicelluloses. This approach comes under sustainable "green" biotechnology.

KEY WORDS: LIGNOCELLULOSE, RICE STRAW, FERMENTATION, CELLULASES AND HEMICELLULASES

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INTRODUCTION

Lignocellulosic biomass consists mainly of cellulose (35–50%), hemicellulose (25–30%) and lignin (25–30%). The plant biomass is a carbon source for bio-refinery industry, considered as sustainable and environmental friendly substitute to the current petroleum platform (Kamm *et al.,* 2004). The composition of lignocelluloses not only depends upon the species but also on the different parts of the plant, their age and growth conditions (Jorgensen, 2003). The large quantity of lignocellulosic materials create them potentially inexpensive and easily available natural resources for the production of high value compounds and biofuels. The degradation of cellulose, hemicelluloses and lignin are extremely slow. The various microorganisms are capable of growing on lignocellulosic materials and produce a wide range of enzymes that could be of scientific or industrial importance, (Varma et al. 2017 Tolalpa et al. 2018) .

Cellulose

It is homopolysaccharide constituent of the fiber wall, consists of D-glucose linked together by ß-1, 4-glycosidic bonds. Cellulose form intra- or intermolecular hydrogen bonds resulting in the formation of cellulose microfibrils. Due to hydrogen bond it is not soluble in most solvents and resistance against microbial degradation (Jorgensen, 2003). It is hydrolyzed by cellulase, 1, 4-ß-cellobiosidase and ß-glucosidase (Schmidt, 2006). Cellulases hydrolyses cellulose consists of cellobiohydrolases, endoglucanases and ß-glucosidase. The cellobiohydrolase and endoglucanase function together for hydrolyzing 1, 4-ß-D-glycosidic linkages in cellulose, cello-oligomers and other ß-D-glucans and to form cellobiose from the non-reducing ends, which are degraded by ß-glucosidase to glucose units.

Hemicellulose

It consists of monomeric residues, like D-glucuronic acid, D-mannose, D-arabinose, D-glucose and D-xylose. They have lesser degree of polymerization in comparison to cellulose, with side chains that can be acetylated. They are classified according to the monomeric sugar in the backbone of the polymer, e.g. mannan (ß-1, 4-linked mannose) or xylan (ß-1, 4-linked xylose) hemicelluloses. The main chains of glucose and mannose residues are generally associated with ß-(1, 4) bond. The side chain is attached to main chain via a-(1, 6) bonds in galactoglucomannan. Xylan can be degraded by endo-1, 4-ß-xylanase and 1, 4-ß-xylosidase to xylose (Jorgensen, 2003). Due to more heterogenous nature of hemicellulose, a combination of enzymes is necessary for its degradation, such as endoxylanases, ß-xylosidases, endomannanases, ß-mannosidases, a-L-arabinofuranosidases and a-galactosidases.

Lignin

It is very much hydrophobic and forms an amorphous complex with hemicelluloses enclosing cellulose and make unavailable to microbial utilization of available carbohydrates within the wood cell wall. It is jointing component to attach cells and harden the cell wall of xylem, which is accountable for the smooth movement of water from roots to leaves. Its aromatic rings are responsible to create it trickier to degrade (Schmidt, 2006). Ester linkages take place among the free carboxy group in hemicellulose and the benzyl groups in lignin, the lignicarbohydrate complex (LCC), embeds the cellulose, which is resposibe for giving protection against microbial and chemical degradation (Jeffries, 1994). The lignin is not to be utilized by most of the microorganisms due to its phenylpropane units in the structure (Schmidt, 2006). Now days there are huge attention in isolating organisms able to degrade lignin, in the cleaving of the chemical bonds that are present between lignin and hemicelluloses. The continuous utilization of these compounds is extremely slow. The microorganisms isolated from from soil and rumens are capable to degrade in to sugars, which can be utilized as energy and carbon source by a variety of microorganisms for the production of different products.

Role of microbial consortium in utilization of lignocellulose biomass

Kumar et al. (2001) isolated *Branhamella catarrhal*, *Brochothrix sp., Micrococcus luteus* and *Bacillus firmus* from cane sugar factory effluent contaminated soil. All microbes can use cinnamic acid as sole carbon source with significant inhibition after addition of glucose. The *B. catarrhalis* and *Brochothrix sp*. were able of metabolizing ferulic acid but not in the presence of glucose. The lignocellulolytic enzyme profiles of five strains of *Agaricus* and four strains of *Pleurotus* were determined by Rana and Rana in 2004. The crude enzyme from the colonized substrate was used to analysis the enzymatic activities. All strains of *Agaricus* showed higher level of cellulases activities in comparison of *Pleurotus* strains. The *Pleurotus sp*. exhibits high ligninases activity. The difference in lignocellulolytic enzyme summary was present both at interspecies and intraspecies point. The lignocellulose utilization in solid state fermentation in sugarcane bagasse and rice straw by *Aspergillus* tamari was isolated by Umasaravanan *et al*. (2010). The microorganisms isolated from marine environment which can utilize the lignocellulosic biomass were showed by Sethi et al. (2013).

The organisms showing maximum degradation were recognized as *Bacillus pumilus* and *Mesorhizzobium sp.,* as well two fungal sp. recognized as *Aspergillus niger*

and *Trichodermaviride*. The organisms showed different levels of utilization at different time point in a diversity of substrates. The thermophilic microbial consortium was isolated by Malik et al. (2015) from sugarcane industry mature compost. The consortium was able to degrade rice straw. The lignin can be productively degraded by white-rot fungi, producing a number of oxidoreductases, enzymes able to attack phenolic structures. Varma et al. (2017) studied about those microbes which were actively involved in lignocelluloses degradation during drum composting of mixed organic waste i.e. saw dust, cattle manure, dry leaves and vegetable waste in a 550 L rotary drum composter.

An anaerobic thermophilic bacterial strains for ethanol production from D-xylose was reported by Sommer et al. (2004). Lu et al. (2004) reported *Clostridium phytofermentans*, responsible for the maximum number of enzymes for the utilization of lignocellulosic biomass between sequenced *Clostridial* genomes. The *Clostridium thermocellum* generate a collection of cellulolytic and hemicellulolytic enzymes in a multienzyme complex system are known as cellulosome. The cellulosome was firstly reported in anaerobic bacteria such *as Clostridium thermocellum* in 1983 and then in anaerobic fungi in 1992 (Wilson et al., 1992). Abd-Elsalam et al. (2009) isolated bacterial strains KafAH19 degrade synthetic lignin and use as a carbon source. The strain was biochemically characterized as gram-positive rod. By using 16S rDNA sequencing the culture was recognized as *Bacillus sp.*. The lignocellulose utilizing microbes by using a selective media using lignin, xylan and cellulose as selective substrates was isolated by Wahyudi et al. (2010). The isolates were recognized as *Enterococcus casseliflavus/gallinarum* sp. Wongwilaiwalin et al. (2010) worked on thermophilic lignocellulose utilizing microbial consortium and multi-species lignocellulolytic enzymatic involvement. Gontikaki et al. (2015) observed that the proficient degradation of terr OC in the marine environment could be fuelled by labile marine based objects by treating coastal sediments with 13C-lignocellulose, as a proxy for terrOC, in the presence and absence of unlabelled diatom detritus that acted as the important inducer. The amount of priming was viewed by the differentiation in lignocellulose mineralisation between diatom-amended treatments and controls in aerobic sediment slurries.

Ransom-Jones et al. (2017) have reported that landfill sites symbolize a repository of uncultivated lignocellulose-degrading Microbes such as *Firmicutes*, *Bacteroidetes*, *Spirochaetes*, and *Fibrobacteres,* which are rich source for biomass degradation. According to Cortes-Tolalpa et al. (2017), Lignocellulosic biomass (LCB) is a striking source of carbon for the production of sugars and other useful chemicals. Due to its innate density and heterogeneity, proficient biodegradation requires the actions of diverse and various types of hydrolytic enzymes. So, they observed that the wheat straw degradation potential of synthetic microbial consortia composed of bacteria and fungi.

Alessi et al. (2018) explored the enzymatic degradation of lignin which is complicated due to its structural complexity, lack of hydrolysable linkages and insoluble nature and also discuss the developments in the degradation of lignin by microorganisms and the catabolic pathways for degradation of lignin. Mohn et al. (2018) illustrate that by applying stable isotope probing (SIP) coupled with amplicon and shotgun metagenomics, it is possible to recognize and describe the functional attributes of cellulose, hemicelluloses and lignin - degrading bacteria and fungi. The halotolerant lignocellulose degrading microbial consortia was isolated by Cortes-Tolalpa et al. (2018) by feeding a salt marsh soil microbiome on an intractable carbon and energy source, i.e., wheat straw.

Conclusion and future prospects

Lignocellulose compounds are most abundant agricultural residues present in the world. There are various reports that lignocellulose compounds can be utilized by fungi, action mycetes and bacteria. The lingo cellulytic utilizing microbial consortium can be used for the conversion of biomass feedstock to useful bio-based products. The use of farming crop wastes involves a separation of the polymeric compounds - cellulose and hemicelluloses. This approach comes under sustainable “green” biotechnology.

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Satisfaction of occupational and physical therapy students during their internship program

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ABSTRACT

The internship year is considered to be a golden period for students' training, as they are exposed during that time to a majority of medical specialties and will gain more knowledge and clinical skills. it is the time they convert their taught information and knowledge into skills. However the quality of training and the appropriateness of environment as well as the satisfaction of trainee is a major issue. Our study aimed to assess the satisfaction about internship training of occupational and physical therapy, among interns in Riyadh. A cross-sectional study was conducted including 81 Occupational Therapy (OT) and Physical Therapy (PT) interns from King Abdulaziz Medical City (KAMC) and King Abduallah Specialized Children Hospital (KASCH) (48 females, 33 males; 41 PT, 40 OT; mean age 23.07 yrs, SD-1.17). Interns were excluded if they had a repeated rotation either due to weak performance or as disciplinary action from the department, faced problems in the department that required further investigation, and spent less than two months in the hospital. A survey previously used in Al Dammam study. It has 21 items divided across four domains that are either related to preparatory work towards internship, satisfaction, and comfort with the supervisor and internship site, attainment of intended learning outcomes, or evaluation and feedback during internship. Three interns were excluded as they spent less than two months in the hospital. Interns had a satisfaction rate of 83.05% where PT interns had a satisfaction rate of 86.15%, and OT interns had 78.50%. Additionally, PT interns had a higher satisfaction rate than OT interns across all domains. However, the only significant difference between the two programs was on the items related to evaluation and feedback during the internship. Interns in the main hospital had a satisfaction rate of 84.15%, whereas interns in KASCH had a satisfaction rate of 80.94%.We found a high satisfaction rate of OT and PT interns in this study, and higher satisfaction of PT interns compared to OT interns. However, further research can be conducted using the same questionnaire to explore different hospitals in Riyadh and different region within Saudi Arabia to have an overall estimation of satisfaction rate among the PT and OT at the national level.

KEY WORDS: INTERNSHIP, OCCUPATIONAL THERAPY, PHYSIOTHERAPY, SATISFACTION

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INTRODUCTION

Internship training is known to be one mandatory year after completing the undergraduate study where the interns are required to apply their academic (theoretical) learning to practical (clinical) experiences. The internship year is considered to be a golden period for students' training, as they are exposed during that time to a majority of medical specialties and will gain more knowledge and clinical skills, (Finucane and O'Dowd, 2005). As stated by Davis (1990), intern training is an experiential learning which gives the students the opportunity to apply what they have learned from schools in real world situation, and it gives the interns a chance to consolidate and combine thinking and action, (Cheong et al., 2014, Sasnett and Rose, 2016; Yasser et al., 2016).

It is a valuable component of academic programs, which is beneficial for the student, employer as well as the university. Internship training has a major role in improving learning professional skills, applying what have been learned theoretically in real life, improving communication and teamwork skills, gaining more experiences which increase interns' quality to deal with different situations in the future, identifying strengths and weakness, and preparing interns' mindset for their future careers, (Hart et al., 1989; Sasnett and Rose, 2016 and Phua 2017).

Training programs might also help to improve immediate skills that could eventually improve course performances. For example, better time management, heightened initiative, and better self-discipline and an overall better self-concept (Dennis, 1996). The interns gain tremendous advantages other than the experience they obtain during their internship. This opportunity allows them to discover their areas of interest and scout about the environment. However, future professional jobs must be available for the interns in order to achieve the best possible outcomes, (Wurfel and Walter 1985, Berger, 1991; Ramus, 1997; Verney et al., 2009 and Phua 2017).

The strengths \ advantages of internship confirm the importance of developing an efficient training program. Earlier studies reported that the majority of interns improved their knowledge about the field as a result of the training/internship program (Al Thukair, 2014)interns, alumni, coordinators, and supervisors of the Health Information Management and Technology (HIMT. Sufficient contact with a training coordinator may promote the benefits that interns could gain from internship (Yafang and Gongyong, 2008). The contentment of interns with the training may be a chance to raise their interests in full-time jobs (D'abate et al., 2009). In contrast, according to Taylor (1988), "poor supervision was the most likely condition to lead to dissatisfaction with internships" (Taylor, 1988). Nelson (1994) stated that repetitious work with low freedom to test knowledge along with the high risk that accompanies insufficient feedback would relate to dissatisfaction with supervision (Nelson, 1994).

Al-Muhanna (2009), observed that the major issue facing medical and health profession interns is the gap between what is offered by the training institution and what the interns want or expect. Another problem in internship training is the stress linked with the internship year due to its importance to the interns' professional lives (Al-Muhanna, 2009). Another issue mentioned by Hannon (2000) is the lack of adequate preparation for internship. Graduates thought they haven't yet developed the acquired attitudes and skills, therefore, it was perceived as being unfair to the patient and eventually decreeing the interns' benefit from clinical training. Additionally, some stated that some clinical practices may be more effective than the ones actually used (Hannon, 2000). In one study, the lowest rate of satisfaction was given to the feedback level from the trainer as well the number of available trainers in relation to the number of students (Serwah, Sulimani, Samy, & Serwah, 2015). In another study, interns have reported a few incidents of mistreatment especially verbal attacks (Daugherty, Baldwin, & Rowley, 1998). Also, another study which was concerned with pharmacy interns stated that the training period in a certain area \ ward is too short (Phua et al., 2017).

Having knowledge about these issues and any other issues that may face medical and health profession interns will help in improving the interns' experience in clinical training and improving their performance. The present study was proposed to overcome the lack of data on the satisfaction of interns in occupational therapy and physical therapy about their training program. The results of the study will give insight to strengths and weaknesses and chances for improvement of the current internship programs with respect to OT and PT.

METHODS

Subjects of this study were occupational and physical therapy interns in King Abdulaziz Medical City (KAMC) and King Abduallah Specialized Children Hospital (KASCH). Institutional review board (IRB) approval was obtained before conducting the study, and informed consent was obtained from all participants before the data collection. Participants' confidentiality was strictly observed throughout the study by using an anonymous unique serial number for each subject and restricting data only to the investigators. The inclusion criteria were occupational and physical therapy interns, male and female, who passed at least one rotation of 2 months duration in the designated setting. The exclusion criteria were occupational and physical therapy interns who have had a repeated rotation either due to weak performance or as disciplinary action from the department, faced problems

in the department that required further investigation, and spent less than two months in the hospital.

The data was collected by a structured questionnaire which measures the satisfaction of interns in the following areas: preparation, internship posting, professional skills and knowledge, and evaluation and feedback. It had been previously used in Al Dammam study, with slight modifications including questions about the duration of the internship, any conflict the interns have experienced in the current setting, and the number of passed rotations. In this questionnaire, the subjects filled out variables about their age, gender, program, a name of the hospital, and the start and end date of the internship. However, the focus of this survey was converted to fit the clinical internship program rather than NCAAA. This questionnaire, adapted from the previously mentioned study, was used in Saudi Arabia with similar objectives and subjects to this study. Therefore, this questionnaire was used to broaden the scope of measuring interns' satisfaction in KAMC and KASCH in Riyadh.

The measurement tool used was the Likert scale where a score of 0 was assigned to strongly disagree which is the lowest grading, and a score of 3 was assigned to strongly agree which is the maximum score. A score of 1 was assigned to disagree, and a score of 2 was assigned to agree. Whereas in yes/no questions at the end of the questionnaire, a score of 1 was assigned to 'yes' and a score of 2 is assigned to 'no.' At the end of the survey, the interns had the chance to write any suggestions or recommendations the could have made their internship experience better which can be used by the institution for future improvements in their internship program.

The collected data was entered into Microsoft Excel after ensuring the removal of confidential information and after being coded and cleared. It was then exported to SPSS version 22 where categorical variables were represented in frequencies and percentages, and the continuous variable was represented in mean and standard deviation. Percentages of overall satisfaction, satisfaction in each domain, satisfaction between programs, and satisfaction between hospitals were calculated. Independent t sample test was used to measure any significant difference in satisfaction between programs.

RESULTS AND DISCUSSION

DEMOGRAPHICS OF PARTICIPANTS

A total of 100 surveys were distributed, and 84 responded. 3 participants were excluded as they have spent less than two months in the hospital. 81 participants entered analysis including 40 (49.4%) from occupational therapy program and 41 (50.6%) from physical therapy program. 45 (55.6%) respondents were in King Abdulaziz Medical City, 26 (32.1%) respondents were in King Abduallah Specialized Children Hospital, and 10 (12.3%) respondents were in both hospitals. Table 1 shows the detailed backgrounds of the respondents

Table 1. Demographics of Participants

	Occupational Therapy	Physical Therapy	All
Age Mean (Std.Deviation)	22.97 yrs (1.20)	23.13 yrs (1.14)	23.07 yrs (1.17)
Gender			
Male	17	16	33
% Within Program	42.5%	39.0%	40.7%
Female	23	25	48
% Within Program	57.5%	61.0%	59.3%
Total	40	41	81
% Within Program	100.0%	100.0%	100.0%
Hospital			
KAMC	21	24	45
% Within Program	52.5%	58.5%	55.6%
KASCH	15	11	26
%Within Program	57.5%	26.8%	32.1%
Both	4	6	10
% Within Program	10.6%	14.6%	12.3%
Total	40	41	81
%Within Program	100.0%	100.0%	100.0%
Total of Program	40 (49.4)	41 (50.6)	81 (100)

FIGURE 1. Satisfaction of interns in all four domains between Occupational Therapy and Physiotherapy Department

The present study was proposed to determine data on the satisfaction of interns in occupational therapy and physical therapy department in King Abdulaziz Medical City (KAMC) and King Abduallah Specialized Children Hospital (KASCH) in Saudi Arabia at Riyadh. The result shows the satisfaction rate in 4 domains (Preparation, Internship Posting, Professional Skills and Knowledge, and Evaluation and Feedback by the program) according to OT & PT Program. PT interns had a satisfaction rate of 86.15%, whereas OT interns had a satisfaction rate of 78.50%. In comparison to each domain, PT interns had a satisfaction rate of 94%, 92%, 95%, and 64% respectively to the domains of preparation, internship posting, professional skills and knowledge, and evaluation and feedback. OT interns had a satisfaction rate of 87%, 88%, 90%, and 48% respectively to the previously mentioned domains as shown in figure 1. Additionally, figure 2,3,4 and 5 shows level on each item related to preparatory work towards internship, comfort with the supervisor and internship site, attainment of intended learning outcomes and evaluation and feedback during the internship. The p-value for all domains was measured. However, the only significant difference between the programs was in the evaluation and feedback domain (p-value > 0.05).

Table 2 shows the detailed scoring of the survey in each of the four domains by hospitals. In the comparison between KAMC and KASCH, interns in KAMC had a satisfaction rate of 84.15%, whereas interns in KASCH had a satisfaction rate of 80.94%. The difference in some interns between KAMC and KASCH should acknowledge which may affect the precision of our estimates in this study.

This study aimed to assess the satisfaction of occupational and physical therapy interns about their internship experience in KAMC and KASCH. We asked the interns to fill out a survey with four domains and a total of 21 items. The results of this study indicate that OT and PT interns are generally satisfied with their internship experience. Items related to preparatory work towards internship, satisfaction, and comfort with the supervisor, internship site and attainment of intended learning outcomes had a high satisfaction rate of over 90%, whereas items related to evaluation and feedback during the internship had the lowest satisfaction rate. Therefore the feedback in a regular short period is very important and considered excellent mechanism for student to rectify, (Serwah et al., 2015).

The five items with the highest agreement level include: learning new skills and knowledge during the

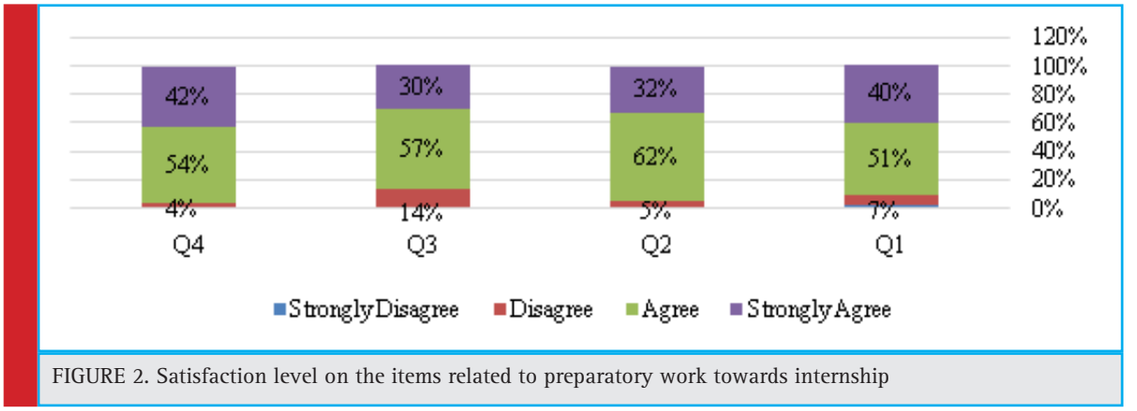

FIGURE 2. Satisfaction level on the items related to preparatory work towards internship

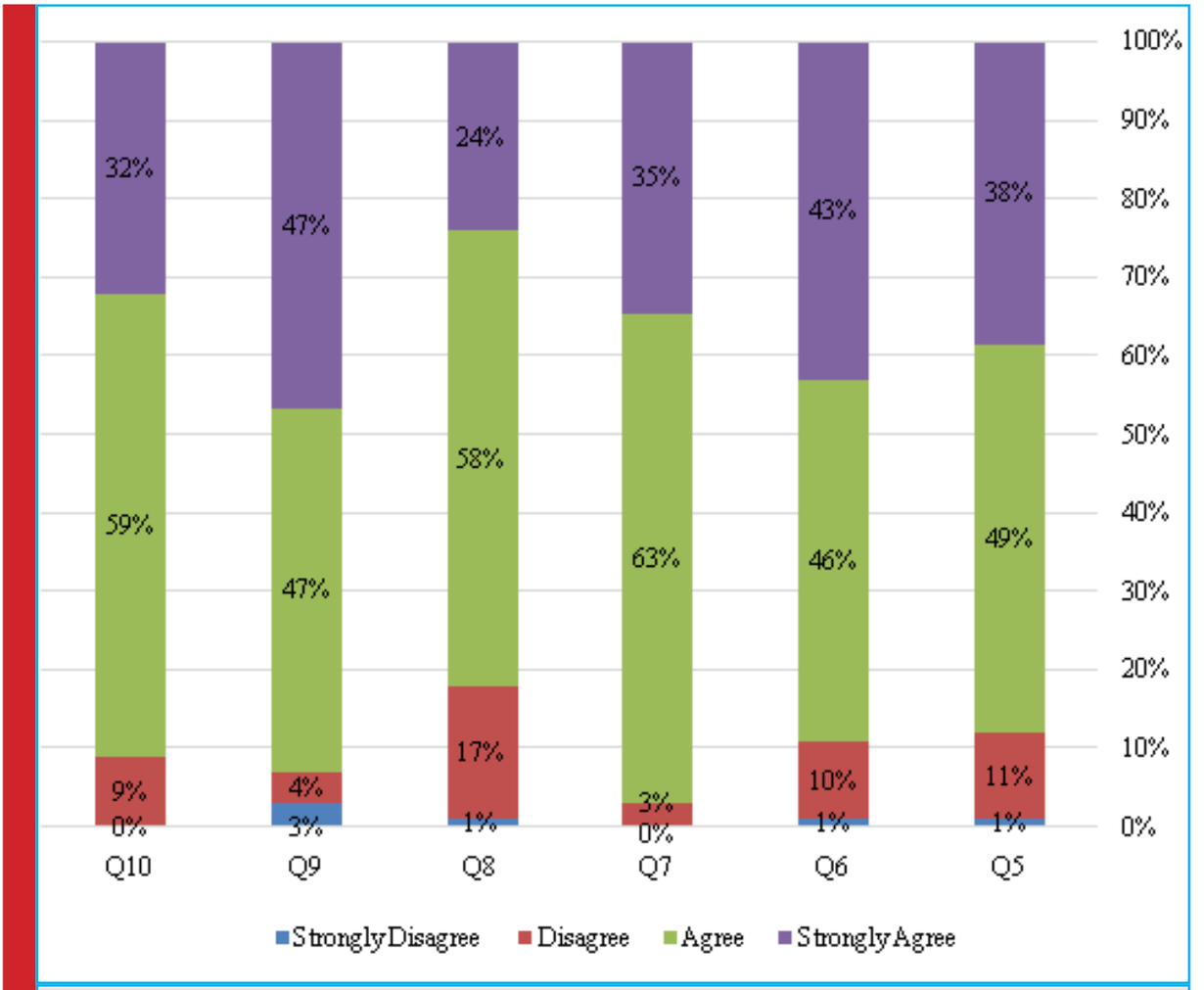

FIGURE 3. Satisfaction level on the items related to comfort with the supervisor and internship site

internship (item 11), the interns feeling their presence at the hospital made a positive contribution to professionals at internship site (item 7), followed by the interns gaining insight into the work environment and field of their interest (item 13), Alshomrani et al. 2016, Sasnett et al. 2016, Finucane et al. 2009). Being well prepared for the internship (item 4), developing an awareness of the areas in which the interns needed growth (item 16), and finding the internship handbook to be clear and very useful (item 2) followed by applying to the job in the hospital if an open opportunity was available (item 9).

The five items with the lowest agreement level include: being asked to prepare a report at the end of each internship rotation (item 19), having arranged meeting by the university to reflect on the internship experience (item 20), visitations from internship coordinator at field location for observation (item 17), having the opportunity to freely evaluate the experience at the hospital (item 18), and being heard and responded to when raising issues about the internship process with the internship coordinator (item 21) (Finucane et al. 2005).

PT interns were generally more satisfied than OT interns. On each domain of the survey, PT interns also had a higher satisfaction rate than OT interns. However, the only significant difference between the two programs was on the items related to the evaluation and feedback domain. This might be attributed to individual differences in the supervisors' evaluation of the interns toward the end of the internship. Furthermore, interns who had their internship in KAMC had a slightly higher satisfaction rate than interns in KASCH generally and on each domain. The only significant domain was the satisfaction and comfort of the supervisor and intern-

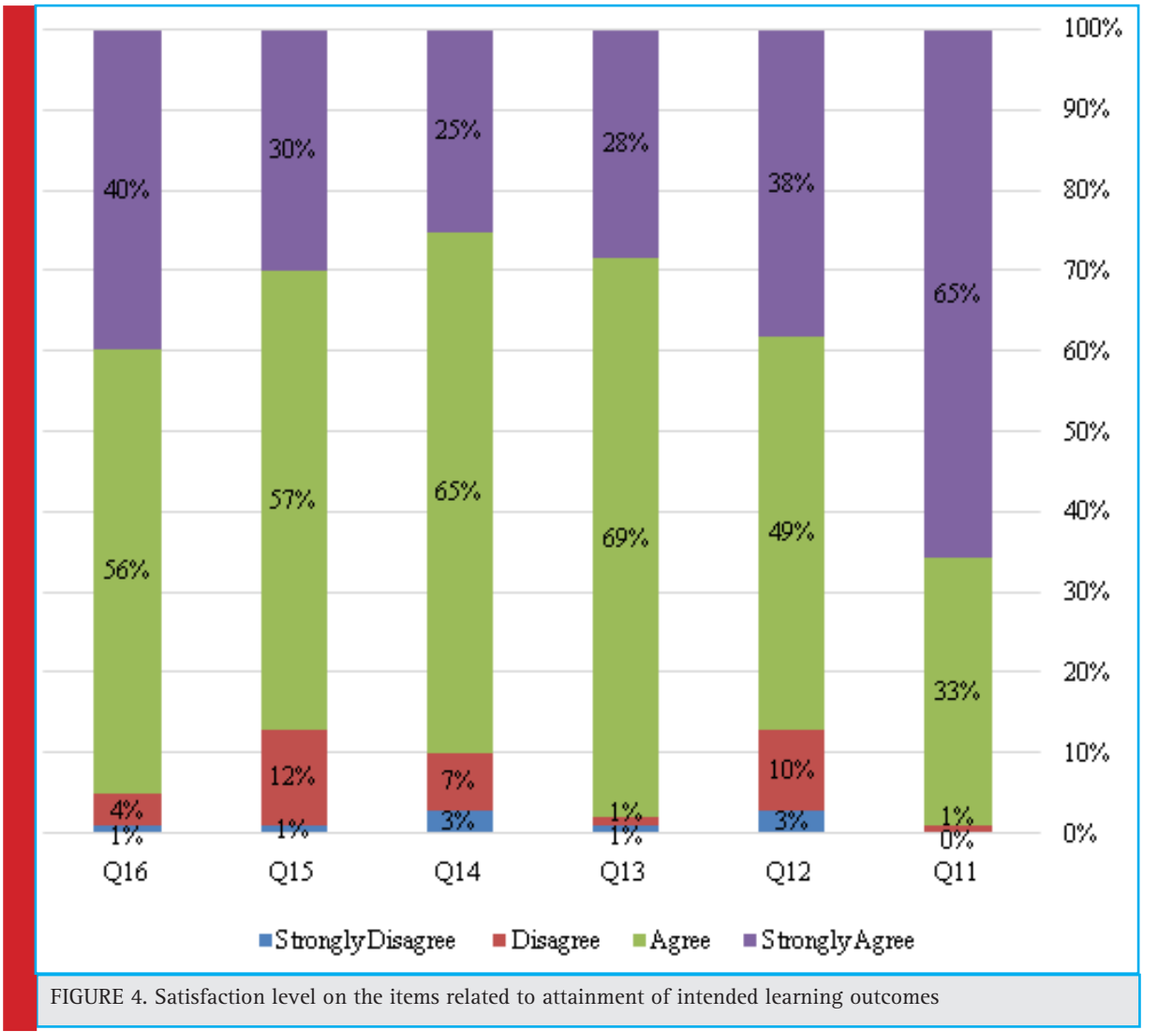

FIGURE 4. Satisfaction level on the items related to attainment of intended learning outcomes

ship site,). Taylor 1988 highlighted the importance of supervision. Also, giving the interns the opportunity to choose the internship site could help in increasing their satisfaction level.

Through examining the literature, we found one study that has been carried out in Damam in which we can compare our finding to their results. Al Thukair conducted a study in 2014, at Al Dammam comparing the satisfaction in PT and HIMT interns, and found that items related to preparatory work towards internship, satisfaction, the comfort with the supervisor, the internship site, and attainment of intended learning outcomes had the highest satisfaction rate, whereas items related to evaluation and feedback during the internship had the lowest satisfaction rate which was consistent with our study, (Al Thukair 2014). This Items identified as having the lowest satisfaction rate were inconsistent with our research as not all items related to evaluation and feedback during internship were identified as having the lowest satisfaction rate.

Strengths of this study include that the data was collected from KAMC and KASCH which are tertiary care hospitals with high bed capacity and high-quality rehabilitation services that cover most patient population in the central region of Riyadh. Secondly, we collected a sample size that should be representative of all OT and PT interns in the central region of Riyadh. Thirdly, the questionnaire had been previously used in Al Dammam study by Al Thukair (2014) as a valid and a reliable questionnaire. Finally, this study, up to our knowledge, is the first study that assesses the satisfaction of OT interns, or OT and PT interns combined in Saudi Arabia. However, limitations of the study include the limited hospital sittings with no further expansion to more hospitals. Additionally, the difference in a number of interns between KAMC and KASCH should be acknowledged which may

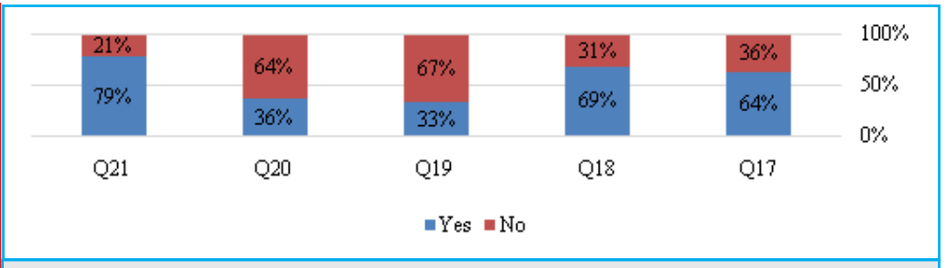

FIGURE 5. Satisfaction level on the items related to evaluation and feedback during internship

Table 2. Satisfaction of interns in four domains between KAMC and KASCH hospital

Items		Response	KAMC	KASCH	Both	Total
Q1	Preparation: The orientation for internship increased my level of comfort with the process, and, with my responsibilities as an intern.	Strongly Disagree	2 (4.4%)	0 (0.00%)	0 (0.0%)	2 (2.5%)
		Disagree	3 (6.7%)	3 (11.5%)	0 (0.0%)	6 (7.4%)
		Agree	17 (37.8%)	18 (69.2%)	6 (60.0%)	41 (50.6%)
		Strongly Agree	23 (51.1%)	5 (19.2%)	4 (40.0%)	32 (39.5%)
Q2	Internship handbook was clear and very useful.	Strongly Disagree	1 (2.2%)	0 (0.0%)	0 (0.0%)	1 (1.3%)
		Disagree	2 (4.4%)	2 (7.7%)	0 (0.0%)	4 (4.9%)
		Agree	27 (60.0%)	20 (76.9%)	3 (30.0%)	50 (61.7%)
		Strongly Agree	15 (33.3%)	4 (15.4%)	7 (70.0%)	26 (32.1%)
Q3	Learning outcomes were clear.	Strongly Disagree	-	-	-	-
		Disagree	7 (15.6%)	3 (11.5%)	1 (10.0%)	11 (13.6%)
		Agree	23 (51.1%)	18 (69.2%)	5 (50.0%)	46 (56.8%)
		Strongly Agree	15 (33.3%)	5 (19.2%)	4 (40.0%)	24 (29.6%)
Q4	Overall, I was well prepared for my internship.	Strongly Disagree	-	-	-	-
		Disagree	1 (2.2%)	2 (7.7%)	0 (0.0%)	3 (3.7%)
		Agree	24 (53.3%)	16 (61.5%)	4 (40.0%)	44 (54.3%)
		Strongly Agree	20 (44.4%)	8 (30.8%)	6 (60.0%)	34 (42.0%)
Q5	Internship posting: My internship supervisor provided an orientation to the internship department.	Strongly Disagree	0 (0.0%)	1 (3.8%)	0 (0.0%)	1 (1.2%)
		Disagree	3 (6.7%)	6 (23.1%)	0 (0.0%)	9 (11.1%)
		Agree	24 (53.3%)	12 (46.2%)	4 (40.0%)	40 (49.4%)
		Strongly Agree	18 (40.0%)	7 (26.9%)	6 (60/0%)	31 (38.3%)
Q6	When I started my internship, I felt accepted and welcomed by my co-workers.	Strongly Disagree	0 (0.0%)	1 (3.8%)	0 (0.0%)	1 (1.2%)
		Disagree	3 (6.7%)	5 (19.2%)	0 (0.0%)	8 (9.9%)
		Agree	17 (37.8%)	13 (50.0%)	7 (70.0%)	37 (45.7%)
		Strongly Agree	25 (55.6%)	7 (26.9%)	3 (30.0%)	35 (43.2%)
Q7	I felt that my presence at the organization made a positive contribution to professionals at internship site.	Strongly Disagree	-	-	-	-
		Disagree	1 (2.2%)	1 (3.8%)	0 (0.0%)	2 (2.5%)
		Agree	23 (51.1%)	20 (76.9%)	8 (80.0%)	51 (63.0%)
		Strongly Agree	21 (46.7%)	4 (19.2%)	2 (20.0%)	28 (34.5%)
Q8	Internship tasks were relevant to my academic course work.	Strongly Disagree	0 (0.0%)	1 (3.8%)	0 (0.0%)	1 (1.2%)
		Disagree	7 (15.6%)	6 (23.1%)	1 (10.0%)	14 (17.3%)
		Agree	25 (55.6%)	16 (61.5%)	6 (60.0%)	47 (58.0%)
		Strongly Agree	13 (28.9%)	3 (11.5%)	3 (30.0%)	19 (23.5%)

Q9	If an appropriate job were open at this department, I would apply for it.	Strongly Disagree	2 (4.4%)	0 (0.0%)	0 (0.0%)	2 (2.5%)
		Disagree	1 (2.2%)	1 (3.8%)	1 (10.0%)	3 (3.7%)
		Agree	19 (42.2%)	17 (65.4%)	2 (20.0%)	38 (46.9%)
		Strongly Agree	23 (51.1%)	8 (30.8%)	7 (70.0%)	38 (46.9%)
Q10	Overall, my experience as an intern met my expectations.	Strongly Disagree	-	-	-	-
		Disagree	2 (4.4%)	3 (11.5%)	2 (20.0%)	7 (8.6%)
		Agree	26 (57.8%)	17 (65.4%)	5 (40.0%)	48 (59.3%)
		Strongly Agree	17 (37.8%)	6 (23.1%)	3 (30.0%)	26 (32.1%)
Q11	Professional Skills and Knowledge I learned new skills and knowledge in my internship	Strongly Disagree	-	-	-	-
		Disagree	0 (0.0%)	1 (3.8%)	0 (0.0%)	1 (1.3%)
		Agree	12 (26.7%)	12 (46.2%)	3 (30.0%)	27 (33.3%)
		Strongly Agree	33 (73.3%)	13 (50.00%)	7 (70.0%)	53 (65.4%)
Q12	My internship supervisor showed interest in my suggestions/ideas. .	Strongly Disagree	0 (0.0%)	1 (3.8%)	1 (10.0%)	2 (2.5%)
		Disagree	6 (13.3%)	2 (7.7%)	0 (0.0%)	8 (9.9%)
		Agree	22 (48.9%)	14 (53.8%)	4 (40.0%)	40 (49.3%)
		Strongly Agree	17 (37.8%)	9 (34.6%)	5 (50.0%)	31 (38.3%)
Q13	I gained insight into the work environment and field of my interest.	Strongly Disagree	1 (2.2%)	0 (0.0%)	0 (0.0%)	1 (1.3%)
		Disagree	1 (2.2%)	0 (0.0%)	0 (0.0%)	1 (1.2%)
		Agree	30 (66.7%)	18 (69.2%)	8 (80.0%)	56 (69.1%)
		Strongly Agree	13 (28.9%)	8 (30.8%)	2 (20.0%)	23 (28.4%)
Q14	I was able to integrate theory and practice.	Strongly Disagree	1 (2.2%)	1 (3.8%)	0 (0.0%)	2 (2.5%)
		Disagree	3 (6.7%)	3 (11.5%)	0 (0.0%)	6 (7.4%)
		Agree	29 (64.4%)	17 (65.4%)	7 (70.0%)	53 (65.4%)
		Strongly Agree	12 (26.7%)	5 (19.2%)	3 (30.0%)	20 (24.7%)
Q15	My classes became more meaningful after my internship.	Strongly Disagree	0 (0.0%)	1 (3.8%)	0 (0.0%)	1 (1.3%)
		Disagree	4 (8.9%)	5 (19.2%)	1 (10.0%)	10 (12.3%)
		Agree	25 (55.6%)	18 (69.2%)	3 (30.0%)	46 (56.8%)
		Strongly Agree	16 (35.6%)	2 (7.7%)	6(60.0%)	24 (29.6%)
Q16	I developed an awareness of areas in which I needed growth.	Strongly Disagree	0 (0.0%)	1 (3.8%)	0 (0.0%)	1 (1.2%)
		Disagree	3 (6.7%)	0 (0.0%)	0 (0.0%)	3 (3.7%)
		Agree	23 (51.1%)	17 (65.4%)	5 (50.0%)	45 (55.6%)
		Strongly Agree	19 (42.2%)	8 (30.8%)	5 (50.0%)	32 (39.5%)
Q17	Evaluation and Feedback Internship coordinator visited me at field location for observation.	Yes	30 (66.7%)	15 (57.7%)	7 (70.0%)	52 (64.2%)
		No	15 (33.3%)	11 (42.3%)	3 (30.0%)	29 (35.8%)
Q18	I have the opportunity to freely evaluate my experience at the hospital.	Yes	34 (75.6%)	15 (57.7%)	7 (70.0%)	56 (69.1%)
		No	11 (24.4%)	11 (42.3%)	3 (30.0%)	25 (30.9%)
Q19	I am asked to prepare a report at the end of each internship rotation.	Yes	14 (31.1%)	12 (46.2%)	1 (10.0%)	27 (33.3%)
		No	31 (68.9%)	14 (53.8%)	9 (90.0%)	54 (66.7%)
Q20	Meetings were arranged by my university to reflect and share my internship experience throughout the course.	Yes	19 (42.2%)	9 (34.6%)	1 (10.0%)	29 (35.8%)
		No	26 (57.8%)	17 (65.4%)	9 (19.0%)	52 (64.2%)
Q21	I was heard and responded to when I raised issues about the internship process with internship coordinator.	Yes	37 (82.2%)	18 (69.2%)	9 (19.0%)	64 (79.0%)
		No	8 (17.8%)	8 (30.8%)	1 (10.0%)	17 (21.0%)

affect the precision of our estimates in this study. It is worth mentioning that some of the preceptor deviated from the training framework to address more specialized advanced skills or techniques which is match the level of the intern and track them out of the training scope. There was a notable feedback on the divergence of the training content versus the written training manual, since some trainer tends to address a higher level content above the interns level.

CONCLUSION

We found a high satisfaction rate of OT and PT interns in this study, and higher satisfaction of PT interns compared to OT interns, a regular short term feedback between intern student and their preceptors is of a great importance for student to rectify and maintain focuses on the training framework and avoid expanding to advanced techniques beyond student level will assure better outcomes. It is obvious that a further research can be conducted using the same questionnaire to explore different hospitals in Riyadh and different regions within Saudi Arabia to have an overall picture of satisfaction rate among the PTs and OTs on the national level preceptors must maintain training to the level of the interns.

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Bioassay guided isolation of α-glucosidase inhibitory compound, *in vivo* postprandial anti hyperglycemia and docking study of the isolated compound from the leaves of the methanolic extract of *Quercus serrata*

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ABSTRACT

Diabetes mellitus is rapidly emerging creating major health problem worldwide. Though synthetic drugs are available, due to their association with side effect, there is always interest for search of herbal formulation. *Quercus serrata* is a plant used by traditional healers of Manipur, India as a folk remedy to treat diabetes mellitus. The objective of this study is bioassay guided isolation of α-glucosidase inhibitory compound from the leaves of Quercus serrata and to check the postprandial antihyperglycaemic effect of the isolated compound in STZ-induced diabetic albino mice. And to perform molecular docking studies to predict the bind-

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ing interaction of the isolated compounds with α-glucosidase.The methanolic extract of *Quercus serrata* leaves was screened for α-glucosidase inhibitory activity and fractionated into n-butanol, ethyl acetate and water fraction to isolate the active compound. Quantification of isolated compound was done by HPLC-PDA. Postprandial antihyperglycaemia effect was check in normal and STZ (100mg/kg) + nicotinamide (120mg/kg) induced diabetic mice after sucrose load (2g/kg). Molecular docking study was done using AutoDock 4.2.6. Rosmarinic acid was identified as the active compound present in *Quercus serrata* leaves responsible for the inhibition of α-glucosidase with IC50 0.23 ± 0.02 µgmL-1 (0.636µmolmL-1). Postprandial blood sugar and Area Under Curve were significantly ($p<0.05$) reduced by treatment with rosmarinic acid in normal and diabetic mice. Additionally, *in silico* docking study elaborated the possible binding between rosmarinic acid and α-glucosidase. Above finding confirmed the anti-diabetic potential of traditionally used *Quercus serrata* leaves and the isolated compound, rosmarinic acid.

KEY WORDS: α-GLUCOSIDASE, DIABETES MELLITUS, MOLECULAR DOCKING, POSTPRANDIAL HYPERGLYCEMIA, QUERCUS SERRATA, ROSMARINIC ACID

INTRODUCTION

Diabetes mellitus and the spectrum of complications associated with it impose uncertain healthcare challenges and economic burdens to the global population. The catastrophic prevalence of diabetes mellitus, predominantly type 2 diabetes, has become a global healthcare problem affecting 425 million people worldwide, (Zimmet et al., 2014, IDF Diabetes Atlas, 2017). In India, it is reaching extreme epidemic level. A recent study revealed that prolongation of diabetes leads to neuropathy (24.6%), the most common complication, followed by cardiovascular complications (23.6%), renal problems (21.1%), and retinopathy (16.6%) (Kaveeshwar and Cornwall, 2014).

Diabetes is a life style disease and food habit plays a major role in the management of diabetes. Control of postprandial hyperglycemia is also one of the measures to control severity of the disease. Acarbose, miglitol and voglibose are available clinically used drug to control postprandial absorption of glucose by inhibiting the enzyme α-glucosidase and α-amylase. Although substantial quantum of efforts has been made towards conquering the disease, it is still imperative to put rigorous effort in research to mitigate the disease. There is always search for new herbal formulation to avoid the side effect associated with the synthetic drug, (Polonsky, 2012, Nakatsu et al., 2017, Brito-Arias et al., 2018).

Quercus serrata Murray is widely used by traditional healers of Manipur, India as a folk remedy to treat diabetes mellitus (Sheikh et al 2015). No elaborate study has been reported on the medicinal use of *Quercus serrata* leaves and the chemical constituent present. So, the present study is aim at bioassay guided isolation of the active constituent responsible for the inhibition of the enzyme α-glucosidase, checking its antihyperglycaemic activity and to do molecular docking study of the isolated compound to predict the possible binding interaction of the isolated compound with the enzyme, α-glucosidase.

MATERIALS AND METHODS

Leaves of *Quercus serrata,* Murray were collected from Kangla Siphai, Manipur, India and authenticated by Dr. Biseshwori Thongam, Scientist D, Pant Taxonomist, IBSD, Manipur. Voucher specimen (No. IBSD/M-202) was deposited in the IBSD herbarium. Analytical or HPLC grade organic solvents (Merck Millipore, India) were used for experiments. For column chromatography, 100-200 mesh size silica gel (Merck) was used. α-Glucosidase (Maltase, EC 3.2.1.20), *p*-nitrophenyl-α-D-glucopyranoside and streptozotocin were purchased from Sisco Research Laboratory. HPLC (Shimadzu LC-20AD) was performed using Photo diode array (PDA) detector. NMR spectra were recorded on a Bruker Avance 500 MHz instrument with TMS as an internal standard. Chemical shifts were expressed in α values. Agilent 6520 Accurate mass Q-TOF/LC-MS was used to determine molecular weight. Absorbance was measured by Thermo Scientific Multiskan spectrometer.

Extraction and isolation of active compound: Air-dried leaves of *Quercus serrata* (3.5 kg) were extracted three times with methanol (MeOH) (15 L). Then 150 mL water was added to the concentrated methanolic extract (3.0 L) to make hydro-alcoholic solution. This solution was washed with 3.0 L of petroleum ether to remove fatty matter present in the leaves and then concentrated to yield 400 g of dried extract. A suspension was prepared from this using 500 mL of water and fractionated into ethylacetate , *n*-butanol and water to yield 152 g of ethyl acetate fraction, 103 g of butanol fractions and 135g of water fraction respectively. The butanol fraction was found to be most active and was subjected to silica gel column chromatography with increasing polarity: petroleum ether-chloroform 1:1 (Fr 1, 1 L), chloroform (Fr 2, 1 L), and chloroform – methanol 9:1 (Fr 3, 2 L), chloroform – methanol 4:1 (Fr 4, 2 L), chloroform – methanol 1:1 (Fr 5, 2.5 L) and chloroform – methanol 3:7 (Fr 6, 2.5 L). The sub-fraction 6 (SFr 6) was found to be most active in inhibition of α-glucosidase. Further, SFr 6 was

subjected to semi- preparative HPLC to yield compound 1 (rosmarinic acid).

Analytical and semi-preparative HPLC: Shimadzu LC-20AD with PDA detector (SPD-M20A) fitted with either an analytical (CAPCELL PAK C18 MGII S5, 5µ C18 250 × 4.6 mm) or semi-preparative (CAPCELL PAK C18 MGII S5, 5µ C18 250 × 10.00 mm) column was used for HPLC analysis. A solution of 10 mg/mL of enriched fraction (SFr 6) was prepared in HPLC grade water and methanol (7:3) and filtered through 0.45µm Millex-HN syringe filter. A 20µL or 200µL aliquot of filtered sample solution was injected for analysis for analytical and semi preparative separation respectively. The mobile phase used consisted of 1% acetic acid in water (solvent A) and methanol (solvent B) following linear gradient over a total run time of 23min: initially a linear increase of B up to 100% in 21 min and back to 100% A in 23 min. Flow rate was 1 mL/min and 3 mL/min for analytical and semi-preparative respectively. Individual peaks eluting from the column were carefully collected and the solvents were evaporated in a rotary vacuum evaporator. Instrument control and data handling were performed with the LC solution software on a PC.

Quantitative analysis by HPLC-PDA: Quantitative analysis was done by analytical HPLC in the protocol described above. Standard stock solution of 1mg/mL of rosmarinic acid was prepared and then diluted to yield the desired test concentrations. Quantitative estimation of the major active compound present in the methanolic extract, *n*-butanol fraction and enriched fraction (SFr6) was done using calibration curve of the standard solution and plotted concentration versus area using excel 2007 (Sheikh et al., 2016).

α-Glucosidase inhibitory assay: A 0.5U/mL stock solution of readily available α-glucosidase (from *Saccharomyces cerevisiae*) was prepared in 0.1M phosphate buffer (pH 6.8) and diluted with the same buffer to desired test concentration. Stock solution of plant extract, fractions and compounds were prepared in dimethyl sulfoxide (DMSO) and diluted for assay to the required concentration with same buffer maintaining DMSO concentration below 1% v/v. α-glucosidase inhibitory activities were determined spectrophotometrically based on an earlier reported method by using *p*-nitrophenyl-α-D-glucopyranoside as substrate (Laishram et al., 2014) Acarbose was used as positive control and the uninhibited enzyme was taken as negative control (DMSO control). The assay was performed in three independent experiments.

Experimental animals: Swiss albino mice (25-30 g) were used in all the experiment and were procured from Regional Institute of Medical Sciences (RIMS), Manipur, India. Ethical clearance was obtained from the Institutional Animals Ethical Committee (Approval No.–RIMS.171/IAEC/2011) prior to the experiments. Animals were acclimatized in laboratory condition for 7 days in polypropylene cages lined with husk under standard environmental conditions. Animals had free access to water and were fed on pelleted diet.

Acute oral toxicity study : The acute toxicity study was performed as per Organisation for Economic Co-operation and Development (OECD) guideline no. 423 (Annexure-2c) adopted by the Committee for the purpose of control and supervision of experiments on animals (CPCSEA), Government of India (Veeraraghavan, 2000). Mice were kept under observation for 14 days with special attention for the first 24 h after dosing.

Induction of diabetes: Diabetes was induced in mice by single intraperitoneal (i.p.) injection of nicotinamide (120mg/kg i.p.) and then 100 mg/kg b.w. of streptozotocin (freshly dissolved in 0.1M citrate buffer, pH 4). Mice showing marked hyperglycemia (fasting blood glucose ≥ 250 mg/dl) at 48 h after streptozotocin treatment were considered as diabetic and selected for the study, (Nakatsu et al., 2017)

Effect of rosmarinic acid on postprandial hyperglycemia : Both normal and diabetic mice were fasted overnight and divided into five groups (n=6). Group I served as normal control and received vehicle, group II served as positive control and received acarbose (10 mg/kg b.w.). Group III, IV and V received rosmarinic acid (5, 10 and 20 mg/kg) suspended in gum acacia (2% w/v) **(**Chandramohon et al., 2015**).** All groups received 2 g/kg b.w. sucrose orally along with their respective test samples (Miura et al., 2004). Blood glucose levels were estimated from the tail vein from each group before (0 min) and at 30, 60 and 120 min after sucrose load. Blood glucose was measured by using Accu-Check Active, Roche Diagnostic Mannheim, Germany. AUC was calculated by using the software, GraphPad Prism 5.

***In silico* structure prediction of α-glucosidase:** *S. cerevisiae* α-glucosidase protein sequence (MAL12, Accession Number: P53341) was retrieved from UniProt (www.uniprot.org). NCBI CDD (Marchler-Bauer et al., 2015) was used to find out the conserved domain and other important catalytic sites. Structural 3D model for the protein was built using automated comparative protein modeling server SWISS-MODEL (https://swissmodel.expasy.org). Homology based search was performed by the server in Protein Data Bank (PDB) and SWISS-MODEL template library (SMTL) repositories for template structures (Biasini et al., 2014). Suitable template with similar biological property and highest similarity was selected for creating the 3D model. Further checking accuracy of

the newly generated structure was done by using PROCHECK (Laskowski et al.,1993) and ProSA-web (Sippl, 1993; Wiederstein and Sippl, 2007) for evaluation of Ramachadran plot.

Docking study: 3D structure of rosmarinic acid (PubChem CID: 5281792) was obtained from NCBI PubChem database (https://pubchem.ncbi.nlm.nih.gov/). Surface topology was calculated using CASTp server (Dundas et al., 2016) for potential binding pockets in the structural model of α-glucosidase. Initially whole protein was considered for docking. Polar hydrogen atoms and Gasteiger charge were added to the protein molecule. Grid size of 96 Å × 120 Å × 110 Å with a spacing of 0.619 Å was set to cover the entire protein. Lamarckian genetic algorithm search was considered to generate 100 conformations and rest of the parameter was set to default. Later, a cluster analysis was carried out by considering the root mean square deviation of conformations to identify major occupied binding sites in the protein. On the basis of major CASTp pockets and top AutoDock clusters information, a smaller grid box, 62 Å × 60 Å × 70 Å with a spacing of 0.464 Å was determined for the second set of docking simulation. All other parameters and procedureof the docking program were kept as earlier.

The best docked conformation of rosmarinic acid was selected based on lowest binding energy. Minimization of the docked complex was then performed using CHARMm force-field with smart minimizer (2000 steps) by considering Generalized Born with Molecular Volume (GBMV) implicit solvent model. Binding free energy (ΔG) of the docked complex was calculated by using the same GBMV model. Implicit Distance-Dependent Dielectric model was used to calculate total and individual interaction energy (IE) possessed by the amino acid residues. Molecular graphics, analysis and depiction were performed using UCSF Chimera (Pettersen et al., 2004) and BIOVIA Discovery Studio Visualizer Version 4.5 (http://www.3dsbiovia.com).

Statistical analysis: Results were expressed as Mean ± S.D., where n=6. Differences among data were determined using one way ANOVA followed by Tukey's Multiple Comparison test (Graph Pad Prism software, version 7). $^{*}p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Quantitative HPLC analysis: Rosmarinic acid (**Fig. 1**) was found to be the major compound present in the enriched fraction Sfr 6. **Fig. 2** showed the HLPC chromatogram of rosmarinic acid in methanolic extract, *n*-butanol fraction, enriched fraction (SFr 6) as well as

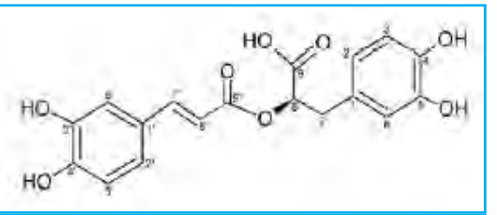


FIGURE 1. Structure of Rosmarinic acid

that of isolated rosmarinic acid. The calibration curve (**Fig. 3**) of the standard rosmarinic acid was found to be linear (r^2=0.993) in the concentration range of 0.01 to 0.09 mg/ml. 54.28±4.14 mg, 239±1.21 mg and 426±8.36 mg of rosmarinic acid was present per 1gm each of methanol extract, *n*-butanol fraction and enriched fraction (SFr 6) respectively.

Rosmarinic acid NMR interpretation: Compound 1 (**Fig. 1**) gave a molecular ion peak in HRMS at m/z = 360.3212 $[M+H]^+$ corresponding to the molecular formula $C_{18}H_{16}O_8$. The ^{13}C NMR in CD_3OD indicated the presence of signals attributed to all 18 carbon atoms. One ester carbonyl at δ = 167.06 displayed HMBC correlation with H-7' (δ 7.58), coupled to a doublet of *J* = 14.5 Hz at δ 6.34 indicative of a cinnamoyl ester. Another carbonyl at δ 172.06 showed HMBC correlation with signal of H-7 (δ 3.13, H-7_a and δ 3.05, H-7_b), coupled to a methene signal at δ 5.20, agreeing with the presence of a phenyl lactic acid unit. Two *ABX* systems were noted in the aromatic region of the ^1H NMR vouching for 3,4-dihydroxylation pattern of both the rings. The linkage of the cinnamoyl ester to the phenyl lactic acid moiety was inferred from HMBC correlations displayed by the H-8 signal to both the carbonyl peaks. Signals at δ 6.98 (1H, d, *J* = 2.0 Hz, H-6') and δ 7.06 (1H, dd, *J* = 8.5, 2.0 Hz, H-2') showing *meta* coupling between them and *ortho* coupling between H-2' and H-3' with signal at δ 6.79 (1H, d, *J* = 8.5 Hz). Another *ABX* system with signal at δ 6.64 (1H, d, *J* = 2.0 Hz, H-2), δ 6.70 (1H, dd, *J* = 9.0, 2.0 Hz, H-6) and δ 6.69 (1H, d, *J* = 9.0 Hz, H-5) is observed. All signals of ^1H and ^{13}C, and the observed HMQC, ^1H-^1H COSY and HMBC correlations are in good agreement with literature reports on rosmarinic acid.

^1H NMR (500 MHz, CD_3OD, δ ppm): 7.58 (1H, d, J = 14.5 Hz, H-7'), 7.06 (1H, dd, J = 2.0, 8.5 Hz, H-2'), 6.98 (1H, d, J = 2.0 Hz, H-6'), 6.79 (1H, d, J = 8.5 Hz, H-3'), 6.70 (1H, dd, J = 2.0, 9.0 Hz, H-6), 6.69 (1H, d, J = 9.0 Hz, H-5), 6.64 (1H, d, J = 2.0 Hz, H-2), 6.34(1H, d, J = 14.5 Hz, H-8'), 5.20 (1H, m, H-8), 3.13 (1H, m, H-7_a) and 3.05 (1H, m, H-7_b).13C NMR (125 MHz, CD_3OD, δ ppm): 172.06 (C-9), 167.06 (C-9'), 148.34 (C-4'), 146.35 (C-7'), 145.41 (C-5'), 144.76 (C-4'), 143.89 (C-3), 127.84 (C-1), 126.25 (C-1'), 121.77 (C-2), 120.40 (C-6'), 116.18 (C-6),

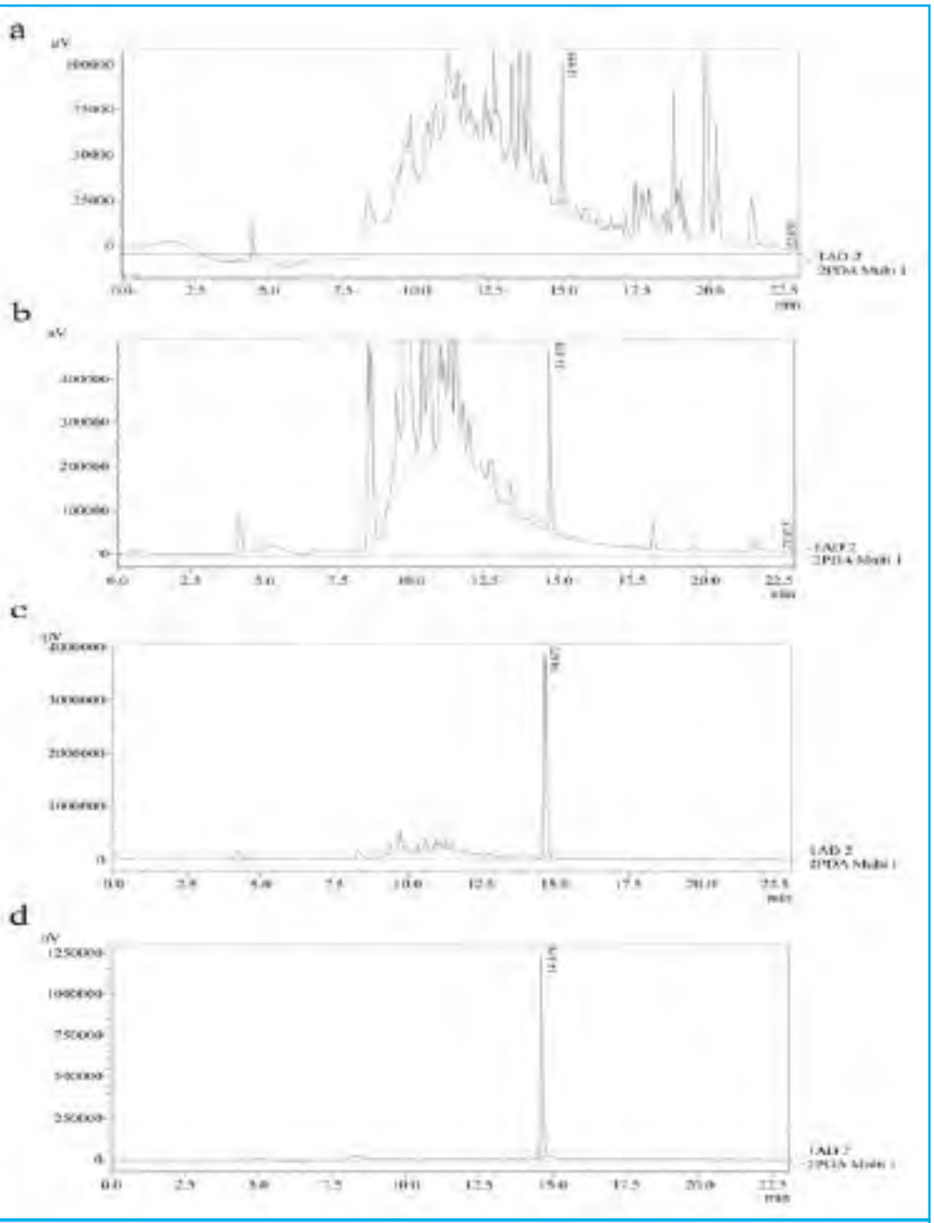


FIGURE 2. HPLC chromatogram of (a) methanol extract (b) n-butanol fraction (c) enriched fraction SFr6 and (d) isolated rosmarinic acid, showing the peak of rosmarinic acid along with the retention time.

115.10 (C-5), 114.89 (C-3′), 113.81 (C-8′), 112.99 (C-2′), 73.18 (C-8) and 36.51 (C-7). **(Supp Fig. 1a, 1b)**

Oral acute toxicity study: No fatality and ethological changes were observed when administered a dose of 2000 mg/kg b.w. of the methanolic extract. No changes in behavioral pattern were observed in the mice in oral toxicity study. So, *Quercus serrata* leaves were non toxic upto a dose of 2000mg/kg.

In vitro δ-glucosidase inhibitory effect: Blood glucose levels are highly affected by the saccharides contained in food which are converted into glucose by the actions of digestive enzymes like δ-glucosidase. Carbohydrates like sucrose are hydrolysed to monosaccharides (glucose and fructose) by δ-glucosidase thereafter caused an increased in blood glucose. The methanolic extract of *Quercus serrata* leaves, ethyl acetate fraction, *n*-butanol

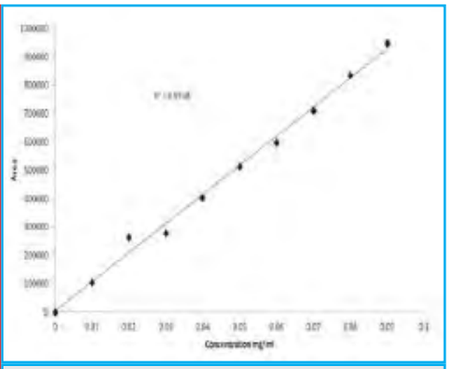


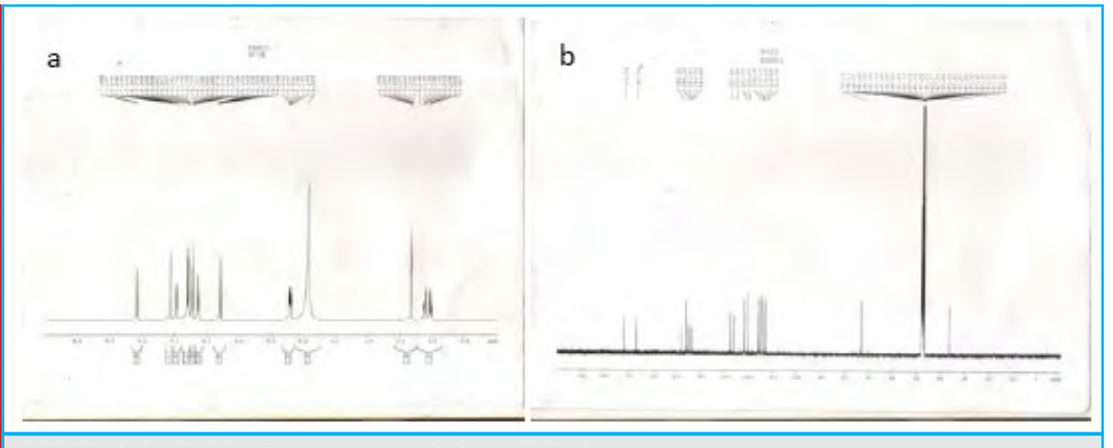
FIGURE 3. Calibration curve of standard rosmarinic acid as obtained from HPLC chromatogram. The graph is plotted, Area against Concentration (mg/ml) from the range 0.01 to 0.09mg/ml.

fraction, enriched fraction (SFr 6) and rosmarinic acid potentially inhibited δ-glucosidase, and the result is shown as **table 1**. The above result signifies *Quercus serrata* as a promising plant for postprandial management of diabetes mellitus type 2. Moreover, rosmarinic acid showed greater potential in inhibition of δ-glucosidase *in vitro* when compared to that of acarbose.

Postprandial antihyperglycaemic effect of rosmarinic acid: Rosmarinic acid is a polyphenol compound. It is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid (Petersen and Simmonds, 2003). The effect of rosmarinic acid on postprandial blood glucose level after sucrose load in overnight fasted normal and diabetic mice were presented in the **Fig 4A & B**. In diabetic mice postprandial blood glucose increased from 19.14±1.01 at 30 min to 20.81±0.62 mmol/L at 60 min and decreased to 18.50±0.51 mmol/L at 120 min. Rosmarinic acid at a dose of 20 mg/kg b.w significantly reduced the postprandial blood glucose to 15.43±0.64, 15.01±0.48 and 14.19±0.74 mmol/L at 30 min, 60 min and 120 min which value was comparable to that of acarbose 15.45±0.23, 14.42±4.01 and 14.21±1.12 mmol/L at 30 min, 60 min and 120 min respectively. Rosmarinic acid at all the doses caused significant fall in AUC (**Table 2**) when compared to diabetic control (56.31±1.50 mmol. min/L) indicating its effectiveness in lowering postprandial glucose absorption. There are studies which reported that medication which can flattens the peak blood glucose level postprandial can reduce the AUC (Inoue et al.1997). Postprandial blood glucose and AUC in normal rats was in consistent with that of diabetic groups.

Docking: To understand the binding interaction between rosmarinic acid and α-glucosidase, molecular docking was performed. Yeast and human glucosidase are similar in their substrate specificity, pH optimum, and inhibitor sensitivity. Thus, the yeast enzyme besides its affordability serves as a good experimental model to learn more about the structure, substrate specificity, and enzymatic mechanism of human glucosidase (Brito-Arias et al., 2018).

AutoDock 4.2.6 (Morris et al., 2009) incorporated in MGL Tools version 1.5.6 (The Scripps Research Institute) was used to execute the docking simulations. In this docking study α-glucosidase was treated as rigid molecule and rosmarinic acid as flexible. Due to non availability of three-dimensional crystal structure of S. cerevisiae α-glucosidase, homology study is done. Based on homology study , the protein structure of S. cerevisiae isomaltase (PDB ID: 3AJ7, Chain A at 1.3Å resolution) which got 72% similarity and 99% query coverage when compare to S. cerevisiae α-glucosidase, MAL12



SUPP FIG. (1a) 1H NRM of rosmarinic acid (1b) 13C NMR of rosmarinic acid

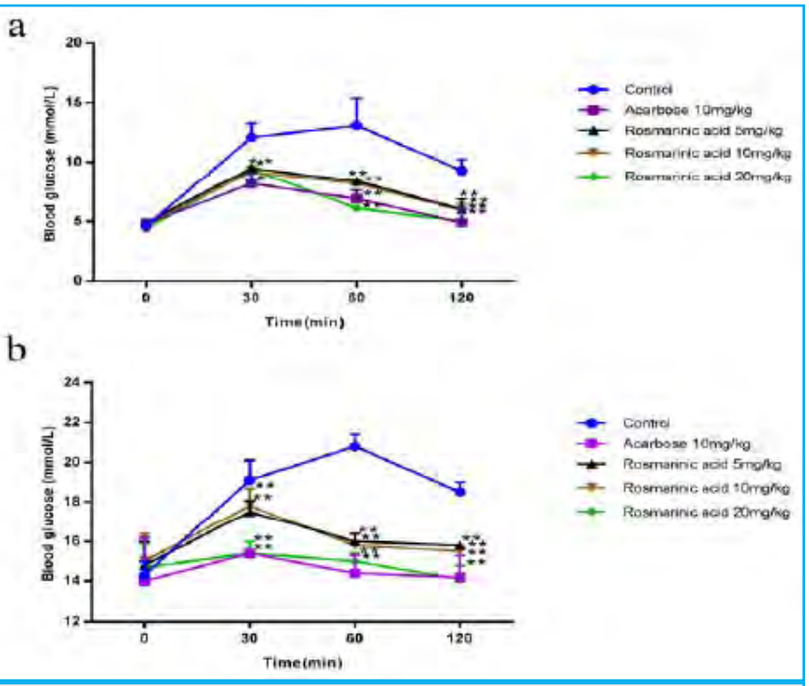


FIGURE 4. Effect of rosmarinic acid on postprandial blood glucose concentration after sucrose load. Graph showed plasma blood glucose concentration (mmol/L) on Y axis against time (min) on X axis. (a) on normal mice, (b) on diabetic mice. Values are the Mean ± SD, n=6, *P < 0.05 statistically significant when comparing to control.

was chosen for our study as the best template to build the 3D structure for S. cerevisiae α-glucosidase. (Fig 5)

Molecular docking study found that Domain 1 of α-glucosidase resembled α-amylase catalytic domain with a gap of 42 amino acids, named here as AamyGap42 **(Fig. 6a)**. These AamyGap42 site residues are shaped as α-helices near the entry point amino acid residues (HIS279, THR307, SER308, PRO309, PHE310, PHE311, and ARG312) of the active site pocket of the enzyme (Yamamoto et al., 2010).

FIGURE 5. Target-template alignment of *MAL12* (α-glucosidase protein sequence of *S. cerevisiae*) and 3AJ7 (Isomaltase protein sequence of *S. cerevisiae* (PDB ID: *3AJ7*, Chain A)), showing high sequence homology of important catalytic site residues.

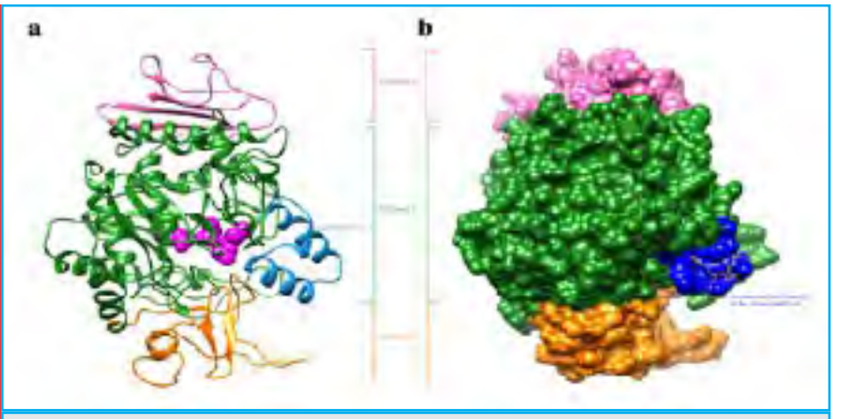


FIGURE 6. (a) 3D homology model predicted by the SWISS-MODEL program presented in rounded ribbon with designated three domains and AamyGap42 site. Catalytic residues are represented in magenta spheres. (b) Rosmarinic acid and α-glucosidase docked complex in solid surfaces, representing AamyGap42 interacting residues in blue color.

According to Benkert et al 2009, GMQE score which is expressed between 0 and 1 is a score where higher value indicate increased stability of the predicted structure. GMQE score for our final 3D model was found to be 0.92 which imply good model accuracy. The evaluation of the obtained models was done using the PROCHECK program through Ramachandran plot. Ramachandran plot revealed 88.8% residues were within the most favoured regions, 10.6% of residues were in additional allowed regions, 0.4% residues were in generously allowed regions, and only 0.2% residues were found in the disallowed regions. The percentage of residues in most favorable regions showed the quality of protein models. The above result obtained indicated that the model developed was qualified to be used for molecular docking process.

Surface topology analysis, was done to know the potential binding pockets in the structural model of α-glucosidase. Surface topology analysis on the protein have identified two major pockets, named here as Pocket

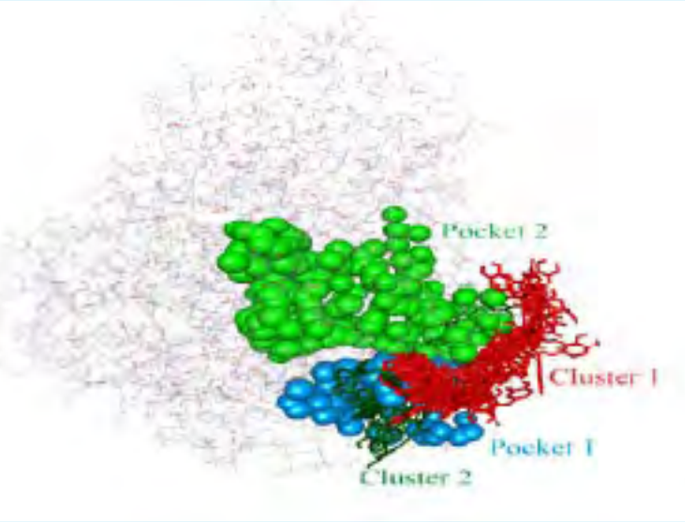


FIGURE 7. CASTp pockets and AutoDock conformation clusters at α-glucosidase. Major two CASTp identified pockets represented in spheres. Top two AutoDock conformation clusters (stick representation) coinciding with Pocket 1.

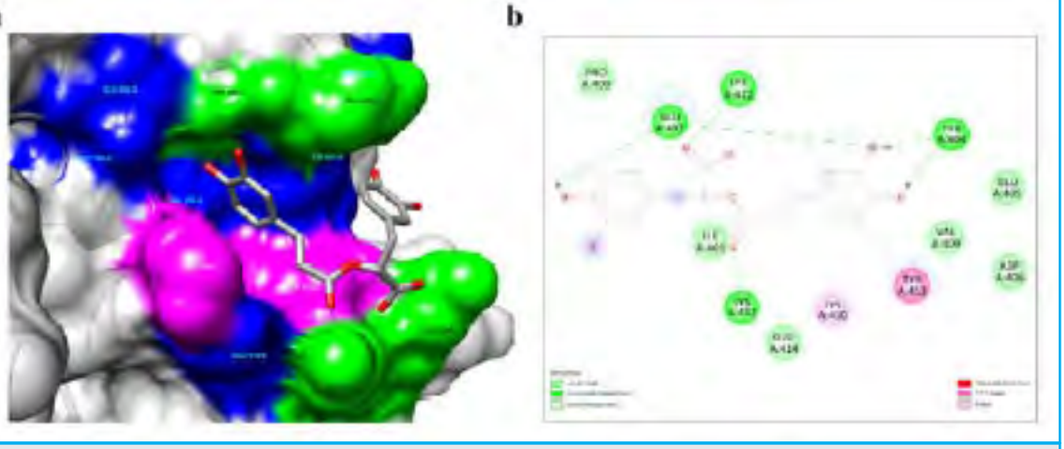


FIGURE 8. Docking analysis: (a) 3D surface representation of rosmarinic acid and α-glucosidase interactions, representing hydrogen bonding sites (light green), Pi-interactions (pink), and van der Waals interactions (blue). (b) Schematic representation of the interaction in detail with covalent and non-covalent bonds.

Table 1. Half maximum inhibitory concentration (IC^{50}) of the tested samples on α-glucosidase

Samples	IC_{50} (μgmL^{-1})
MeOH extract	1.84 ± 0.11
Ethylacetate fraction	2.83 ± 0.07
n-butanol fraction	1.15 ± 0.06
Water fraction	No inhibition up to 500 μgmL^{-1}
n-butanol Sub fraction (SFr 6)	0.66 ± 0.01
Rosmarinic acid	0.23 ± 0.02(0.636 $\mu molmL^{-1}$)
Acarbose	78.2 ± 0.17

Each value is the mean ± SD (n=3).

Table 2. AUC of postprandial glucose after sucrose load in normal and diabetes mice

Group (mg/kg.bw)	AUC mmol.min/L	
	Normal rats	Diabetic rats
Control	32.15±0.23	56.31±1.50
Acarbose(10)	19.95±0.41**	43.90±0.94*
Rosmarinic acid (5)	23.35±0.43*	48.82±0.57*
Rosmarinic acid (10)	22.71±0.25*	48.85±1.21*
Rosmarinic acid (25)	20.20±0.21**	44.80±1.33*

Each value is the mean ± SD (n=6). *p< 0.05 statistically significant comparing to control.

1 and Pocket 2 with volume of 431.6 $Å^3$ and 384.7 $Å^3$ respectively (**Fig. 7**). Two sets of docking study were performed, first by involving the whole protein, resulting in multiple conformations of the rosmarinic Acid. Root mean square deviation cluster analysis of these docked conformations showed two most populated clusters, Cluster 1 and Cluster 2 (**Fig. 7**). Auxiliary observations have shown that all the pockets and clusters were surrounding the AamyGap42 site with Cluster 1 directly falling under it. The catalytic and active sites of the protein were falling under Pocket 2. CASTp pockets (Pocket 1 and Pocket 2) along with AutoDock major clusters (Cluster 1 and Cluster 2) lead the target area in the protein to perform second set of docking experiment. This targeted docking study showed that rosmarinic acid did not directly bind at the active site present in Pocket 2 of the protein, instead it attached to the AamyGap42 site of Domain 1 with the lowest binding energy of -7.72 kcal/mol (**Fig. 6b**).

Binding details showed that rosmarinic acid interacted with 12 amino acids in the AamyGap42 site forming total 6 hydrogen bonds, two each with amino acid residue GLU402 and TYR404, and one each with LYS417 and LYS422. This interaction substantially increased the binding affinity as these amino acid residues were also found to be the major contributor of electrostatic interaction energy (IE). Moreover, the residues TYR413 and LYS410 were involved in Pi-Pi T-shaped and Pi-alkyl interactions respectively (**Fig. 8a,8b**). Pi-Pi T-shaped interaction between TYR413 and first benzene ring of the rosmarinic acid, Pi-Alkyl interaction with LYS410, and van der Waals interaction with PRO400, ILE401, GLU405, ASP406, VAL409, GLU414, strengthened the stability of the enzyme and rosmarinic acid complex. Binding free energy (ΔG) between the receptor and ligand was found to be -19.14 kcal/mol. The energy value sug-

gested the possibility of inhibition of α-glucosidase by rosmarinic acid. The interaction energies (IE) between the protein and ligand analysed for each amino acid residues and overall residues showed electrostatic IE as -44.70 kcal/mol and van der Waals IE as -31.22 kcal/mol which showed good binding energy with the enzyme.

For the first time we have reported the anti-diabetic effect of *Quercus serrata* leaves extract and the dominant active constituent rosmarinic acid. Further, this study reported the *in silico* binding analysis of rosmarinic acid with the enzyme α-glucosidase of *S. cerevisiae*.

Conflict of interest: The authors declare no competing interest.

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Utilization of damaged and spoiled wheat grains for bioethanol production

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ABSTRACT

First generation ethanol from starchy crops particularly maize is an established technology that being a renewable and bio-based resource has advantages over gasoline. The second generation ethanol from lignocellulosics owing to its economic considerations is still at pilot scale and is yet to see commercialization. This has increased the demand of starchy feed-stocks for energy. The recently released National Policy on Biofuels in May, 2018 categorically also emphasizes on the potential of different raw materials for ethanol production by consenting the utilization of damaged food grains like wheat, broken rice etc. that area otherwise unfit for human consumption. As far as wheat is concerned, The Comptroller and Auditor General (CAG), India (2017) reported that Food Corporation of India's (FCI) wheat stock worth 700 crores was damaged solely in Punjab from 2011 to 2016 as the grain was kept in open areas attributed to the lack in storage facility. Development of efficient technology to pretreat and convert damaged starch into fermentable sugars and optimization of enzymatic hydrolysis using commercial as well as indigenous enzyme preparation are the key points for the efficient bioethanol production from damaged wheat. Further, the synergistic action of alpha and glucoamylase in the hydrolysis of wheat mash have been tried that has revealed 96.25% conversion efficiency with an ethanol yield 5.60 % (v/v). The present review discusses research progress in bioethanol production from damaged wheat grains containing higher starch content. Thus, utilization of especially damaged and spoiled wheat grains pave better way for commercialization of bioethanol production from an economical perspective.

KEY WORDS: BIOTHANOL, INDIGENOUS, LIGNOCELLULOSICS, DAMAGED WHEAT

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INTRODUCTION

Global demand for energy sources and their utilization determine the economic status and growth of developing countries all over the world (Xu and Liu 2009). The major energy demand is still supplied from conventional fossil fuels such as oil, coal and natural gas which don't regenerate at sustainable manners (Twidell and Weir 2003). Fossil Fuels have played an indispensible role in development of industry but its indiscriminate use and the resulting environmental pollution led to advent of alternate fuels. The Energy Information Administration (EIA) reported that 70% of oil consumed in the United States was used for transportation (EIA 2015 a). According to EIA's 2014 report, 27% of petroleum consumed in the United State was imported from foreign countries (EIA 2015b). Hence, use of renewable resources to produce liquid biofuels offer attractive solutions to reducing greenhouse gas emissions, decreasing reliance on foreign oils, addressing energy security concerns, strengthening rural and agricultural economies and increasing the sustainability of the world transportation system. Today, Bioethanol has long been regarded as a suitable substitute to fossil fuels. It has immense properties such as higher compression ratio, shorter burn time and leaner burn engine, which lead to theoretical efficiency advantages over gasoline (Hansen *et al* 2005). Brazil has the world's first sustainable bioethanol economy with 6.19 billion gallons produced in 2014, which represents over 25% of the world's ethanol fuel and is ranked second in the world next to the United States (Biofuels 2016).

According to the Renewable Fuels Association (RFA 2018), the global production of bioethanol stood at 27 billion gallons in the year 2017, with the USA (15,800 million gallons) and Brazil (7,600 million gallons) as the largest producers in the world. India ranks eighth in ethanol production next to EU, China, US and Brazil with a total production of 280 million gallons in 2017 (RFA, 2018).

The first and second generation bioethanol are commonly referred as first generation bioethanol resources and third type as second generation bioethanol resources. The shift from first to second generation bioresources is obligatory due to main reasons; one is that first generation bio resources have alternate uses such as food (sugarcane, corn), animal feed (molasses) etc, secondly that these are still unable to meet the global demand of bioethanol. On the other hand, Lignocellulosics need costly steps of pretreatment and range of enzyme requirement for saccharification which is making bioethanol production from lignocellulosics a costly affair. In this scenario, there is need for a suitable economical substitute as an alternative. Therefore the National Policy on Biofuels –2018 categorically emphasis on the potential of different raw materials for ethanol production by consenting the utilization of sugarcane juice, sugar containing materials like sugar beet, sweet sorghum, starch containing materials like corn, cassava, damaged food grains like wheat, broken rice, rotten potatoes, unfit for human consumption. India is an agricultural country where wheat and rice are staple food for its burgeoning population. In terms of production, India stands second after china in wheat and rice production accounting for about 200MT/year out of this about 12MT (6%) of grains are damaged by post-harvest storage due to poor storage facilities and hence damaged by insects, rodents, birds and microbial spoilage (Sharon *et al* 2014). As far as wheat is concerned, Comptroller and Auditor General (CAG) (2017) reported that Food Corporation of India's (FCI) wheat stock worth 700 crore was damaged solely in Punjab from 2011 to 2016 as the grain was kept in open areas attributed to the lack in storage facility (Anonymous 2017). All this damaged grains were disposed off as they were not fit for consumption by human and animals. However, the damaged grains can be put to application by producing ethanol from its starch content which constitutes 70-80% in wheat and rice. Theoretically, 30gal/MT of ethanol from damaged wheat and rice may be obtained (Gawande and Patil 2015). Wheat starch is comprised of one quarter amylose and around three quarters amylopectin with little protein and lipid debasements (0.8% and 0.2% respectively) (Bowler *et al* 1985).

The process of grain bioethanol production involves milling (grinding and pretreatments), mashing (enzymatic or acid hydrolysis, steaming, adding supplements etc) and Fermentation (SHF or SSF) which is followed by distillation and dehydration to produce anhydrous ethanol. The conversion of starch to ethanol can be accomplished by acid hydrolysis, but the generation of by products such as levulinic and formic acid may cause hampered yeast growth hence, lower yields of alcohol (Kerr 1944). The acid hydrolysis has now been largely switched with amylolytic enzymes (α-amylase and glucoamylase) which deliver 95% more yield of glucose (Hua and Yang 2016).

Though using damaged grains will incur lower substrate cost, mashing involves costly commercial saccharification and enzymes which may also be taken care of by using indigenous culture of *Bacillus subtilis*, *Bacillus circulans*, *Bacillus cereus* etc for α-amylase and *Aspergillus* such as *Aspergillus niger, Aspergillus oryzae* etc for glucoamylase. Fermentation is the final stage performed after starch pretreatment (digestion) for bioethanol production The process cost may be further be reduced by using Simultaneous Saccharification and Fermentation as it reduces the time as well as energy by using two different vessels for Saccharification and

Fermentation besides lowering the chances of contamination. This review designed to provide an insight into the process details as well as the update of the damaged cereals particularly damaged wheat for bioethanol production.

FEEDSTOCKS FOR BIOETHANOL PRODUCTION

The raw materials for bioethanol production can broadly be classified as (i) sucrose-containing feedstock (sugarcane, sugar beet and sweet sorghum) (ii) starch-containing feedstock (wheat, corn and cassava) and (iii) cellulosic feedstock (straw, grasses, wood, stovers, agricultural wastes and paper etc) (Table 1) while the bioethanol produced from sucrose-and starch-containing feedstock is classified as 1st generation bioethanol (ethanol from corn and sugarcane) that produced utilizing cellulosic feedstock is referred as 2nd generation bioethanol. The source of third generation biofuel is lipolytic compounds obtained predominantly from algae. Most current bioethanol production processes utilize more readily degradable biomass feedstock such as cereals (corn and grain) and molasses. However, the utilization of edible agricultural crops exclusively for biofuel production conflict with food and feed production (Wheals *et al* 1999). One of the major problems with bioethanol production is the variability in available raw materials as their geographic locations differ from season to season, place to place and price of substrates which affects and hence the production cost of bioethanol (Kumar 2006; Yoosin *et al* 2007).

UTILIZATION OF DAMAGED CEREAL GRAINS FOR BIOETHANOL PRODUCTION

Cereal grains are used mostly for food and feed. However, post heat losses from farm to fork take a significant portion of damaged/infested cereal grains which are not fit for consumption. But there are rich sources of sugar (in the form of starch) just like simple sugar substances such as molasses, sugarcane juice can be fermented. These starch based materials like corn, rice and wheat have infact proved to be promising raw materials for their efficient fermentation into industrial as well as potable ethanol with the help of appropriate fermenting micro-organisms (Awasthi *et al* 2015). As per estimates provided by Food Corporation of India (FCI) huge quantities of cereal grains are getting spoiled every year due to unfavourable climate conditions and become unfit for human and animal consumption and one million tonnes of damaged grains is lying unutilised in FCI stores (Kumar *et al* 1999). The damage includes discoloration, breakage, cracking, attack by fungi, insect damage, chalky grain, partial softening due to dampness, off smell etc (Gawande and Patil 2015).

The damaged grains used for ethanol production are ten times cheaper than fine quality. These damaged or waste cereal grains can be utilized for the effective production of ethanol using fermentation process which will not only meet (partially) our needs but may provide some incentive to the farmers who suffers due to crop damage. The chemical composition of cereal grains is characterized by the high content of carbohydrates mainly starch (56–74%) deposited in the endosperm and fibre in the bran (2–13%). The second important group of constituents is the proteins which fall within an average range of about 8–11% and high content of B-vitamins is, in particular, of nutritional relevance. Hence, spoiled and damaged starchy grains can also be used for bioethanol production.

DAMAGED WHEAT: A PROMISING RAW MATERIAL FOR BIOETHANOL PRODUCTION

Global Scenario of wheat

Wheat is produced in 120 countries and accounts for about 19 per cent of the world's calorie supplies. It is used primarily as flour for making bread, pastry, pasta and noodles etc. It is also used to feed livestock, with the feed accounting for about 17 per cent of global wheat consumption. In addition, the by-products from milling of wheat into flour are also used as feed. The annual global production of dry wheat is about 529 Tg whereby Asia (43%) and Europe (32%) are the primary producers. Like rice, China is the largest producer of wheat with about 18% of global production at an average yield of 3:4 dry mg ha $^{-1}$.The second largest producer is India, where dry wheat production is 71 Tg (12%), and the yield is 2:4 dry mg ha^{-1} (Seungdo *et al* 2004).

India produces wheat in appreciable amount that can be a very good raw material for bioethanol production. Secondly, a huge quantity of wheat is wasted every year due to mismanagement; lack of proper storing facilities in the warehouses and spoiled wheat can also be utilized for bioethanol production. In the present Indian scenario as per estimates provided by Food Corporation of India (FCI) huge quantities of cereal grains are getting spoiled every year due to unfavorable climatic conditions and become unfit for human and animal consumption. There are about one million tons of damaged and spoiled grains lying unutilized in FCI stores (Kumar *et al* 1999).

As far as wheat is concerned, Comptroller and Auditor General (CAG) (2017) reported that Food Corporation of India's (FCI) wheat stock worth 700 crores was damaged solely in Punjab from 2011 to 2016 as the grain was kept in open areas attributed to the lack in storage facility (Anonymous 2017). All this damaged grains were disposed off as they were not fit for consumption

by human and animals. Belboom *et al* (2015) reported that the consumption of 1 MJ bioethanol produced from wheat instead of 1 MJ gasoline can reduce greenhouse gas emissions by 42.5 - 61.2%.

Structure and composition of wheat grain

Wheat, derived from the wild (*Triticum aestivum* L.) is today the fifth major cereal plant cultivated in the world (Fig 2.1). Besides growing wheat for food purposes, there is also an interest in wheat cultivars for non-food and technical uses starch and bioethanol production (Kust and Potmesilova 2014). Damaged wheat grains could be an economical carbon source for ethanol fermentation in the industry, because of high starch content and low commercial value. Yan *et al* (2010) tested field-sprouted-sorghum and concluded that the use of these kernels significantly reduced fermentation time and yielded higher ethanol. Starch or amylum is a polymeric carbohydrate comprising of a large number of glucose units joined by glycosidic bonds and contains two main structural components, amylose and amylopectin. Amylose is essentially a linear polymer in which the glucose residues are connected by the α-1, 4 linkages. The other main component of starch is amylopectin, which is a larger and branched molecule with both α-1, 4 and α-1, 6 linkages. Most native wheat starch is a mixture of amylose and amylopectin, in the ratio of 1:3 by weight. The content of amylose in wheat starch generally ranges from 20–26% (Buresova *et al* 2010).

STARCH HYDROLYSIS

In industry, starch is converted into sugars or fermented to produce ethanol. Starch cannot be metabolized directly by yeast, but must first be broken down into simple six carbon sugars (glucose) prior to fermentation. The conversion of starch-containing feedstock to obtain fermentable sugars is mainly comprised of three operations which are: (i) milling, (ii) liquefaction and (iii) saccharification using enzymes.

The first stage of starch hydrolysis is gelatinization which is to break down the intermolecular bonds of starch with heat in the presence of water. Starch granules are quite resistant to penetration by both water and hydrolytic enzymes due to the formation of hydrogen bonds within the molecule and other molecules. However, these intra and inter- hydrogen bonds are weakened during gelatinization. During this stage, the temperature of aqueous suspension of starch is elevated, the water absorption and expanded granules dissolving starch granules to form a viscous suspension or slurry. This allows disruption or burst of the starch granules and exposes it to enzyme attack. This process is known as gelatinization and the temperature at which starch properties are changed is named as gelatinization temperatures (Albani 2008). The susceptibility of starch to amylase attack depends on the properties of the specific starch, such as e.g. degree of gelatinization and the characteristics of the specific amylase (Bijttebier *et al* 2008). Different starches have different gelatinization temperatures, implying different ease of cooking. Cassava starch has a lower temperature, relatively to cereal starches; the pasting temperatures for cassava, corn, wheat and rice are 60-65°C, 75-80°C, 80-85°C and 73-75°C, respectively (Swinkels 1998; Thirathumthavorn and Charoenrein 2005). The physicochemical properties of starch impose limitations in the use of higher starch concentrations as a result of gelatinization of the starch which causes undesirable viscosity development.

Liquefaction is a step that starch is degraded by an endo-acting enzyme namely alpha –amylase (EC 3.2.1.1) which hydrolyzes only α-1, 4 and causes dramatically drop in viscosity of cooked starch. Typically, liquefying enzymes can have an activity at a high temperature (> 85°C) so that the enzyme can help reduce paste viscosity of starch during cooking. The dextrins, i.e. products

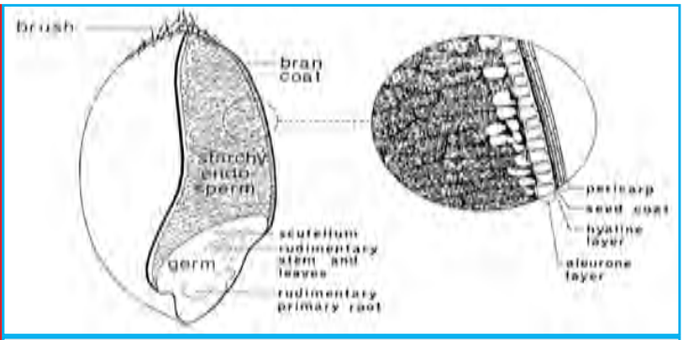

FIGURE 1. Wheat grain cut lengthwise through crease (Pomeranz 1988)

obtained after liquefaction, is further hydrolyzed ultimately to glucose by glucoamylase enzyme which can hydrolyze both α-1,4 and α-1,6 glycosidic linkage in amylase and amylopectin branches of starch. Glucoamylase (GA), also known as amyloglucosidase (EC 3.2.1.3), is an inverting type and exo-acting enzyme, capable of hydrolyzing α-1,4 glycosidic linkages in raw or soluble starches and related oligosaccharides with the inversion of the anomeric configuration to produce glucose.

The starch-based bioethanol industry has been commercially viable for about 30 years; in that time, tremendous improvements have been made in enzyme efficiency, reducing process costs, time, increasing hydrolysis and bioethanol productivity. Hydrolysis of starch may be considered as a key step in the processing of starch-based feedstock for the bioethanol production. Starch can be hydrolyzed by acid, acid-enzyme and enzyme-enzyme techniques.

Acid hydrolysis (lintnerization)

Acid hydrolysis is an important chemical modification that can significantly change the structural and functional properties of starch without disrupting its granular morphology. During acid hydrolysis, amorphous regions are hydrolysed preferentially, enhancing the crystallinity and double helical content of acid hydrolyzed starch (Wang and Copeland 2013). According to Dziedzic and Kearsley (2012) acid hydrolysis was discovered at the beginning of the 19th century by boiling wheat starch with dilute sulphuric acid results in a sweet syrup. Later, potato starch was used as the starch source and sulphuric acid was replaced by hydrochloric acid and indirect heating of the reaction vessel was common practice. Since then, acid has been used to a great extent for the breakdown of starch into glucose particularly in industry. Bej *et al* (2008) had investigated on concentrated acid hydrolysis (H_2SO_4) of wheat flour in a batch reactor at different temperatures and acid concentrations. A maximum conversion (42%) of starch to the reducing sugars was obtained at 95°C and pH 3.

Similarly, Hoseinpour *et al* (2010) showed that hydrolysis of starch using dilute sulphuric acid leads to complete conversion to glucose under optimum conditions of 130°C, 1% acid and 7.5% solids loading for 30 minutes. The mineral acid or acid-base involved in the hydrolysis can be of diluted or concentrated form and dilute acid process at 1-5% concentration is conducted under high temperature, pressure and has fast reaction. The concentrated acid process on the other hand uses relatively mild temperatures and reaction times are typically much longer as compared to dilute acid hydrolysis. The biggest advantage of dilute acid processes is their fast reaction rate, which facilitates continuous processing for hydrolysis of both starch and cellulosic materials. Their prime disadvantage however is the low sugar yield and this has opened up a new challenge to increase glucose yields higher than 70% (especially in cellulosic material) in an economically viable industrial process while maintaining high hydrolysis rate and minimizing glucose decomposition (Xiang *et al* 2004; McConnell 2008). The concentrated acid hydrolysis offers high sugar recovery efficiency, up to 90% of both hemicelluloses and cellulose sugars. However, this technique does have a number of drawbacks such as relatively low yield and formation of undesirable by-products (Ramprakash and Muthukumar 2014).

Enzymatic hydrolysis

In the last decade, the starch industry has transformed from using acid in the hydrolysis process to enzyme. The acid was largely replaced by enzyme which gives 95% more yield of glucose (Hua and Yang 2016). Enzymatic hydrolysis of starch requires two types of enzymes due to the fact that starch or amylum comprises of two major components, namely amylose, a mainly linear polysaccharide consisting of α-1,4-linked α-glucopyranose units and the highly branched amylopectin fraction that consists of α-1,4 and α-1,6-linked α-glucopyranose units (Knox *et al* 2004). These two types of linkages, α-1, 4 and α-1,6-linked required an efficient starch hydrolysis agent or enzyme that can fraction α-1,4 and promote α-1,6 debranching activity which leads to a reduction in viscosity of gelatinized starch in the liquefaction process. There are certain type of carbohydrate-degrading enzymes include a -amylases, b -amylases, debranching enzymes, cellulases, b -glucanases and glucosidases etc. The process of enzyme hydrolysis involves hydration of starch by heating the starch in aqueous suspension to give α-amylase an access to hydrolyze the starch (Fig 2). Exoamylases such as glucoamylase is added during saccharafication which hydrolyses 1,4 and 1,6-alpha linkages in liquefied starch(Maarel *et al* 2002).The important advantages of the saccharification of starch by the amylase mode include higher yield and purity, easy crystallization, better process control, lower cost of production, ion exchange capacity, significant reduction in energy requirement, elimination of heavy depreciations on expensive corrosion resistant equipment, production of new products and formation of lower by-products (Barfoed 1967; Madsen and Norman 1973; Fullbrook 1984).

MICROBIAL DIVERSITY INVOLVED IN AMYLASE PRODUCTION

The amylolytic microorganisms have immense applications in industries as well as in scientific research as they are more stable when compared with plant and ani-

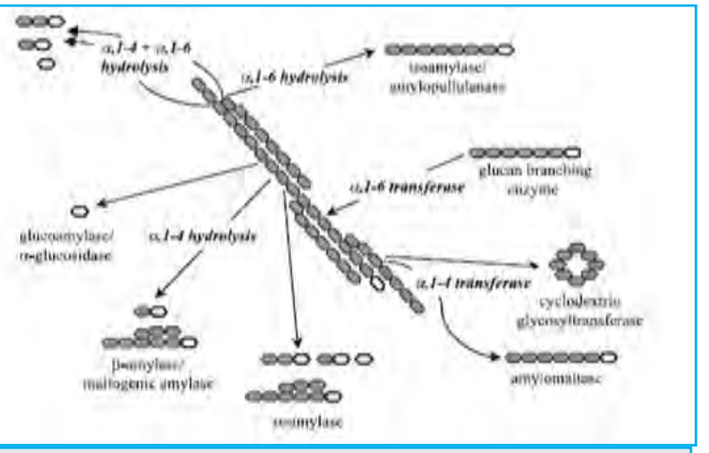

FIGURE 2. Different enzymes involved in the degradation of starch. The open ring structure symbolizes the reducing end of a polyglucose molecule (Maarel et al 2002).

mal amylase. The major advantage of using microorganisms for the production of amylases is the economical bulk production capacity and the fact that microbes are easy to manipulate to obtain enzymes of desired characteristics. Though amylases are produced by several fungi, yeast, bacteria and actinomycetes, but only a few selected strains of fungi and bacteria meet the criteria for commercial amylase production (Table 1).

Microbial production of alpha amylase

Profiling microorganisms with high potential for amylase production in submerged fermentation (SmF) using synthetic media has been widely recognized due to their myriad applicability in bioethanol production. Raplong *et al* (2014) identified *Bacillus* using mannitol egg yolk polymyxin B (MYP) agar a highly selective media.They reported that *Bacillus cereus* strain SB2 had largest zone of hydrolysis of 12mm on nutrient agar supplemented with starch. Amylase activity of 2.56U/ml was obtained at pH (6.5), temperature (35°C), incubation time (24 hr) and inoculum concentration (4%) in submerged fermentation. Singh and Kumari (2016) isolated starch degrading bacteria from soil samples collected from different environment sources (Banana, Potato and Sugarcane field samples). Out of 10 isolated bacterial strains, *Bacillus* sp. B3 gave positive starch hydrolysis and thus was suggested for industrial application like starch modification with better efficiency with the increase in temperature.

Similarly, Rehman and Saeed (2015) investigated 39 amylase producing *Bacillus* sp. from soil of which *Bacillus* sp. stain AS-2 was reported to have highest enzyme activity (3179.62 IU/ml/min). Vaseekaran *et al* (2015) isolated, identified and characterised thermostable amylolytic bacteria from contaminated soil with decaying materials *i.e.* kitchen waste and bakery waste soil etc. Their investigation revealed one strain identified as *Bacillus licheniformis* with highest α-amylase activity (7.0±0.21 Um/L) at 24 h and enzyme showed neutral optimum pH and temperature (90°C) without additives.

Dash *et al* (2015) also identified and optimized new *B. subtilis* strain BI19 that produced appreciable amount of amylase. Singh *et al* (2012) produced extracellular amylase by *Bacillus* sp. which was optimized in a submerged fermentation as maximum enzyme activity was obtained at 35°C and pH 7 and after 10 h inoculation. In submerged fermentation, contents of a synthetic medium are very expensive and uneconomical, so there is urgent need of these to be replaced with more economically available agricultural, industrial and domestic by products which are used as substrates for SSF to produce enzymes in economical way.

SSF holds tremendous potential for the production of enzymes in view of its economic and engineering advantages. It can be of particular relevance in those processes where a crude fermented product may be used as an enzyme source (Pandey *et al* 1999). The major critical factors affecting microbial synthesis of enzymes in a SSF system include selection of a suitable substrate and strains, particle size of the substrate, inoculum concentration, moisture level of the substrate, temperature and pH. Selection of an appropriate solid substrate plays an important role in the development of efficient SSF processes (Lonsane *et al* 1985). Sexena and Singh (2011) carried out solid state fermentation using various agro-industrial wastes with best amylase producing strain isolated from soil. Different physicochemical conditions were varied for maximum enzyme production. The iso-

Table 1. Over view of amylase producing bacterial and fungal strains (Sundarram *et al* 2014)

Amylolytic Microorganism type	Fermentation type	Reference
B.amyloliquefaciens	SSF	Oboh (2005)
Bacillus licheniformis	SSF	Babu *et al* (1995)
Bacillus coagulans	SSF	Prakash *et al* (2009)
B. polymyxa	SSF	Prakash *et al* (2009)
B. mesentericus	SSF	Prakash *et al* (2009)
B. vulgarus	SSF	Prakash *et al* (2009)
B. megaterium	SSF	Prakash *et al* (2009)
Bacillus licheniformis GCB-U8	SmF	Sodhi *et al* (2005)
Bacillus sp. PS-7	SSF	Ramesh and Lonsane (1990)
Bacillus licheniformis M27	SSF	Amoozegar *et al* (2003)
Halobacillus sp MA-2	SmF	Gomes and Gomes (2003)
Halomonas meridiana	SmF	Kathiresan and Manivannan (2006)
Rhodothermus marinus	SmF	Anto *et al* (2006)
Bacillus cereus MTCC 1305	SSF	Sivaramakrishnan *et al* (2007)
Fungi		
Aspergillus oryzae	SSF	Leveque *et al* (2000)
Penicillium fellutanum	SmF	Erdal *et al* (2010)
Thermomyces lanuginosus	SSF	Upgade *et al* (2011)
Aspergillus niger	SSF, Smf	Yang and Wang (1999)
Penicillium roquefortii	SSF	Sivaramakrishnan *et al* (2006)
Streptomyces rimosus	SSF, Smf	Sudo *et al* (1994)
Aspergillus kawachii	SSF, Smf	Balkan and Ertan (2007)
Penicillium chrysogenumm	SSF	Sindhu *et al* (2009)
Penicillium janthinellum (NCIM 4960)	SSF	Prakasham *et al* (2007)
Aspergillus awamori	SmF	Siqueira *et al* (1997)
Pycnoporus sanguineus	SSF	Saito *et al* (1975)

*SSF-Solid state Fermentation ; * SmF- Submerged Fermentation

late produced about 5400 units/g of amylase at 1:3 moisture content, 20% inoculum concentration, temperature (50°C), pH 6.0 and after 72 h of incubation with Mustard Oil seed cake as the substrate. Similarly, Maity *et al* (2015) utilized *Bacillus subtilis* (ATCC 6633) for production of alpha amylase by optimization of the fermentation media. They also reported that 80% retention of alpha amylase activity comparable to purified porcine pancreatic amylase in the presence of drastic conditions of temperature (60°C), pH (6-11), detergents and utilized various industries like detergent, food and paper industries.

RSM is a statistical and mathematical tool for designing experiments, building models, evaluating the combined effect of many variables to investigate the optimum conditions for desirable response with reduced number of required experiments. Tanyildizi *et al* (2005) combined effects of macronutrients of media on α-amylase production by *Bacillus* sp. using response surface methodology. The results showed that yeast extract had no effect on α-amylase production. The optimal combinations of media constituents for maximum α-amylase production were determined as 17.58 g/l starch, 12.37%

(v/v) glycerin and 8.77 g/l peptone. Similarly, Sun *et al* (2011) optimized the process parameters through the statistical approach for the production of alpha amylase by *Bacillus subtilis* ZJF-1A5 in submerged fermentation. Among the variables screened, the temperature and time were most significant and also showed a positive interaction. The optimum levels were: temperature (35.8°C), pH (5.03) and time (54hrs). Under these conditions α-amylase yield was 191.15 U/ml.

Purification is a key step in the enzymes production where residual cell proteins and contaminants are removed. The methods used to purify amylases can vary considerably, but most purification protocols involve a series of steps (Sun *et al* 2010). Aassar *et al* (1992) performed the acetone fractionation of *Bacillus lentus* culture filtrate yielded the highest α-amylase activity and 66.6% fraction reached 13-fold that of the crude enzyme preparation. α-amylase from *Bacillus licheniformis* was purified 6-fold with a yield of 38% using by two gel filtration chromatography steps on Sephadex G-100 and Superose 12 column (Bozic *et al* 2011). In addition to the classical chromatographic techniques, immunoaffinity chromatography has been applied for the preparation of highly purified amylases (Jang *et al* 1994). Abdu *et al* (2011) identified a novel *Bacillus cereus* MS6 strain, which could produce extra cellular amylase that was purified by DEAE-Cellulose anion exchange and sepharose gel filtration chromatography, resulting in high yield of enzyme. The native protein showed a molecular mass of 149 kDa being composed of a homo dimer of 78 kDa polypeptide by SDS–PAGE.

Biochemical characterization of alpha amylase

When defining the proposed unit of activity for any enzyme, the International Unit of Biochemistry stated that reaction conditions should be specified as optimal. This implies that enzyme activities are only valid within a range of physical properties. Therefore, optimum conditions for producing maximum enzyme activities need to be determined.

Amenaghawon *et al* (2016) conducted a study of enzymatic hydrolysis towards cocoyam starch and found that the rate of hydrolysis was faster at a higher temperature. Therefore, there has been a need and continual search for more thermophilic and thermostable α-amylase (Burhan *et al* 2003). Aassar *et al* (1992) observed that the pure enzyme from *Bacillus lentus* was stable at higher temperatures in the presence of its substrate. It exhibited an optimum reaction temperature of 70°C and retained about 42°-70°C of its activity at 85°C and even at higher temperatures the enzyme still showed some activity. Weemaes *et al* (1996) studied stability of α-amylases produced by *B. amyloliquefaciens*, *B. licheniformis* and *B. stearothermophilus* under combined high temperature and pressure and the results indicated that α-amylase produced by *B. licheniformis* was the most stable enzyme.

The pH of a solution affects the structure and activity of enzymes. Khanna (2010) explained that pH has an effect on the state of ionization of acidic or basic amino acids. If the state of ionization of amino acids in a protein is altered then the ionic bonds that help to determine the 3D shape of the protein got changed. Sodhi *et al* (2005) reported that α-amylase of *Bacillus* sp. PS-7 strain showed pH optima at pH 6.5 and displayed 87 and 52% of peak activity at pH 6.0 and 5.0, respectively. Elkhalil and Gaffar (2011) analysed the pH activity profile of *Bacillus sterothermophilus* which showed an optimum activity at pH 7 compared to the *B. acidocaldarius*, with an activity optimum at pH 6. The relative activities of *Bacillus sterothermophilus* at pH 9 and 10 were about 1.5 and 4.5 times higher than those of the *B. acidocaldarius*. Similary, Qader *et al* (2006), who stated that the optimum pH of Bacillus sp. AS-1 was around 7.5. Most of amylases are known to be metal ion-dependent enzymes, namely divalent ions like Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+} and Fe^{2+} etc (Pandey *et al* 2000). Najafi and Kembhavi (2005) studied the effects of chemical modifiers on α-amylase enzyme activity from marine *Vibrio* sp. The results suggested the involvement of amino acids such as Lys, Trp, Asp/Glu and His in enzyme activity. It also has been reported that heavy metal ions such as Hg^{2+}, Ag^{2+} and Cu^{2+} inhibited amylase activity (Dey *et al* 2002).

Asoodeh *et al* (2013) studied the effect of metal ions (K^{+}, Na^{+}, Zn^{2+}, $Ba^{2+,}$ Mg^{2+}, Ca^{2+}, Fe^{2+} and Hg^{2+}), on the enzyme activity. Among the testified metal ions, Mg^{2+}, Fe^{2+} and Ba^{2+} increased the amylase activity, while Hg^{2+} and Zn^{2+}were established to inhibit enzyme activity. Asoodeh *et al* (2013) determined the kinetic parameters by incubating 0.1 ml of enzyme (0.1 mg/ml) in the presence of 0.9 ml starch at different concentrations (0.1–1.2 % w/v). As estimated from Michaelis–Menten equation the values of Km and Vmax for starch as substrate were 4.5 ± 0.13 mg/ml and 307 ± 12 lM/min/mg, respectively.

SIGNIFICANCE OF FUNGAL GLUCOAMYLASE (GA) IN STARCH HYDROLYSIS

Glucoamylase (GA), also known as amyloglucosidase (EC 3.2.1.3), is an inverting and exo-acting enzyme, capable of hydrolyzing α-1,4 glycosidic linkages in soluble starches and related oligosaccharides with the inversion of the anomeric configuration to produce glucose. In addition to acting on α-1,4 linkages, the enzyme slowly hydrolyzes α-1,6 glycosidic linkages of starch (Weil *et al* 1954; Fierobe *et al* 1998). The widely accepted mechanism of hydrolysis involves proton transfer from the catalyst to the glycosidic oxygen of the scissile bond.

A general acid–base catalyst (McCarter and Withers, 1994; Sinnot, 1990; Tanaka *et al* 1994) donates hydrogen to the glucosidic oxygen and a catalytic base guiding the nucleophilic attack by a water molecule on the C-1 carbon of the glucose moiety.

Microbial production of glucoamylase

Traditionally, glucoamylase have been produced by SmF. The development of microbial strains, media composition and process control has contributed to the achievement of high levels of extracellular glucoamylase. Banakar *et al* (2012) investigated the amylase production from fungal species by submerged fermentation (SmF). The Production medium was supplemented with 2% (w/v) soluble starch incubated under shake culture at a temperature of 28±1°C, pH-7.0 for 7 days. Maximum amylolytic activity was recorded with crude enzyme at 3rd day of incubation by *Penicillium* sp. (0.87±0.05 U/mL) followed by *Penicillium chrysogenum* (0.69±0.05 U/mL), *Aspergillus candidus* (0.67±0.03 U/mL), *Aspergillus fumigatus* (0.066±0.06 U/mL) and at 7th day of incubation was by *Penicillium* sp. (1.13±0.03 U/mL) followed by *Penicillium chrysogenum* (1.12±0.004 U/mL). Wang *et al* (2008) investigated food waste (FW) as potential substrate for the glucoamylase production by *Aspergillus niger* UV-60 under submerged fermentation. They reported that optimum concentration of 2.50% (dry basis), smashed food waste (smashed-FW) produced glucoamylase of 126 U/ml after 96 h of incubation, whereas 137 U/ml of glucoamylase could be achieved within the same time from raw food waste (raw-FW) of 3.75%.

Recently, Okwuenu *et al* (2017) optimized the production of glucoamylase from *Aspergillus niger* in a submerged fermentation process using amylopectin from guinea corn starch as the sole carbon source. Specific activities for crude enzymes were found to be 729.45 U/mg and 1046.82 U/mg at five and twelve days harvested enzymes, respectively. Benassi *et al* (2014) investigated the production of glucoamylase from *Aspergillus phoenicis* in Machado Benassi (MB) medium using 1% maltose as carbon source. The maximum amylase activity was recorded with temperature (60–65 °C) and pH (4.5) after 4 days of incubation in static conditions. However, the glucoamylase costs are still too high for the establishment of a cost effective production of energy syrup.

The SSF process has potential to significantly reduce the enzyme production costs because of lower energy requirements, increased productivity, smaller effluent volumes and simpler fermentation equipment (Ellaiah *et al* 2002). Cereal bran flours, potato residue and other starchy waste materials have been utilized as fermentation substrate for glucoamylase production by filamentous fungi (Joshi *et al* 1999; Biesebeke *et al* 2005). Glucoamylase production by *A. niger* was extensively studied using wheat bran in SmF and SSF by Kaur *et al* (2003). Wheat bran, paddy husk, rice processing wastes or other starch containing wastes have gained importance as supports for fungal growth during glucoamylase production (Arasartnam *et al* 2001).

Sethi and Gupta (2015) isolated amylolytic fungi from soil and identified them as *Aspergillus niger, Pencillium chrysogenum, Microsporium sp. and Fusarium* sp on the basis of morphological, biochemical characterization and starch hydrolysis assay, of these *Pencillium chrysogenum* was most potent alkaline amylase producing fungi with highest enzyme activity under optimised conditions *i.e* pH (8.0), temperature (45°C), wheat bran (1%) and peptone incubated for 7 days. Indriati *et al* (2018) reported that 3, out of 16 thermophile bacteria produced high amylase activity in media supplemented with wheat flour @ 2% at 40-50°C.

Zambare (2010) employed response surface methodology to optimize SSF medium and various parameters for production of glucoamylase by *Aspergillus oryzae* on the solid surface of rice husk, wheat bran, rice bran, cotton seed powder, corn steep solids, bagasse powder, coconut oil cake, and groundnut oil cake as substrates which resulted in a 24% increase in the glucoamylase activity. Optimum glucoamylase production (1986 µmoles of glucose/min/g of fermented substrate) was observed on wheat bran supplemented with 1%, (w/w) starch, 0.25%, (w/w) urea at pH 6, 100%, (v/w) initial moisture and 300°C after incubation of 120 hrs.

Kiran *et al* (2014) utilized food wastes such as waste bread, waste cakes, cafeteria waste, fruits, vegetables and potatoes for glucoamylase production by solid state fermentation. Response surface methodology was used to optimize the fermentation conditions for improving enzyme production and waste cake was the best substrate for glucoamylase production. The highest glucoamylase activity (108.47 U/gds) was achieved at initial pH (7.9), moisture content (69.6% wt) and inoculum loading 5.2×105 cells/g of substrate and incubation time of 6 days. Kumar and Satyanarayana (2004) improved the glucoamylase production by a thermophilic mold *Thermomucor indicae* seudaticae in solid-state fermentation (SSF) by applying response surface methodology (RSM). The glucoamylase production containing wheat bran as substrate, under the conditions optimized by RSM, was 455 ± 23 U/g of dry moldy bran (DMB) is higher than those reported in the literature.

Similarly, Banerjee and Ghosh (2017) applied response surface methodology, a statistical tool for the optimization of glucoamylase production by Aspergillus niger in solid state fermentation using garden pea peel as a substrate. The optimized fermentation composition was incubation time: 5 days; incubation temperature: 30°C; and substrate amount: 3g, which resulted in GA produc-

tion of 90.1728 Ugds-1. In literature also, Alam et al (2014) examined the effect of process parameters (pH, inoculum concentration and agitation speed) on glucoamylase production from bitter cassava by *Aspergillus niger* using response surface methodology (RSM).Utmost glucoamylase production of 38.30 U/ml was attained under optimized conditions of pH, inoculum concentration and agitation speed of 4.8, 3.7 % (v/v) and 260 rpm, respectively. Both the experimental and predicted results were in agreement with each other as values of 38.30 U/ml and 38.07 U/ml were obtained respectively thus, confirmed the validity of the developed model as well as attainment of the optimal points.

Glucoamylase from various sources have been purified extensively by the procedures using several types of column fractionations including ion-exchange, hydrophobic and gel filtration chromatographic steps. Bagheri *et al* (2014) investigated a glucoamylase enzyme from *Aspergillus niger* and purified it using fractionation, followed by anion-exchange chromatography. The results revealed that molecular mass of glucoamylase enzyme was estimated to be 62,000 Da, using SDS–PAGE and 57151 Da, based on mass spectrometry. Slivinski *et al* (2011) produced glucoamylase by *Aspergillus niger* in solid-state fermentation. The enzyme was partially purified by ammonium sulphate precipitation and ion exchange and gel filtration chromatographies. Its molecular mass was estimated as 118.17 kDa by electrophoresis. Okwuenu *et al* (2017) investigated the production of glucoamylase from *Aspergillus niger* in a submerged fermentation process using amylopectin fractionated from guinea corn starch as the carbon source. The crude enzyme with specific activity 729.45 U/mg was purified to the level of gel filtration (using sephadex G-100) via ammonium sulphate (70%) precipitation and specific activities were found to be 65.98 U/mg and 180.52 U/mg respectively.

Biochemical characterization of glucoamylase

Many glucoamylases derived from fungi were functionally active at thermophilic temperatures, usually 50 to 60 °C. The enzymes from *Aspergillus niger* NRRL 330 and *Aspergillus awamori* var. *kawachi* were optimally active at 50 °C and 60°C, respectively whereas GAs of *Arthrobotrys amerospora* were optimally active at 55.8°C (Spinelli *et al* 1996; Norouzian *et al* 2000). Ali and Hossain (1991) reported that the optimum temperature for the action of the glucoamylase was 60°C. The enzyme was stable at temperatures between 40 and 60°C with essentially no loss of activity in 30 min.

The rate of an enzyme catalysed reaction varies with pH of the system. Slivinski *et al* (2011) produced glucoamylase enzymes by *Aspergillus niger* in SSF and partially purified and characterized them biochemically. The partially purified enzyme had an optimum pH (4.5-5.0) and temperature (60 °C), with an average activity 152.85 U ml^{-1}. Jebor *et al* (2014) purified and characterized glucoamylase enzyme from *Aspergillus niger* The purified glucoamylase (A&B) had a maximum activity at pH (8 and6.5) and temperature (40°C and 30°C) respectively. It was also found that the K_m and V_{max} value of glucoamylase (B) were (2.8 mM and 9.8 mM/min) respectively using different concentration of starch. Banerjee and Ghosh (2017) used garden pea peel as a substrate in SSF by *Aspergillus niger* for the production of glucoamylase. The K_m and V_{max} for glucoamylase were 0.387 mg of soluble starch ml^{-1} and 35.03 $U^{-1}\mu l^{-1}min^{-1}$ respectively.

Okwuenu *et al* (2017) obtained Lineweaver-Burk plot of initial velocity at different substrate concentrations and K_m and V_{max} of the enzyme were found to be 770.75 mg/ml and 2500 µmol/min respectively. Vivian *et al* (2014) reported the activation of glucoamylase from *Aspergillus phoenicis* by manganese (Mn^{2+}) and calcium (Ca^{2+}) ions. The rise in glucoamylase activity caused by these metal ions (Ca^{2+}, Zn^{2+}, Co^{2+}, Fe^{2+} and Mn^{2+} ions) could be attributed to the ability of these metals ions to serve as an electron donor or Lewis acid as they participate directly in the catalytic mechanism of the enzyme.

SYNERGISTIC USE OF ALPHA AMYLASE AND GLUCOAMYLASE IN STARCH HYDROLYSIS

Kunamneni and Singh (2005) prepared crude amylases from *Bacillus subtilis* ATCC 23350 and *Thermomyces lanuginosus* ATCC 58160 under SSF. The effect of various process variables was studied for maximum conversion efficiency of maize starch to glucose using crude amylase preparations. Doses of pre-cooking and post-cooking amylase, glucoamylase and saccharification temperature were found to produce maximum conversion efficiency and were optimization of fermentation process. Maximum conversion efficiency (96.25%) were recorded at pre-cooking and post-cooking α-amylase (2.243 and 3.383 U/mg solids) respectively and glucoamylase (0.073 U/mg solids) at saccharification temperature (55.1 °C). Soni *et al* (2003) isolated *Bacillus* sp. AS-1 and *Aspergillus* sp. AS-2, producing very high titres of thermostable α-amylase and glucoamylase (198 950 and 3426 U/g fermented dry matter, respectively), during SSF of wheat bran. Both enzymes were active and stable over a wide range of temperature and pH. α-Amylase exhibited a high liquefying efficiency (96%) while glucoamylase revealed high saccharification efficiency (87%), in a 15% starch solution, at 50.8°C. When used in combination, these enzymes could effectively hydrolyzed wheat mash revealing a maximum conversion efficiency (96%).

Sodhi *et al* (2005) used alpha amylase from *Bacillus* sp. PS-7 in combination with a standard commercial amyloglucosidase (AMG), BioglucanaseTM in the hydrolysis of malt starch for alcohol production. It was found that the laboratory alpha amylase preparation worked very well in the synergistic use with AMG, with over all mashing efficiency (89.0 %), overall efficiency (79.5 %) and alcohol yields (25.43 %) and also competed well with the commercial alpha -amylase preparation, PromaltTM, commonly used in combination with BioglucanaseTM, a commercial amyloglucosidase, for malt starch hydrolysis in Indian breweries and distilleries.

ETHANOLIC FERMENTATION OF WHEAT HYDROLYSATE

Direct fermentation of starch using amylolytic microorganism offers a better alternative to the conventional multistage employing commercial amylases for liquefaction and saccharification followed by yeast fermentation (Verma *et al* 2000; Knox *et al* 2004). By using this amylolytic microorganism in direct fermentation, the ethanol production cost can be reduced via recycling of microorganism back to fermenters, thereby maintaining a high cell density, which facilitates rapid conversion of substrate into ethanol. However, there are very few types of amylolytic yeasts that are capable of efficiently hydrolyzing starch. Recombinant microbes and mix of amylolytic microorganism with glucose fermenting yeast in co-culture fermentation can be used to enhance starch hydrolysis and fermenting efficiency.

Review of literature has revealed that ability of yeast strains to achieve high level of ethanol strongly depends on the nutritional conditions and protective functions. The immobilization of fermenting organism for the bioethanol production has been greatly explored as a strategy to overcome substrate and product inhibition and to improve the ethanol tolerance (Ljiljanamojovic *et al* 2009 In Separate Hydrolysis and Fermentation (SHF) configuration, the enzyme production, hydrolysis of biomass, hexose and pentose fermentation are carried out in separate reactors and at their optimum fermenting conditions (Lynd *et al* 2002).

The disadvantages of SHF led to the development of Simultaneous Saccharification and Fermentation (SSF) process (Wright *et al* 1988). It is generally accepted that integration of the enzymatic saccharification and fermentation step which are carried out in one vessel so called simultaneous saccharification and fermentation (SSF) process could reduce the production cost and process time compared to conventional separate hydrolysis and fermentation (SHF) process (Mojovic *et al* 2006). The presence of yeast or bacteria along with enzymes minimizes the sugar accumulation in the vessel because the fermenting organism immediately consumes the released sugars. Since sugar produced during starch breakdown slows down α-amylase action, higher rates, yields and concentrations of ethanol are possible using SSF rather than SHF, at lower enzyme loading. Additionally, the presence of ethanol makes the mixture less vulnerable to contamination by unwanted microorganisms, which is a frequent burden in case of industrial processes (Bai *et al* 2008).

In literature, Kumar *et al* (1999) used simultaneous saccharification and fermentation to produce ethanol from starch of damaged quality wheat and sorghum grains by employing crude amylase preparation from *B. subtilis* VB2 and an amylolytic yeast strain *S. cerevisiae* VSJ4. They reported that 25% concentration of damaged wheat and sorghum starch was found to be optimum for damaged wheat and sorghum starch yielding 4.40%V/V and 3.50%V/V ethanol respectively. Whereas 25% raw starch of fine quality wheat and sorghum grains gave an yield of 5.60%V/V and 5.00%V/V respectively. Similarly, simultaneous saccharification and fermentation (SSF) of damaged grains of sorghum and rice was conducted using *Aspergillus niger* (NCIM 1248) and Saccharomyces cerevisiae VSJl. More yield of ethanol was produced from the damaged sorghum (2.90% v/v) than damaged rice (2.09% v/v) under optimal fermentation conditions (Kumar *et al* 1998). Recent research studies on Simultaneous Saccharification and Fermentation (SSF) of damaged corn grains using symbiotic strains of starch digesting *Aspergillus niger* (NCIM 1248 and sugar fermenting *Saccharomyces cerevisiae* (MTCC 170) revealed that SSF of damaged corn grains yielded maximum ethanol concentration of 4.24 (g/100ml) whereas fine corn grains yielded (6.3 g/100ml) ethanol (Gawande and Patil 2018).

Waste potato mash was chosen as a renewable carbon source for ethanol fermentation because it is relatively inexpensive compared with other feedstock considered as food sources. Izmirlioglu *et al* (2012) optimized the parameters for ethanol fermentation using response surface methodology to achieve maximum ethanol production. The study revealed that pH (5.5) and 3% inoculum size were optimum for maximum ethanol concentration. The maximum bio-ethanol production rate was attained at the optimum conditions of 30.99 g/L ethanol. Hence, waste potato mash was found as a promising carbon.

Current and future perspectives

This review paper investigated the potential for utilization of spoiled wheat grains for bioethanol. The main source for ethanol production in India is still molasses which single handedly cannot sustain the demand. Hence, there is need to look for alternate substrates for meeting the increasing ethanol production. Secondly,

thermostable alpha amylases are a more recent research which may reduce energy on cooling the mash prior to saccharification. It is also imperative to standardize the mash composition by optimizing solid-liquid ratio, addition of protease etc and mash environment (optimum temperature and pH). Further, to lower down the cost of fermentation recombinant glucoamylase-expressing yeasts were utilized to improve the efficiency of starch fermentation. The process cost may be further reduced by using this process as it reduces the time as well as energy by using two different vessels for saccharification and fermentation besides lowering the chances of contamination.

Genetic engineering approaches should be more focused on developing new improved strains with higher substrate tolerance and improved production kinetics. Though using damaged grains will incur lower substrate cost, mashing involves costly commercial saccharification and enzymes which may also be taken care of by using indigenous culture of *Bacillus subtilis, Bacillus circulans, Bacillus cereus* etc for α-amylase and *Aspergillus* sp. such as *Aspergillus niger, Aspergillus oryzae* etc for glucoamylase. Thus, utilization of especially damaged and spoiled wheat grains pave better way for commercialization of bioethanol production from an economical perspective.

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Studies on NOx removal using *Dunaliella salina* algae in photobioreactors

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ABSTRACT

The capability of an algal species to remove NO2 and NO in the simulated flue gas was established using *Dunaliella salina* in Photobioreactors under two variants of NOx sources. The concentrations studies were in the range between 25ppm to 150ppm. The diffusion of NOx and subsequent reaction with water resulted in NO3- and NO2- in the growth medium. Algal growth by absorption of NO3- and NO2- created a nitrate gradient in the bulk medium resulting in NOx uptake rates from the gas phase of up to 96%, leaving the unconsumed nitrogen of up to 7 mg-N/L in the growth medium. Algal species having an initial cell density of 2.8x105 cells/mL grew to the cell density of 1.73x107 cells/mL and dry weight of 262 mg/L. The Nitrogen content of cells varied from 3-6%. The treatment of NOx in Photobioreactors was investigated with reference to the gas removal efficiency, cell growth and total nitrogen content in the biomass

KEY WORDS: *DUNALIELLA SALINA*, PHOTOBIOREACTORS, ALGAL GROWTH

INTRODUCTION

Disproportionate usage of fossil fuels has been considered as the source for manmade toxic emissions comprising carbon dioxide, sulfur dioxide, nitrogen oxides, volatile organic compounds and heavy metals (Mulholland, 2008; Attilo et al., 2009). The by-products of fossil fuels have been identified as one of the major anthropogenic sources of this gas, contributing to global warming by the greenhouse effect. Therefore, it has become obligatory to reduce these toxic emissions before they are disposed into the environment. Nitric oxide (NO) and

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Nitrogen dioxide (NO2) are the two main components that make up NOx. These components are toxic and have various environmental hazards as per Environmental Protection Agency (Ronda, 2014). The nitrogen removal is 94.9 % in the ammonium form nitrogen group using biofilm (Yuxuan Zhu 2018).

There are several methods for treatment of NOx. Selective catalytic reduction (SCR) is used, however it is more expensive when applied for large-scale power plants (Miller et al., 2010). Another way to treat NOx from stationary sources is to use scrubbers to transfer the risk into an aqueous solution, which still must be treated or disposed of (Raja et al., 2007). Hence, to develop an economical and practical process to handle NOx exists. Cultivation of an algae to take up dissolved NOx from a scrubber as a nitrogen source, and oxygen only released as a by-product. This concept has been worked to estimate that, algal strains and conditions, algae can take up nitrogen from dissolved NOx (Nagase et al., 1997). Toxic compounds present in the flue gas inhibit the growth rate. NO2 has high solubility in water and therefore, reacts with water to form aqueous nitrates (NO3) and nitrites (NO2), some of the unaccounted-for nitrogen was lost due to volatization of gaseous nitrogen species, (Kaitlyan 2018).

Aqueous nitrate and nitrites are used by the algae as a source of nitrogen for cell synthesis, (Mulholland & Lomas, 2008). The dissolved NO2 and NO react to form dissolved nitrogen compounds which are available to ingest, biological conditions for the uptake of nitrate or nitrite by the algae, (Lee & Schwartz 1981). The nitrate or nitrite uptake by the algae reduce the soluble NO2, thus increases the concentration gradient of NO2 between liquid and the air. Thus, apparent solubility of NO2 is proportional to the NO2 gradient in the bulk medium, (Skalska, et al 2010). Nitrogen mono-oxide and sulfur dioxide can be removed by simultaneous absorption into aqueous mixed solutions of sulfite and [Fe II (edta)]H2O)]2–, ferrous ion coordinated to an anion of ethylene-diaminetetraacetic acid, (Tomasz et al 2016). A sequential process for the recovery and purification of multiple products was used on a mixture of algal biomass comprised of *Spirulina platensis* and *Dunaliella salina* (Kethineni 2017).

Dunaliella salina is a green algae known to with stand high salinity and accumulate carotenes. The nitrate requirement for algae is more for accumulation of biomass than for accumulation of carotene. Nitrates in the range of 1- 10mM is suitable for algal growth (Tafreshi et al., 2009). Harter et al, (2012) performed a mass balance for nitrogen from NOx for *Dunaliella* cultures in a column reactor. In a lab scale trail under simulated flue gas the results indicate that with an inflow of 150 µg N/L day NOX along with CO2 the alga could able to maintain a net influx of 0.52.73 µg N L-1 d-1 which amounts to 35% NOx removal. Nagase et al, (1997) studied the removal rate of nitric oxide by *Dunaliella tertiolacta* supplied in the range between 25-500ppm. At an inlet concentration of 500ppm NO in addition to CO2, it was shown to remove 110 µmoles per hour at a flow rate of 150mL/min. Also, it was shown that within a range of 100 to 400mL/min gas flow rate, a maximum of 60% of the NO was removed. These results suggest that *D. salina* is a potential algal species for NOx removal. the ability of the green algae, *Chlorella* to acclimate to high level of NOx and the potential usage of *Chlorella* strains in biological NOx removal (DeNOx) from industrial flue gases, (Tianpei and Gang Xu 2016). To understand the NOX removal process and to increase its range of applicability. The use of microalgae for simultaneous removal of CO_2, SO_x and NO_x from flue gas is an environmentally benign process, (Hong-Wei Yen et al, 2016), 75% decrease of the nitrogen concentration in the medium, with respect to the optimal values for growth, increased the lipid fractions of algal species, (Attilio Converti et al 2009).

It is very important to undertake biological NO_X fixation. Therefore, in this work, two individual experiments were conducted to productively remove the NOx from simulated flue gas with varying NOx loading rates by estimating the optimal growth parameters. Different NOx concentrations were supplied to each photobioreactor inoculated with *Dunaliella salina*. NOx removal efficiency and algal growth were determined in each experiment.

MATERIAL AND METHODS

DUNALIELLA CULTURE

All three reactors were inoculated with 600 mL of pure *Dunaliella salina* (SAG:42.88) grown in *Dunaliella* medium (=Dun) at 250C and a pH of 7.0. The inoculum was grown to a 1 x107 cells/mL, with an initial cell density of 2.8x105 cells/mL

GROWTH MEDIUM

The modified *Dunaliella* growth medium was used for inoculum and algae growth experimentation. All the nitrogen uptake by the algal cells was provided through inlet simulated gas. *Dunaliella salina* was grown in modified *Dunaliella* medium. A nitrogen free stock solution was prepared with K2HPO4, 0.1 g/ 100 mL. 20 mL of this nutrient was mixed with 30 mL of the soil extract and 930 mL artificial seawater to make a liter solution. The growth medium was given in Table 1.

Table1. Modified *Dunaliella* growth medium		
Component	**Stock sol. (g/100mL)**	**Nutrient concentrate ion (mL)**
KOH	0.1	20
Soil extract	30	-
Artificial seawater	930	-

Operating conditions of the reactor

Case1: pure NO2 feed source:

NO2 gas diluted with ambient air was used as the simulated flue gas for the first run. As the boiling point of NO_2 is approximately 20°C at atmospheric pressure; NO_2 was initially released as a liquid in the tubing. NO2 was blended with 3Lpm of air to get NOx concentrations of 100ppm, 200ppm,350 ppm in photobioreactors 1a, 1b, and 1c respectively. Experimental conditions were given in Table 2.

Table 2. Experimental conditions for case 1			
Setting	**Reactor 1a**	**Reactor 1b**	**Reactor 1c**
Inlet NO_x (ppm)	100	200	350
Inlet Gas Flow Rate (Lpm)	3	3	3
pH	7-8	7-8	7-8
Temperature (°C)	20	20	20

The simulated gas entered each photobioreactor through a sparger, pH was maintained in the range of 7.0 to 8.0 using CO2. The carbon dioxide feed was monitored by separate valves to each reactor which was controlled based on the pH in the reactor. NOx removal rates were monitored for four days. The reactors were illuminated with three 1 m long fluorescent white lights emitting, a total of 2700 Klux.

Case 2: Gas feed source calibration:

For the case 2, the reactors were inoculated before to the start of NOx loading and left for two days during which, only ambient air was supplied to the system. NOx was given from NO2 calibration gas cylinders comprising 5000 ppm and 9000 ppm NO2 concentration. Thus, in this case, the need for the liquid NO2 trap was ignored as the gas mixture was already in a vapor state. Thus, pumping calibration gas achieved steady inlet concentrations. Experimental conditions were given in table3. The calibrated NO2 is blended using air for the required NOX concentrations. The calibrated gases were supplied to the reactor at 3 Lpm, having NO2 concentrations of 25 ppm, 50 ppm, 120 ppm respectively. The concentration range was chosen to resemble real-time power plant NOx concentrations. Two aquarium stones of 12cm were used to diffuse the gas into the reactor. CO2 was supplied in the system, at a concentration of 400ppm until the pH remains 7 However when the pH is below 7, the pH 7 was maintained using 1.0 g/L solution of sodium bicarbonate (NaHCO3). The system was run for six days after loading of NOx. Influent and effluent concentrations of NO and NO2 in gas samples were measured using an analyser, (Testo 350-S/-XL, USA).

Table 3. Experimental conditions for case 2			
Setting	**Reactor 2a**	**Reactor 2b**	**Reactor 2c**
Inlet NO_x (ppm)	25	50	120
Inlet Gas Flow Rate (Lpm)	3	3	3
Influent CO_2(g) (ppm)	400	400	400
pH	7-8	7-8	7-8
Temperature (°C)	20	20	20

RESULTS AND DISCUSSION

NOX REMOVAL EFFICIENCY

Case 1: pure NO2 feed source

In this case, the reactors were fed with pure NO2 which is delivered as a liquid at room temperature and then the collected vapors were diluted with air. Using pure NO2 gas, actual average inlet concentrations for reactors 1a, 1b, and 1c were 108 ppm, 35ppm, 70 ppm respectively (Table 4). Fig. 1 shows the *Dunaliella* growth curve in reactors 1a, 1b, and 1c during 90-hour run.

Table 4. Influent and effluent NO_x concentrations in reactors				
Reactor	**Ratio (NO:NO2)**	**Inlet NOx (g) Conc (ppm)**	**Outlet NO_x (g) Conc (ppm)**	**Average removal Conc (%)**
1a	0.47	108±55	47±33	49
1b	0.15	35±16	15±12	51
1c	0.35	70±32	8±7	81
2a	0.038	27±6	11±4	59
2b	0.035	57±9	2±8	96
2c	0.043	126±12	7±11	95

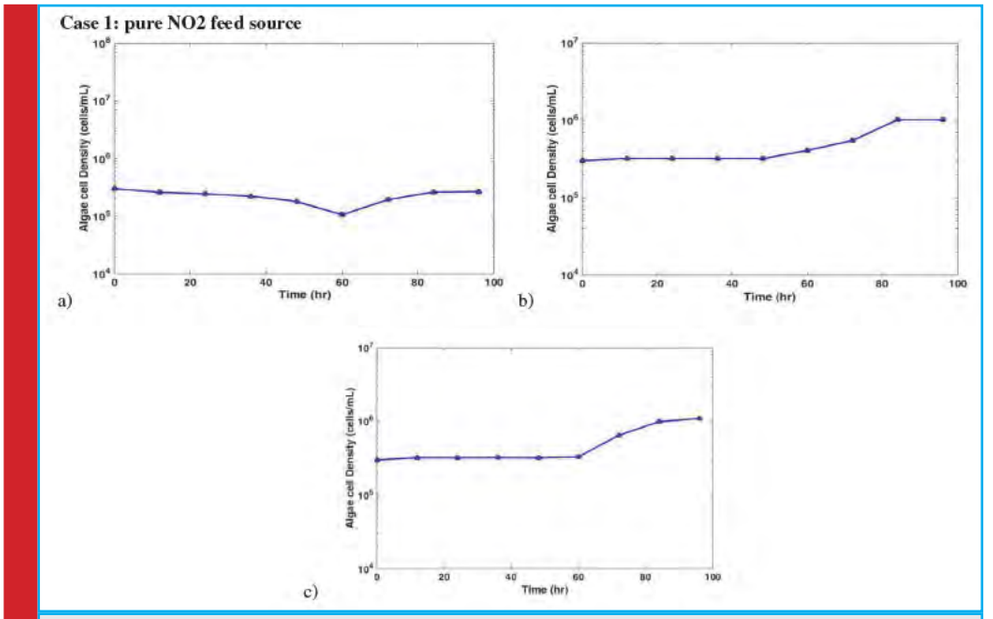

FIGURE 1. Growth curves in Photobioreactors for case 1

All three reactors began with $2.8x10^5$ cells/ml. As shown in Fig. 1, the culture in reactor 1a began exhaustive during the first 24 hours, therefore, the cells never attained a density greater than the initial. The maximum cell densities, for 1b, 1c were 1.46x106 cells/mL and 1.48x106 cells/mL respectively. Table 3 presents the NOx removal data for the reactors under case 1. Inlet and outlet NOx in Table 3 is the summation of measured NO and NO2 concentrations in the inlet and outlet streams. The efficiency of NOx removal by the algal system is based on total nitrogen through the reactor system and not on any particular NOx component. Therefore, removal percent of total NOx is only considered in the analysis. Reactor 1a obtained an average NOx removal of 49%,

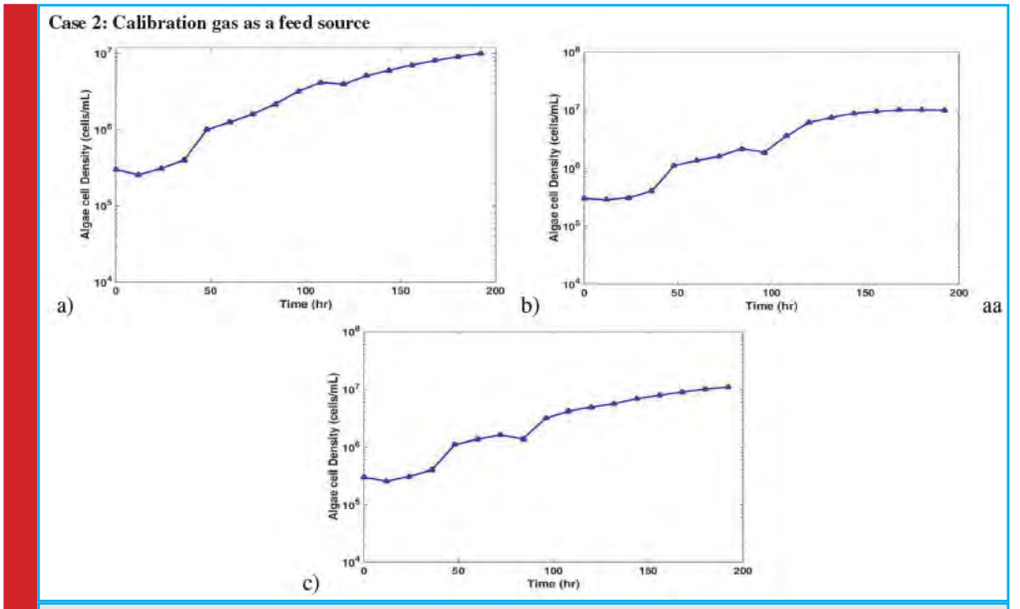

FIGURE 2. Growth curves in Photobioreactors for case 2

Table 5. Total suspended solids of Photobioreactors in Case 2		
Photo-bioreactor	**TSS Initial (mg/L)**	**TSS Final (mg/L)**
2a	6.0	210
2b	5.7	243
2c	6.3	222

Reactor 1b achieved an average 51% NOx removal, and Reactor 1c has removed an average of 81% of inlet NOx. The average NOx removal efficiency for the first 60 h was 39%, but the average removal for the last 25 hours was 52%. As the algal culture was declining, removal of NOx could be associated with the dissolution of NO2 into the medium.

Reactors 1b and 1c presented moderate growth following an extended lag phase, as shown in the figure1. Algae in reactors 1b, 1c has not truly experienced log phase, and the run was ended after 90 hours.

Case 2: Calibration gas as a feed source

As shown in Fig. 2 all three reactors began with a cell concentration of 2.8x105 cells/mL for three reactors 2a, 2b, and 2c .Maximum cell densities were 1.24x107 cells/mL, 1.46x107 cells/mL and 1.72x107 cells/mL respectively. To overcome some of the problems that appeared from using pure NO2 gas in case 1, NO2 calibration gases of 5000ppm and 10000 ppm NO2 were used to supply NOx for case 2. This allowed for accurate NOx loading concentrations. Actual NOx Loading rates for case 2 were 27 ppm, 57 ppm and 126 ppm for reactors 2a, 2b, and 2c respectively.

The data in Table 4 presents the removal of NOx for the reactors (case 2). Inlet and outlet NOx in Table 4 is the summation of measured NO and NO2 concentrations in the inlet and outlet streams. Reactor 2a got an average NOx removal of 59% reactor 2b got an average 96%, and reactor 2c was able to remove an average of 95% of inlet NOx. The lag phase was reduced to less than 24 hours due to delay in NOx loading, and log phase was attained between 24 and 48 hours. NOx loading started at 48 hours, and three reactors showed continued growth, but with a significant decline in growth rate (Fig. 2).

Table 6. Nitrogen mass balance data			
	Reactor 2a	**Reactor 2b**	**Reactor 2c**
NO_x Input	890	1740	3450
NO_x Output	450	60	90
N Consumed from NO_x gas phase	440	1680	3360
Initial N in growth medium	23	22	23
Final NO_3-	27	14	48
Final NO_2-	74	13	163
Net Accumulation of NO_3- +NO_2-	78	5	188
Initial Organic N	0	0	0
Final Organic N	310	750	1140
N Accumulated in Algal Cells	310	750	1140
Total N Accumulated	388	755	1228
Mass Balance (% NO_x uptake accounted for)	88%	45%	36%

Total suspended solids (TSS) for initial and final samples were taken to quantify algal growth and to estimate the nitrogen content of the cells. The results were summarized in the Table 5. Total suspended solid results show that 37 fold average mass growth was accomplished over the 190-hour run. For nitrate and nitrite concentrations, liquid samples from case 2 were analyzed, and the results were shown in Fig.3. Nitrate was completely drained in all three reactors before NOx was loaded into the system at 49 hours. Nitrogen source available for algal growth only after that point was from dissolved NO2. Initial and final total organic nitrogen content of the algal cultures was used to estimate the uptake of nitrogen by algae and to determine the nitrogen content of the cells. These analyses summarized in the table: the cells in reactors 2a, 2b, and 2c were found to contain 6.2%, 4.1%, and 7.9% nitrogen respectively.

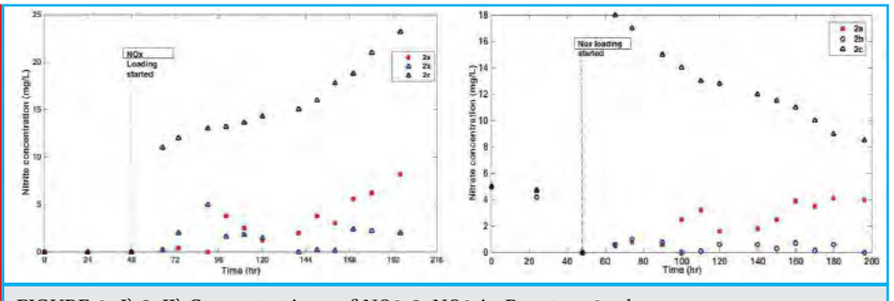

FIGURE 3. I) & II) Concentrations of NO3 & NO2 in Reactors 2a, b, c

A mass balance of nitrogen was conducted to assess the effect of NOx through the system. In reactor 2a, the mass balance alleged for 88% of the observed NOx removal, as slightly more nitrogen was found in the cells and growth medium than entering the system.

For reactors 2b and 2c, only 45% and 36% respectively of the nitrogen that entered the system was found in the cells and growth medium. NOx feed stream: Loading NOx at a particular concentration tested to be very difficult for the first case, as can see by more standard deviations in the table 6:. In the first case, NOx was given from a pure NO2 cylinder, and vapors from this liquid were pumped using peristaltic pumps into the reactors. To overcome large deviations in concentration, case 2 was operated using calibration gases. Data analysis considered only in case 2 because of the NOx feed was not consistent during case1.Nitrogen mass balance data summarized in Table 6. The only difference in the three reactors in case 2 was the loading rates of NOx.

NOx removal: In case 2, NOx removal rates are 59%, 95%, 96% for reactors 2a, 2b, and 2c respectively. Only 59% NOx removal was achieved in reactor 2a, which had the lowest NOx loading rate. NOx loading rates were different in three reactors. Aqueous nitrate and nitrite concentrations in the reactors are shown in Figure3.

Cell growth: The average specific growth rate for the first 2 days in reactors 2a, 2b, and 2c was 0.03, 0.06, 0.12 respectively, the average specific growth rates for the same period in reactors 1a, 1b were both negative, the growth curves of case 2 presented in Fig. 3, after loading NOx, growth rates started to decline in all three reactors and never achieved the value as before NOx loading, and this declining growth is proportional to the NOx concentration entering the system. Approximately 48 hours of NOx loading, the inlet concentrations of NOx do not affect algal growth. Algae took nearly 48 hours to adopt new nitrogen source.

Nitrate /Nitrite: The algae was able to take nitrate in solution before the loading of into the reactors. After 48 hours Nitrogen source is available only from NOx. The steady fall in NO3- concentrations and NO2- accumulation in reactor 2c suggests that algae preferred NO3- as its nitrogen source over NO2-.

CONCLUSION

The primary purpose of this study was to test the hypothesis that *Dunaliella* can grow on nitrogen from dissolved NO2 as its only nitrogen source for cell synthesis. *Dunaliella* grew used only nitrate /nitrite generated by the NOx dissolution for cell synthesis, reaching a maximum cell density of 1.75x107cell/ml. A reactor with lower NO2 loading concentrations resulted in lower NOx removal rates, for this reactor, nitrate was not accumulated as efficiently as a similar with grated NOx loading. In case 2: cell growth of mass between 1850mg/L to 198mg/L. Nitrogen was removed from gaseous NOx at a rate of 0.06-0.45 mg N/mg cell growth. Assuming a 700 MW natural gas fired power plant can produce up to 1,70,000m3/h of flue gas with approx. 50ppm NOx concentrations, growth of a minimun110 kg algal cell/h would be required to treat this stream.

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The effectiveness of pelvic floor exercises on symptoms in females with stress urinary incontinence

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ABSTRACT

The aim of the present investigation is to depict the interventions of pelvic floor exercises in urinary incontinence. The present study deals with the employment of pelvic floor exercises in subjects of varying age group from 30-60 years. Tools employed for the study are B-SAQ validation, the Bladder Control Self-Assessment Questionnaire (B-SAQ) and 1 Hr pad test which was working as outcome measure. The study was designed to investigate the effect of pelvic floor exercises on symptoms and botherness caused by urinary incontinence and achieving continence level was marked through pad test. Results confirmed that there was a significant improvement in the symptoms scores of experimental group in which pelvic floor muscles were employed in contrast with the control group. Pelvic floor muscles should be done under the supervision of physiotherapist as geriatric population needs some external support in order to complete the pelvic floor exercise protocol.

INTRODUCTION

Urinary incontinence is commonest problem in old age affecting social, psychological and physical daily activities. The most prevalent urinary incontinence amongst females is Stress urinary incontinence especially at the age between 30-60 years. Generally it is believed these reduced in muscle strength of urethra and muscle around the sphincter is responsible for causing incontinence. The intension of doing pelvic floor exercises is to increase maximal urethral pressure and also increase in reflex contractions sphincteric unit which can sustain the rise in the intraabdominal pressure. Pelvic floor muscle training exercises is the recognized and preferred

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conservative treatment for urinary incontinence, (Sapsford et al. 2001, Ferreira et al. 2004, Sinclair et al. 2011, Kılıcin et al. 2016 and Aarthi et al., 2018).

Exercises for pelvic floor were introduced in 1948 by Kegel, till date many randomized controlled studies and systematic reviews have supported the efficacy of these exercises, (Cavkaytar et al. 2014). Many researchers have reported that more than 68.4% of the women suffered from SUI while 41.2% of the women reported with mixed urinary incontinence, both showed improvement after 8 weeks of Kegel's exercises. The incidence of urinary incontinence in female subjects in a Turkish community was determined and stress urinary incontinence was found to be higher i.e. 33.7 % than any other type of incontinences in females of different age groups. This was explained through observational studies, based on questionnaires, where it was found that out of total of 192 subjects having incontinence, 45.5% had stress urinary incontinence (SUI), 19.8 % had urge urinary incontinence and 34.6% had mixed incontinence (Bhanupriya et al. 2015 and Kılıcin et al. 2016).

In the previous studies as explained with different outcome measures and exercise protocols including other intervention to enhance the muscle contraction like biofeedback, duration and number of contraction per day can also be a cause for difference in results output. Therefore pelvic floor exercises are accepted as an efficient intervention for SUI, many queries related to protocol adapted are still not yet explained. Other adjunct treatment like interferential therapies did not prove beneficial over pelvic floor exercises in SUI as recently reported by Aarthi et al. 2018).

For application of a competent treatment concern should be not only towards the pathology but also towards the social, socio-economical status and emotional aspect. Earlier surgical intervention was usually considered as foremost choice of treatment, last few years inclination towards conservative treatment has increased. As per the opinion from The International Continence Society, conservative treatment should be the first choice in incontinent patients (Dumoulin et al. 2016).

Researchers also employed different type of exercises protocol for primiparous women and their sexual efficacy by employing 8 weeks of pelvic floor exercises and found improvement in the outcome measure and strength of pelvic floor muscles (Luginbuehl et al. 2015, Malhotra et al 2018). Aforementioned studies employed for Pelvic floor exercises combination with additional treatment but there is limited study on the age group 30 – 60 years of ages. Therefore, there is need to determine that whether the stress urinary incontinence can alone be treated with PFMT on different age groups. The aim of the study is to evaluate the effectiveness of pelvic floor muscle exercise in female subjects with different age groups having stress urinary incontinence

MATERIAL AND METHODS

The 42 female subjects between the ages of 30-60 were included in the study and were divided into two groups, Control (Group A) & Experimental (Group B) and. The study was performed at RLJT Hospital & Research Centre, Jhunjhunu. The group allocation was done blinded through randomly and data acquisition, data reduction, data analysis was been blinded, the demographic data of name, age, height weight and BMI were recorded subjects present with stress urinary incontinence based on patients history ,both parous and nulliparous , pre and post menopausal and hemodynamicaly stable and physically fit for therapeutic exercises these subjects who were included in study, subjects having any history of organ prolapse ,suffering from vaginal or urinary tract infection, tumors or infectious disease or under some kind of medications affecting incontinence were excluded. Total study period was for 8 weeks, readings were taken at baseline, on 4th week and 8th week of the study. Experimental groups were asked to come are to come every weekends and perform the exercise and follow up while control group B were only explained the exercises. Also both groups were asked to follow up on 4th and 8th weeks.

B-SAQ validation, the Bladder Control Self-Assessment Questionnaire (B-SAQ) and 1 Hr pad test was employed as outcome measure, following the method of Sahai et al (2014). The B-SAQ contained 8- questions that assessed the symptoms such as number of times a patient is required to void, difficulty in holding urine, nocturia and urine leaks, for which there were associated scores. Responses to both were scored on a 4-point Likert scale. Total of both the score can give a significant illustration of patients to seek help or medical advice.

1 hour pad test: the test was performed on all the female subjects, where they were given pre-weighed pads and were asked to wear the same, few subjects were requested to wear two pads due to increased symptoms of incontinence. All subjects were asked to drink 500 ml of plain water at room temperature in 15 minutes of span, following which they asked to conduct certain activities like simple walking, climbing up and down a flight of stairs, standing up-down from sitting (10 times), cough vigorously (10 times), on spot jog for 1 minute, pick up objects with bending (5 times) and washing hands in running water (1 minute). All patients were strictly asked not to void their bladder for 1 hour and later the pads were weighed, for which the calculations were done on the basis of pad weight in grams i.e.

1-10 gms signified mild incontinence, 11 to 50 gms signified moderate and more than 50 gms indicated severe incontinence respectively.

Patients were explained about the anatomy and physiology of pelvic floor muscle a group class was conducted for both the groups on another day was an live demonstration video to explain how to perform the exercises in the protocol, also exercises in three different position i.e. Lying, sitting and standing an muscle contraction and stopping while passing the urine For experimental group the patients were explained and asked to perform correct pelvic floor muscle exercise in three different positions: Lying down supine with one pillow below the hips, sitting on chair and standing with legs both slightly kept apart. Patients were asked to perform exercises in lying position for first two weeks 10- 15 repetition. Contracting the muscle with a hold of 2-4 seconds of hold in each contraction without holding breath they were also asked to perform 2-3 sets per , with minimum of 20-30 contractions in a day. At 3rd and 4th week there was an addition of sitting position, number of set remained the same, by increasing the number of repetition to 30-40 in each set and 60-120 contractions over all throughout the day. Remaining 4 weeks, the protocol was added with an additional position of standing and keeping the number of sets as constant, the contraction per set was increased to 50-60 per set and minimal number of contractions per day ranged from 150 to 180. Telephonic and message reminders were sent on phone to encourage the subjects twice a week and queries were noted down and answered either on telephone or during the follow up visits.

RESULTS AND DISCUSSION

The data analysis was done with help of SPSS software version 19 was applied , To isolate the variable that differ from the others Turkey test was applied for multiple comparison analyze the mean and standard deviation within group and ANOVAs test were applied to calculate the difference between groups. Forty subjects were included in the study having a mean age of 46.05±6.04 and were divided in experimental and 41.15±7.12 in control group. The mean height in respective groups were 162±6.69 cm and 58.55±5.09 cm, mean weight was 64±6.50 and 67.3±5.54 in kg, The mean BMI was also calculated as 25.23±2.44 and 24.63±2.13 respectively for experimental and control group.

Amongst the 42 participant in the study, 2 women could not complete the study there was no significant difference seen in Group A (Control) before treatment and after treatment, but there was a statistical difference seen amongst Group B (Experimental Group) subjects at 4th week and 8th week of treatment.

As shown in figure I, the pad test demonstrates the significant improvement at 4th and 8th week for patient who was engaged in exercises and with regular follow-ups Group B, there was an improvement seen in the pad test performed for women in control group (Group A) but was not statistically significant.

Explaining through figure 1 & table 2 there was no significant difference found in between baseline data and 4th week and at end of 8th week in Group A (Control group), similarly when compared within intergroups as explained through table 3, there was a significant dif-

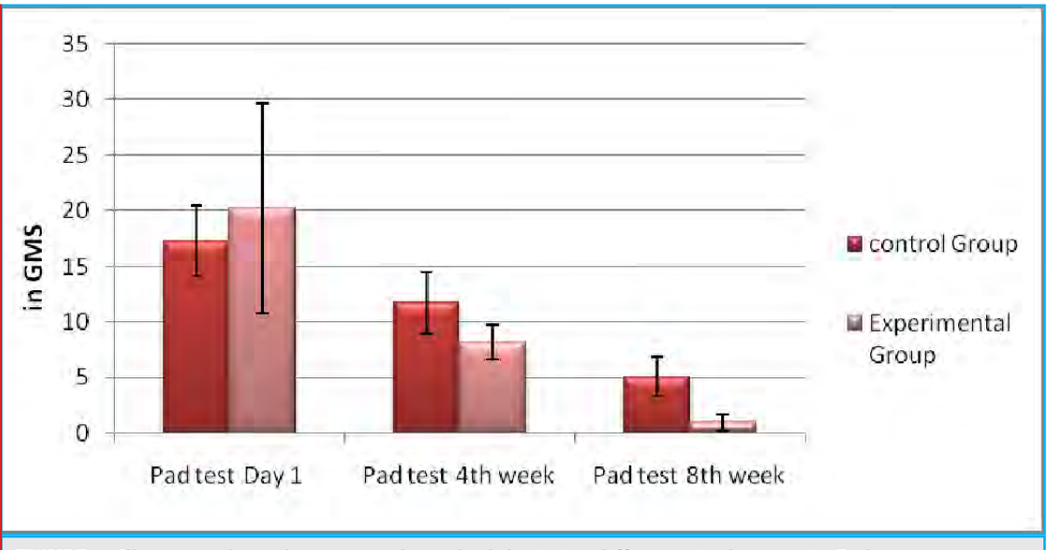

FIGURE 1. Illustrates about the mean and standard deviation difference in the score in Pad test

Table 1. Illustrates the mean and standard deviations in demographic data.

Characteristics	Group A	Group B
Age (Mean ± SD)	41.15±7.12	46.05±6.04
Height (In cm) (Mean ± SD)	158.55±5.09	162±6.69
Weight(In kg) (Mean ± SD)	67.3±5.54	64±6.50
BMI (Mean ± SD)	24.63±2.13	25.23±2.44

ference seen in pad test results for experimental groups, bringing the P at significant value of <0.001.

Explaining through figure 2 the result signifies that symptoms score of B-SAQ were significantly improved from day 1 4th week and at 8th for group B week but there no significant improvement in the scores for control group, figure 3 explains about the bothers score in B-SAQ in shows neither the experimental group nor the control group brings a significant change.

The pelvic floor muscles constitute the core muscles of the pelvis; the study was designed to investigate the effect of pelvic floor exercises on symptoms and bother-

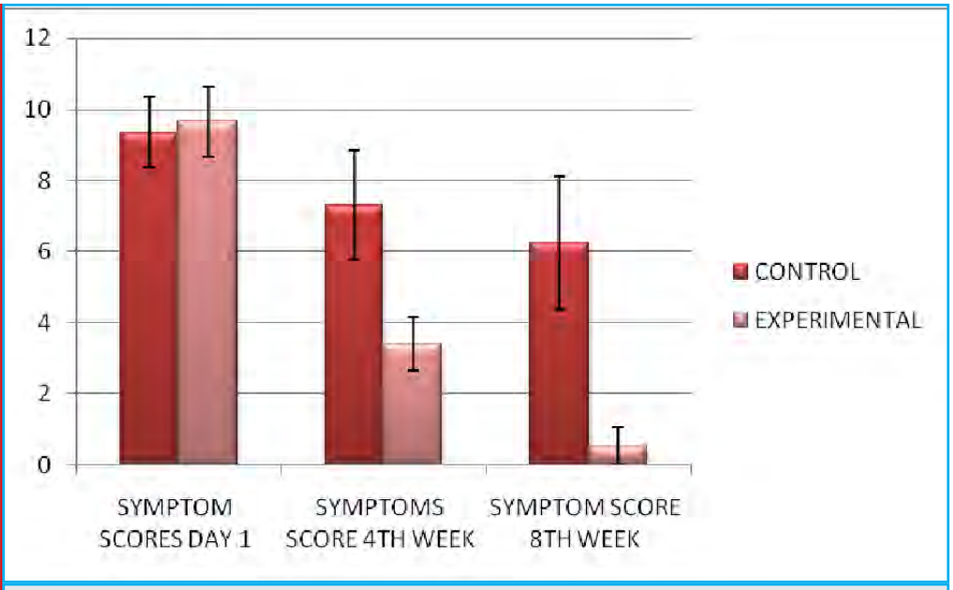

FIGURE 2. Explains about the mean and standard deviation difference in the symptom score in B-SAQ

Table 2. As explained in the above table comparison illustrates the pad test day 1 proved to be statistically significant for 4th week and 8th week

	Statistics	Control group	Experimental group
Day 1	Median (Q1,Q3)	17(15, 18)	19(13, 23)
4th week	Median (Q1,Q3)	11(9,13)	8(7,9) α,@
8th week	Median (Q1,Q3)	5(3,27)	1(0,1.75) α,β,#

Q1: first quartile, Q3: third quartile
α. Statistically significant (p <0.001) reductions at 4th week and 8th week compared to Day 1 in patients with Experimental group
β. Statistically significant (p <0.001) reduction from week 4th to 8th week in patients with Experimental group
@. Statistically significant (p <0.001) differences in mean pad test scores between Control group & Experimental group regimes at 4th week
#. Statistically significant (p <0.001) differences in mean pad test scores between conventional and advance physiotherapy regimes at 8th week

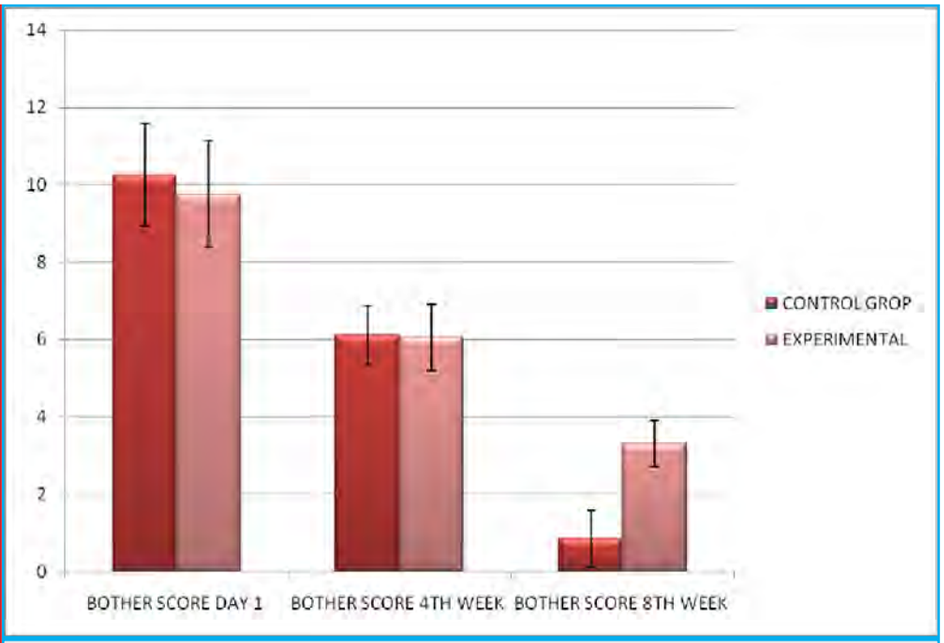

FIGURE 3. Explains about the mean and standard deviation difference in the bother score in B-SAQ

ness caused by urinary incontinence and achieving continence level were marked through pad test. There was a significant improvement in the symptoms scores of experimental group in contrast with the control group. The bother score when compared in both the groups at baseline and 4th week there was no statistical significant difference seen. The present study showed a significant decrease in symptoms and levels of incontinence, also there was an improvement reduction in frequency, urgency and nocturia in experimental group when compared to the control group in a 12 weeks protocol. Supervised protocol for pelvic floor exercises proved to be a most effective method for improvement in the symptoms in urinary incontinence. In a study by Sharon et al. (2010), it has been explained that this severity impacted the quality of life it was found that 30 % of female subjects and 18% of male subjects were reported with micturition dribbling and severity from mild to moderate, also female were more affected than men associated with other variable representing increase in the micturition dribble .this study showed that the increase in symptoms were disturbing quality of life. A significant improvement was seen in quality of life and self esteem frequency of urination and amount of urine, (Johromi et al. 2014).

In a study performed by Ali et al. (2011) the effect of Kegels exercises on women aged between 25-54 years suffering from urinary incontinence was carried out, it was found that the average score before administering the exercises was 53.15 and after the treatment there was a significant improvement to 73.82, bringing the significant level to p=0.0001. A study performed by another researcher on 30 patients affected with urinary incontinence in Imam Reza and Gharazi hospital at Sirjan city Iran with a aim to investigate the effect of kegels exercises on incontinent female subject for 3 month showed

Table 3. Illustrates the mean and standard deviation questionnaire at different time interval for both control and experimental group

Source of Variation	DF	SS	MS	F	P
Between Subjects	19	59.500	3.132		
Between Treatments	5	1254.367	250.873	230.716	<0.001
Residual	95	103.300	1.087		
Total	119	1417.167	11.909		

a significant improvement in reduction frequency of urination and was lessened by 30 % concluded that kegels exercises can become a suitable method to treat urinary incontinence, (Baba et al. 2006). Through the present study we explain that it is better to do pelvic floor exercises under supervision of a physiotherapist, learning of the correct technique of exercises to strengthen the muscle for required results, 2 months of exercises protocol results in the improving the strength and symptoms.

Limitation of the study : Considering small sample size difficult to draw and fırm conclusion Explaining and recognizing the correct pelvic floor muscle and skilled exercises learned by patient as treatment can result in positive effect but if not can result in negative effect increasing frequency of urination and dribbling.

CONCLUSION

Pelvic floor muscle exercises can be considered as an empowerment methods for improvement in the symptoms and bother score also improving on pelvic floor muscle strength. So it is recommended that these types of exercising protocol can be utilized in improving the strength and quality of life in patient suffering from incontinence

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On the spider diversity of Salbardi forest and upper Wardha Dam Amravati India

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ABSTRACT

Present study was carried out to explore the diversity and abundance of spiders from Salbardi Forest and catchment area of Upper Wardha Dam, Dist. Amravati, Maharashtra, India, for consecutive 2 year (2011-2013). The study areas were surveyed by making quadrants of approximately of 10 x 10 meters. The floor, undergrowth, rocks, firewood and stones were systematically searched for spiders. Each site was surveyed from early morning 8 AM to 6 PM at the interval of 7 days. During this survey 142 species of spiders from 62 genera of 21 families were recorded. The most diverse family observed was the Aranidae with 33 species followed by Salticidae 30, Oxyopidae and Thomisidae 11, Lycosidae 9, Gnaphosidae 8, Tertragnathedae 7, Philodromidae 4, Clubionidae, Erasidae, Pholcidae, Pisauridae, Theridiidae and Uloboridae 3 each, Hersilidae, Miturgidae, Scytodiidae and Sparassidae 2 each and Nephilidae, Sicariidae and Theridiosomatidae 1 species each. Abundance and diversity of spiders was recorded highest during August to December while least was recorded during summer. Amongst all families spiders from Salticidae were recorded throughout the year. However Lycosidae, Oxyopidae, Hersilidae and Thomisidae were predominantly observed during late monsoon and early winter. Seasonal variation showed a great impact on diversity and abundance of spiders. Their food consists of common insect pests; they are important predators in nature and may serve as bio-indicator species for specific ecosystem.

KEY WORDS: DIVERSITY, SPIDERS, UPPER WARDHA DAM, SALBARDI FOREST

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INTORDUCTION

Spiders belong to phylum Arthropoda, class Arachnida, a large group of animals with jointed legs and rank seventh in total species diversity among all other group of organisms. Spiders are ancient animals with a history going back over 350 million years. They are abundant and widespread in almost all ecosystems and constitute one of the most important components of global biodiversity. The current global list of Spider fauna is approximately 42,055 belonging to 3821 genera and 110 families (Platnick, 2014). Although the fossils record of spiders is considered poor, almost 1000 spiders have been described from fossils. The oldest known amber that contains fossils arthropods dates from 130 million years ago in the early cretaceous period.

Spiders are generalist feeders with great species richness in every type of terrestrial habitat and play an important role in the structure of communities and food webs, both as an individual numbers and as energy consumers. Spiders acting as ecological indicator are cosmopolitan in distribution and locally abundant in terms of individuals and taxa. Their small body size allows them to maintain their community in small area. Spiders are insectivorous animal and insect fauna changes with the change in vegetation. Spiders play a significant ecological role by being exclusively predatory and thereby maintaining ecological equilibrium.

Spiders have a very significant role to play in ecology by being exclusively predatory and thereby maintaining ecological equilibrium. Bastawade (2004) described arachnid fauna of orders Araneae, Scorpionida and Solifugi from Melghat Tiger Reserve, Amravati, Maharashtra State. Spiders of protected areas in India are studied by Gajbe (1995a) in Indravati Tiger Reserve and recorded 13 species and Gajbe (1995b) 14 species from Kanha Tiger Reserve, Madhya Pradesh. Gajbe (2003) prepared a checklist of 186 species of spiders in 69 genera under 24 families distributed in Madhya Pradesh and Chhattisgarh. Patel (2003) described 91 species belonging to 53 genera from Parabikulum Wildlife Sanctuary, Kerala. **Deshmukh and Raut (2014) reported 92 species from Salbardi forest, Satpura range Maharashtra.** Manju Siliwal *et al*. (2003) recorded 116 species from 66 genera and 25 families of spiders from Purna wildlife Sanctuary, Dangs, Gujarat. Hippargi, *et al*. (2011 b) reported occurrence of spiders from 19, 25, 31 families from Lonar, Melghat and Southern Tropical Thorn forest, Solapur respectively. Hore and Uniyal (2008a, 2008b) worked on the spider assemblage and the diversity and composition of spider assemblages in different vegetation types in Terai Conservation Area (TCA). Hore and Uniyal (2008) worked on spiders as indicator species for monitoring of habitat condition in TCA. They also studied on the effect of prescribed fire on spider assemblages in TCA. **Sebastin and Peter (2009) studied spider fauna from irrigated rice ecosystem in central Kerala.** Spiders provide vital control of the invertebrate population. They are skilled and efficient hunters of insects. Recent studies have investigated the importance of spiders as ecological indicators. Terrestrial arthropods, of which spiders are amongst, have long been monitored for early warning sign of environmental changes. The aim of this study was to investigate the spider species composition in different habitat type within Salbardi forest and Upper wardha Dam area.

MATERIAL AND METHODS

Study area: Present study was carried out to explore the diversity and abundance of spiders from Salbardi Forest (The Latitude and Longitude of salbardi is 21.4183 and 78.0113 respectively) and catchment area of Upper Wardha Dam (The Latitude and Longitude of Upper Wardha Dam is 21.2764 and 78.0572 respectively), Dist. Amravati, Maharashtra, India, (2011-2013). The study areas were surveyed by making quadrants of approximately of 10 x 10 meters. Salbardi is about 8 km. (5 miles) North of Morshi, District Amravati, on the border lying partly in the Betul District of Madhya Pradesh on Madu River. Salbardi is named from its abundance of *Sal* trees and the stony character of its soil, Upper Wardha Dam is situated on Wardha River near to Morshi. Catchment area of both the sites comprises dry deciduous forest.

Field Methods: For adequate sampling the spiders from various spots were collected every weekend. The collection methods such as trapping, sweeping, beating, pitfall trap and visual search for webs or retreats were used. Only mature spiders were collected as well as repetition of collection was avoided. Collected spiders were identified using standard identification keys of Barrion and Litsinger (1995), Biswas and Biswas (2004), Gajbe (1999), Plantnick (1989), Tikader (1974, 1980, 1982 a and b, 1987).

RESULTS AND DISCUSSION

Spiders were sampled from various habitats by making the quadrants approximately of 10x10 m. Consecutive two years survey result shows that total 142 species of spiders belonging to 61 genera and 21 families were identified. Table 1. Abundance and richness of spiders was at the peak during September to January. As spiders feeds exclusively on insects or other arthropods, which were abundantly found during the same season as seasonal flora is also at the peak. Among the specimen most of the individuals were females while very few number

Table 1. Number of genera and number of species recorded from 21 families.

Sr. No.	Family	Number of genera	Number of Species
1	Araneidae	08	33
2	Clubionidae	01	03
3	Eeasidae	01	03
4	Gnaphocidae	05	08
5	Hersilidae	01	02
6	Lycosidae	03	09
7	Miturgidae	01	02
8	Nephilidae	01	01
9	Oxyopidae	02	11
10	Philodromidae	04	04
11	P Pholcidae	02	03
12	Pisuiridae	02	03
13	Salticidae	14	30
14	Scytodidae	01	02
15	Sicariidae	01	01
16	Sparassidae	01	02
17	Tetragnatidae	02	07
18	Theridiidae	03	03
19	Theriosomatidae	01	01
20	Thomisidae	06	11
21	Uloboridae	02	03
	Total	62	142

of males were observed. *Chrysilla lauta* is only male till now recorded from Saltisidae family was also observed during the survey.

Highest number of species were recorded from family Araneidae (33 species from 8 genera) or orb viewers, these spiders were found in all most all spots as well as in all seasons. Spiders from genus *Neoscona* was recorded with high abundance and most of them observed constructing their webs in the evening. Second diverse family was Saltisidae (30 species from 14genera) or jumping spider, spiders of which were recorded throughout the year and in all most all places. Only few species as *Opisthoncus, Rhene, Siler, Telamonia* were found during flowering season. Clubionidae (3 species from a single genus) commonly called as leaf rolling spider were observed through sacs in rolling leaves. Eresidae (3 species from a single genus) or Social spiders were observed through their typical web pattern unkempt irregular and large webs on trees, shrubs from height 4 feet to max 60 feet. In a single web numbers of spiderlings were found. Gnaphosidae (8 species from 5 genera), or ground spider were observed wondering on ground, under litters or in crevices. Hersilidae (2species from 1genus) or tailed spiders found on barks of trees, very much mimicking to habitat, spinnerets of these spiders are long just like tail. Lycosidae (9 species from 3 genera) or Wolf spider were recorded from ground and bushes. Miturgidae (2 species from 1genus) or Dark sac spider observed on vegetation mimicking to environment. Nephilidae (1 species from 1genus) or giant wood spiders, these were observed on huge webs constructed on large trees, with the average diameter of 8-10 meters. Female is large and rest at the centre while 3-4 males comparatively small, were found on web at the periphery. Oxyopidae or lynx spiders, these are colourful spiders observed abundantly during rainy season on seasonal flora, males of these were found in large number along with females. Philodromidae (4 species from 4 genera) or elongated crab spider or running crab spider are dull colored- brown, gray or yellowish spiders and do not build webs, but use silk for draglines and for egg sacs. These are observed running very fast on ground and grass. Pholcidae (3 species from 2 genera) commonly known as daddy long legs, observed in humid and shady areas. Many times females were observed carrying their egg sacs. Pisuiridae (3 species from 2 genera) or Nursery web spiders, carry their egg sacs by means of their jaws and pedipalps these spiders were observed at river site or near water bodies. Scytodidae (2 species from 1 genus) or spitting spiders, have six eyes arranged in three pairs, found under bark and leaf-litter. Sicariidae (1 species from 1 genus) observed in dense forest. Six-eyed, venomous spiders known for their allegedly necrotic bites. Sparasiidae (2 species from 1 genus) or Huntsman spiders found in humid places. Tetragnthidae (7 species from 2 genera) long-jawed orb weavers, mostly observed in vegetation near water bodies. Theridiidae (3 species from 3 genera) or comb-footed spiders, these were collected from on the ground, on plants, in burrows and caves, often webs were found in dark. Theridiosomatidae found in damp habitats, among low-growing vegetation. Thomosidae (11 species from 6 genera) or crab spider / flower crab spider, were observed Wandering on plant and ground, mainly on foliage, camouflages with surrounding. Uloboridae (3 species from 2 genera) cribellate orb weavers or hackled orb weavers. These are non-venomous spiders. These were observed in dry as well as humid habitat on vegetation.

Spiders feeds on insects and other small arthropods, any specific selectivity was not observed in selection of food rather they feed on eggs, larvae of insects along with adults. Depending upon availability of food spiders population was observed varied. However spiders can remain starved for comparatively longer time. Diversity and abundance of spiders depends on population of insects. Diversity of insects is mostly determined by ecological conditions, flora and other environmental

factors. Therefore spiders can be used as bio-indicator species of any ecosystem.

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Statistical optimization of extracellular tannase production by *Streptomyces sp.* AT 13 using response surface methodology and Plackett-Burmen design

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ABSTRACT

Tannase has many important applications in animal feed, chemical, food, and pharmaceutical industries. In the present study, optimization of tannase production by *Streptomyces sp*. AT13 was carried out using statistical experimental designs. Initially, a Plackett-Burmen design (PBD) was employed to screen the preferable nutriments (carbon and nitrogen sources of the medium) to produce tannase. The result showed that only tannic acid was found to be significant for the production of tannase by *Streptomyces sp*. AT 13. The significant factor was further optimized by using Box-Behnken design under response surface methodology (RSM). From among 6 fermentative variables that were studied, 5 significant variables were picked up by PBD. Among 5 variables from PBD, 3 were further optimized by Box-Behnken design. The parameters studied through RSM were 1% Tannic Acid, 0.5 % KCl and 0.1 % KH_2PO_4. Under optimized conditions tannase activity was 18.12 U/ml/min. This activity was almost three times higher as compared to the amount obtained by 'one-at-a-time' approach. (5.19 U/ml/min)

INTRODUCTION

Tannins are polyphenolic compounds with molecular weights ranging from 500 to 3000 daltons which occur widely ina variety of plants including monocots, dicots and ferns (Bate-Smith and Swain 1962; Mcleod 1974; Haslam 1989). They are the fourth most abundant plant constituents after cellulose, hemicellulose and lignin. Based on their structure and properties, tannins are distributed into two major groups – hydrolysable and condensed tannins. Hydrolysable tannins are composed of esters of gallic acid (gallotannins) or ellagic acid (ellagitannins) with a sugar core, which is usually glucose, and are readily hydrolysed by acids and enzymes into monomeric products. Hydrolysable tannins are notably absent in monocots. Commercially, hydrolysable tannins

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are extracted from Chinese gall (*Rhus semialata*), Sumac (*R. coriara*), Turkish gall (*Quercus infectoria*), Tara (*Caesalpina spinosa*), Myrobalan nuts (*Terminalia chebula*) and chestnuts (*Castania sativa*) (Bhat *et al*., 1998).

Tannase (tannin acyl hydrolase) catalyses the hydrolysis of ester and depside bonds of hydrolysable tannins to produce glucose and gallic acid (Barthomeuf *et al*.,1994). Hydrolysis of tannic acid by tannase results in the liberation of glucose, gallic acid and various galloyl esters of glucose (Van de Lagemaat and Pyle, 2006). This enzyme finds widespread applications in food processing, brewing, pharmaceuticals, medicine, textiles, detergents and tea industry (Lekha and Lonsane, 1997). A combination of trimethoprim and sulphonamide is effective against many antibiotic resistant species of bacteria. More than 8000 tonnes per annum of gallic acid is manufactured out of which, almost 70% is used in production of trimethoprim (Beena, 2010).

The use of a sequential experimental design strategy is a useful tool for process optimization. Response surface methodology (RSM) provides important information regarding the optimum level of each variable along with its interactions with other variables and their effects on product yield. It reduces the number of experiments without neglecting the interactions among the parameters. This multivariate approach also improves statistical interpretation possibilities and evaluates the relative significance of several contributing factors even in the presence of complex interactions (Dilipkumar *et al*., 2011). RSM is widely used for multivariable optimization studies in several biotechnological processes such as the optimization of media, process conditions etc. (Mannan *et al*., 2007; Pan *et al*., 2008). Statistical optimization allows the interaction amid possible influencing parameters to be evaluated with a limited number of experiments (Rodriguez *et al*., 2008). Statistical designs such as Plackett-Burman and Response surface methodology are common in practice for testing multiple factors and interactions that can be quantified in an error-free and robust manner (Mohan, 2014).

In order to fully exploit the prospective of this enzyme for various applications, it is imperative to investigate the possibility of enhancing its production by using more efficient production strategies (Rana and Bhat, 2005). Hence in this study the process parameters were optimized for maximum enzyme production adopting Plackett-Burman (PB) and Response Surface Methodology (RSM) with Box Behnken design. The statistical software package DesignExpert® 7.0 (Stat Ease Inc., Minneapolis, and USA) was used.

MATERIALS AND METHODS

Tannic Acid was purchased from Sigma Aldrich, USA. All other chemicals and reagents were used of analytical grade and purchased from Hi-Media, India. *Sterptomyces* sp. AT 13 used for the present study was screened and isolated from Tannery Effluent at G.I.D.C Naroda, Ahmedabad and the organism was maintained on tannic acid agar (TAA) medium. Tannase production by *Sterptomyces* sp. AT 13 was carried out using tannase production medium containing $NaNO_3$ 0.6 % w/v, KCl 0.5 % w/v, $MgSO_4$ 0.05 % w/v, K_2HPO_4 0.05 % w/v, KH_2PO_4 0.05 % w/v and 1 % (w/v) filtered sterilized tannic acid (designated as TA broth). The broth was assayed for tannase activity.

TANNASE ASSAY

The tannase activity was estimated by modified spectrophotometric method of Sharma et al., 2000. Tannic Acid was used as substrate. The basic principle of this assay is based on the formation of chromogen between gallic acid (released by the action of tannase on tannic acid) and rhodanine (2-thio-4-ketothiazolidine). A standard curve was prepared using gallic acid.Crude enzyme was used for the assay. All the tests were performed in triplicates. One unit of tannase activity is defined as the amount of enzyme required to liberate 1µM of gallic acid /min under defined conditions. Enzyme activity was expressed as U/ml/min.

Plackett–Burman design

Plackett–Burman design, an efficient technique for medium component selection (Plackett-Burman, 1946) was employed to establish the factors that significantly enhance the tannase production. Five independent variables (Table 1) were analysed in 12 experimental runs (Table 2) for the production of tannase. Triplicates were maintained for each experimental set up. The response of these factors for the production of tannase was measured by Spectrophotometer method as proposed by Sharma *et al*., (2000). The low level (-1) and the high level (+1) of each factors are demonstrated in Table 2. The statistical software design expert 6.0 was used for analysing the experiment. Total 5 independent variables

Table 1. Screening of variables using a Plackett–Burman design

Variable codes	Variables	Low level (-1)	High level (+1)
A	Tannic Acid	-1	+1
B	KCl	-1	+1
C	$NaNO_3$	-1	+1
D	KH_2PO_4	-1	+1
E	K_2HPO_4	-1	+1

Table 2. Plackett Burman design matrix for the screening of variables

	F1	F2	F3	F4	F5	F 6	F 7	F 8	F 9	F 10	F11	
Run	A:Tannic Acid	B:KCl	C:$NaNO_3$	D:KH_2PO_4	E:K_2HPO_4	F:F	G:G	H:H	J:J	K:K	L:L	R1
1	-1.00	1.00	1.00	1.00	-1.00	-1.00	-1.00	1.00	-1.00	1.00	1.00	1.62
2	1.00	1.00	1.00	-1.00	-1.00	-1.00	1.00	-1.00	1.00	1.00	-1.00	12.99
3	1.00	-1.00	1.00	1.00	-1.00	1.00	1.00	1.00	-1.00	-1.00	-1.00	7.17
4	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	10.12
5	1.00	1.00	-1.00	1.00	1.00	1.00	-1.00	-1.00	-1.00	1.00	-1.00	16.53
6	-1.00	1.00	-1.00	1.00	1.00	-1.00	1.00	1.00	1.00	-1.00	-1.00	5.61
7	-1.00	-1.00	-1.00	1.00	-1.00	1.00	1.00	-1.00	1.00	1.00	1.00	5.91
8	1.00	-1.00	1.00	1.00	1.00	-1.00	-1.00	-1.00	1.00	-1.00	1.00	16.2
9	-1.00	-1.00	1.00	-1.00	1.00	1.00	-1.00	1.00	1.00	1.00	-1.00	2.58
10	-1.00	1.00	1.00	-1.00	1.00	1.00	1.00	-1.00	-1.00	-1.00	1.00	0.3
11	1.00	1.00	-1.00	-1.00	-1.00	1.00	-1.00	1.00	1.00	-1.00	1.00	15.75
12	1.00	-1.00	-1.00	-1.00	1.00	-1.00	1.00	1.00	-1.00	1.00	1.00	0.36

+ "sign indicates high level of the variable a nd" – "sign indicates low level"

along with 5 dummy variables generated by the software. All the experiments were done in triplicate and the average of lipase production was taken as response. The obtained results are matched with the polynomial equation 1 in significant order 1:

$$Y = \beta 0 + \Sigma \beta i Xi \ (i=1, 2, 3.....k) \quad (1)$$

Where Y is the response (Enzyme activity), β0 is model intercept, βi is linear coefficient, Xi is level of the independent variables. The model was statistically analyzed and the overall significance of the model was evaluated by ANOVA (Analysis of variance) involving Fischer's test (F test). P (probability) values and determination of coefficient obtained determines regression model's goodness of fit.

Box-Behenken Design

After optimizing the various nutritional variables by PBD, the 3 most significant variables (Tannic Acid, KCl and KH_2PO_4) were further chosen for response surface methodology using Box-Behnken design. Design-Expert® statistical software was used to analyze the experimental design. A Box-Behnken design with a set of 12 experiments was generated (Table 4). Each variable was stud-

Table 3. ANOVA for the experiment with PBD for Tannase Production by AT 13

Analysis of variance table [Partial sum of squares - Type III]

	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Prob > F	
Model	427.76	10	42.78	285.87	0.0460	significant
A-TANNIC ACID	153.08	1	153.08	1023.04	0.0199	
B-KCl	9.12	1	9.12	60.93	0.0811	
C-$NaNO_3$	15.01	1	15.01	100.30	0.0634	
D-KH_2PO_4	9.97	1	9.97	66.65	0.0776	
E-K_2HPO_4	11.96	1	11.96	79.93	0.0709	
G-G	77.32	1	77.32	516.71	0.0280	
H-H	69.89	1	69.89	467.08	0.0294	
J-J	43.85	1	43.85	293.07	0.0371	
K-K	19.15	1	19.15	127.99	0.0561	
L-L	18.40	1	18.40	122.98	0.0573	
Residual	0.15	1	0.15			
Cor Total	427.91	11				

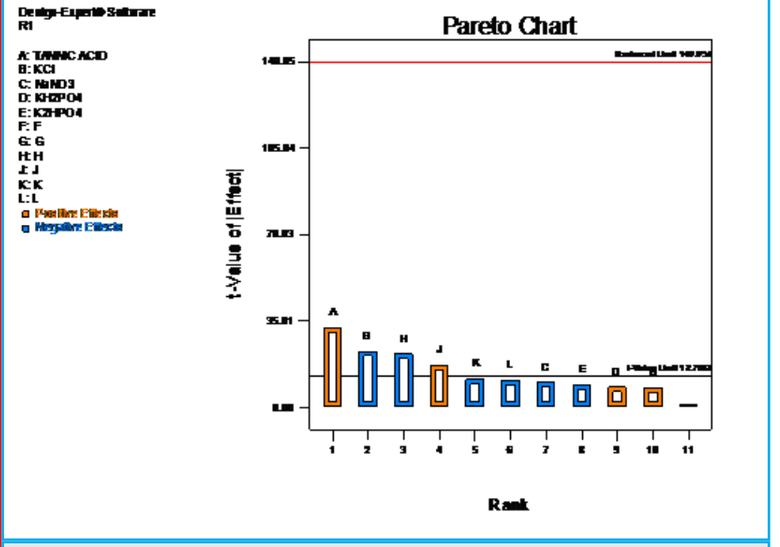

FIGURE 1. Pareto chart showing the effect of media components on tannase activity using AT 13

ied at three levels, +1, 0, -1 where "0" is the central coded value, "+1" high value and "-1" low value. The fermentation was carried out in 250 ml flasks containing 100 ml of the production medium. All experiments were done in triplicate and tannase production obtained was taken as the response.

The obtained results are matched with the polynomial equation 2 in significant order

$$Y = \beta 0 + \Sigma\, \beta iXi + \Sigma\, \beta iiXi\ 2 + \Sigma\, \beta ijXiXj \qquad (2)$$

where Y is the predicted response, β0 is the intercept term, Xi and Xj are the input variables, βi the linear coefficients, βii the squared coefficients and βij the interaction coefficients. The model was statistically analyzed and the overall significance of the model was evaluated by ANOVA (Analysis of variance) involving Fischer's test (F test). P (probability) values and coefficient of determination obtained determines regression model's goodness of fit. The optimum values of each of the 3 significant variables were determined by solving regression equation.

The interactive effects of the variables on tannase production were studied by analyzing the 3D and counter plots which depicted the interactions graphically.

Validation of the model: The statistical model was again tested by carrying out the fermentation under the optimum conditions that were obtained through the Box-Behnken design.

Table 4. Box-Behenken (BB) design matrix of optimizing factors influence for tannase activity

	Tannic Acid	KCl	KH_2PO_4	E.A
Run	A:A	B:B	C:C	R1
1	-1.00	1.00	0.00	12.99
2	-1.00	0.00	-1.00	14.61
3	0.00	1.00	1.00	17.34
4	0.00	-1.00	1.00	12.33
5	0.00	1.00	-1.00	11.52
6	1.00	0.00	1.00	9.27
7	1.00	0.00	-1.00	10.71
8	1.00	1.00	0.00	2.61
9	-1.00	-1.00	0.00	9.66
10	1.00	-1.00	0.00	6.57
11	0.00	-1.00	-1.00	17.79
12	-1.00	0.00	1.00	18.12

RESULTS AND DISCUSSION

SCREENING OF VARIABLES BY PBD

Iimportant fermentative parameters can be effectively and quickly picked up by applying PBD. The use of statistical tools not only saves time by simultaneously optimizing several process variables with few experimental runs but also reduces the cost of fermentation. A total of 5 important variables Viz., tannic acid, KCl,$NaNO_3$, KH_2PO_4 and K_2HPO_4 which were selected for statistical optimization using PBD (Table 1).

Experiments were carried out based on Plackett–Burman design and the results obtained are given in Table 2.

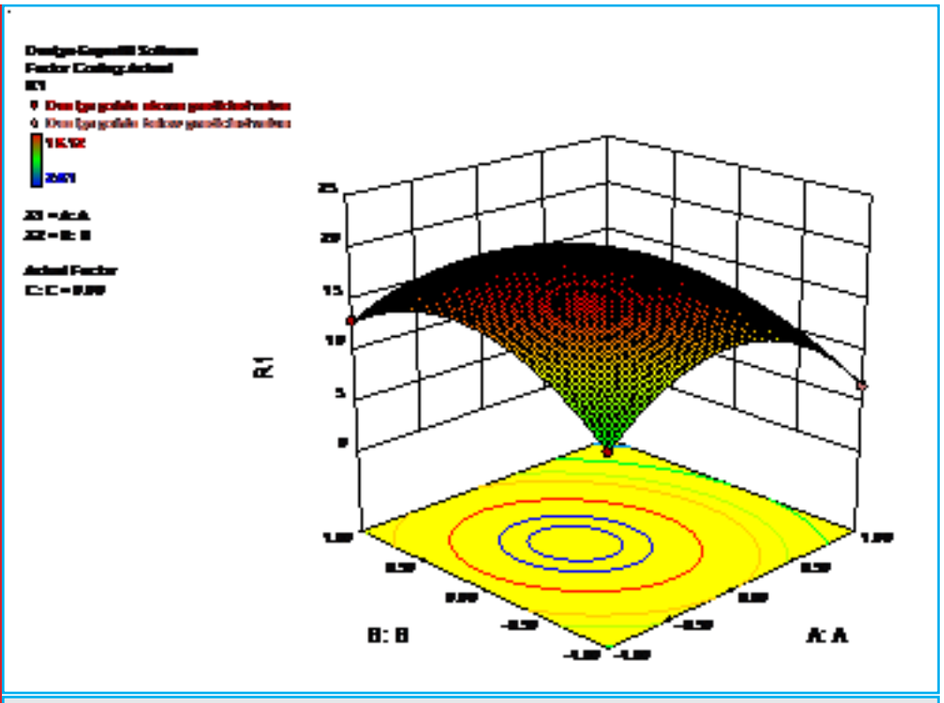

FIGURE 2a. Response surface plot of tannase production by AT 13 showing interaction between tannic acid and KCl

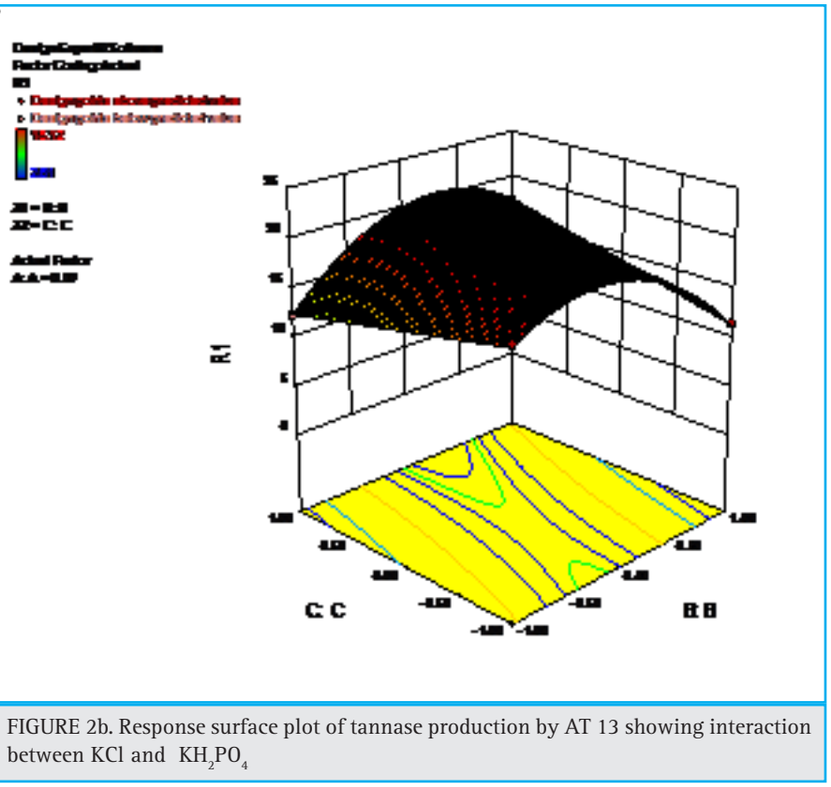

FIGURE 2b. Response surface plot of tannase production by AT 13 showing interaction between KCl and KH_2PO_4

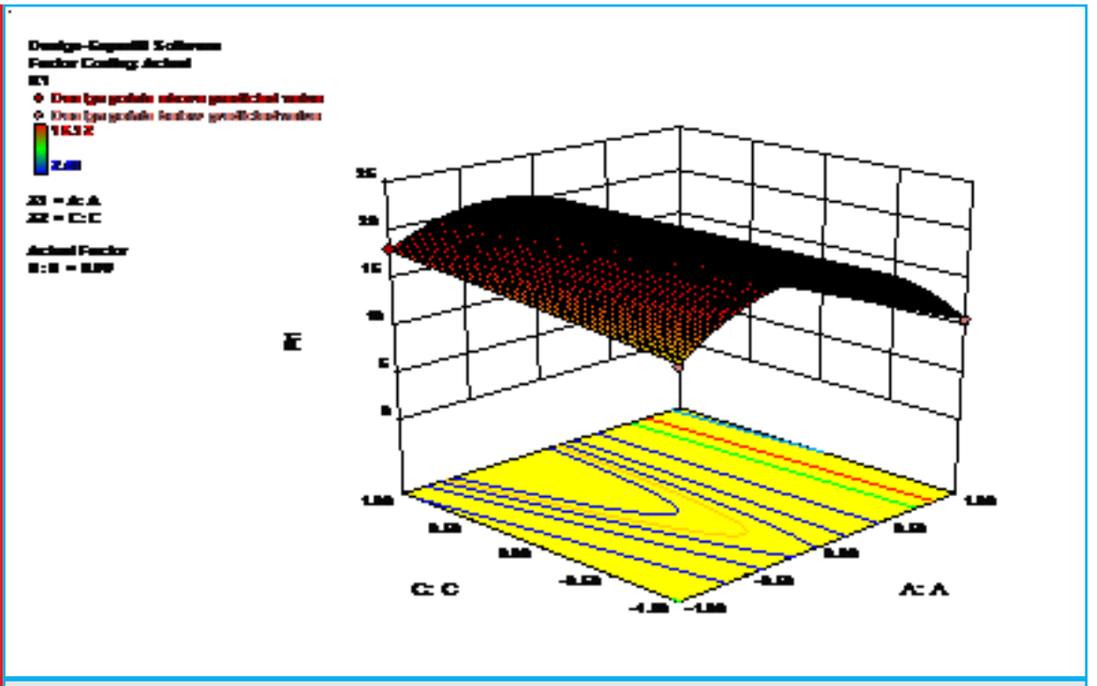

FIGURE 2c. Response surface plot of tannase production by AT 13 showing interaction between KH_2PO_4 and tannic acid

From the table, it was observed that the variation in tannase activity was 0.3–16.53 U/ml/min. From the Pareto chart (Fig. 1), only tannic acid was found to be significant for the production of tannase by AT 13.

Figure 1 show the Pareto chart of effects of variables on tannase production which helps in. identifying important factors that are mainly responsible for enzyme production. From the chart it was evident that the most important contributing factor for tannase production was tannic acid only.

The ANOVA results are given in Table 3. The p-value of ANOVA table serves as a tool for checking the sig-

Table 5. ANOVA for Response Surface Quadratic Model for the production of tannase by AT 13

Analysis of variance table [Partial sum of squares - Type III]

	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Prob > F	
Model	239.38	8	29.92	187.04	0.0006	significant
A-A	85.94	1	85.94	537.18	0.0002	
B-B	0.45	1	0.45	2.79	0.1934	
C-C	0.74	1	0.74	4.61	0.1209	
AB	13.29	1	13.29	83.05	0.0028	
AC	6.13	1	6.13	38.29	0.0085	
BC	31.81	1	31.81	198.84	0.0008	
A2	92.14	1	92.14	575.97	0.0002	
B2	54.50	1	54.50	340.66	0.0003	
C2	0.000	0				
Residual	0.48	3	0.16	187.04		
Cor Total	239.86	11				

nificance of each of the coefficients and is indicative of interaction strength of each independent variable. P-value less than 0.05 indicate that the model terms are significant. In this experiment, the Model F-value of 285.87 implied that the model is significant in enhancing enzyme production In this case A (Tannic acid), G (Dummy), H (Dummy) and J (Dummy) gave significant terms and B, C, D, K and E gave negative significant terms.

Optimization of significant variables by Response surface methodology (RSM)

Tannic acid was found to be significant by PBD was optimized by RSM using Box-Behnken design for tannase production. The response values in terms of tannase activity and the matrix design were represented in Table 4.

The results obtained by Box-Behnken were analyzed by Analysis of variance (ANOVA) with a model F-value of 187.04 indicating that the model was significant (Table 5). Values of Prob > F less than 0.05 indicate that the model terms are significant. In the present model A, AB, BC, AC, A^2, B^2 are significant model terms. The Model F-value of 187.04 implies the model is significant. There is only a 0.06% chance that an F-value this large could occur due to noise. The Values of "Prob > F" less than 0.0500 indicate model terms are significant.This fit of the model was checked with the coefficient of determination R^2, which was calculated to be 0.9680. The determination coefficient (R^2) of the model was 0.9980 indicating that 99.80% of variability in the response could be accounted by the model.

The ANOVA gave the following regression equation:

$$R1 = +19.97 - 3.28^{*} A - 0.24^{*} B + 0.30^{*} C - 1.82^{*} AB - 1.24^{*} AC + 2.82^{*} BC - 6.79^{*} A^2 - 5.22^{*} + 0.000^{*} C^2$$

Where R1 is the tannase activity; A is tannic acid; B is KCl; C is KH_2PO_4 and the interactions AB, BC, AC, A^2, B^2 are significant.

3D Surface Plots

The three-dimensional response surface curves were plotted to study the interaction among different physicochemical factors used and to find out the optimum concentration of each factor for maximum tannase production.

For the construction of 3D plots, effect of two variables was considered while the other variable was held at zero. These plots were helpful in understanding both the individual and the interaction effects of the two factors. For the construction of 3Dplots, effect of two variables was considered while the other variable was held at zero. From the 3D plots, it was clear that the tannase activity significantly increased with increase in the concentration of tannic acid (A) reaching to its maximum at 18.12 U/ml/min, where as the enzyme activity gradually increased with increase KCl (B) concentration and then declined (Figure 2a).Significant interactions were also present between KCl (B) and KH_2PO_4(C)with a low p-value (0.0008) and between KH_2PO_4(C) and tannic acid(A) with a low p-value (0.0085) (Figure 2b and 2c).

Validation of the Model: Validation of the response surface model was confirmed by using optimum conditions obtained by the model. The experimental values were very close to the predicted values and hence it was concluded that the model was successfully validated.

CONCLUSION

The response surface methodology was applicable for the production of tannase from *Streptomyces sp*. AT13. In the present study,optimum conditions for tannase production by *Streptomyces sp*. AT13 was done using Plackett-Burmen design followed by Response surface methodology using Box-Behnken design. From among 6 fermentative variables that were studied, 5 significant variables were picked up by PBD. Among 5 variables from PBD, 3 were further optimized by Box-Behnken design. Analysis of variance (ANOVA) showed the significance of the model and the validity of the model was confirmed by the verification experiments. P-value less than 0.05 indicate that the model terms are significant. The optimum values of parameters obtained through RSM were 1 % tannic acid, 0.5 % KCl and 0.1% KH_2PO_4 to achieve maximum tannase production.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interests: There are no conflicts of interest.

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Deep learning techniques and their applications: A short review

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ABSTRACT

In recent years, there is a revolution in the applications of machine learning which is because of advancement and introduction of deep learning. With the increased layers of learning and a higher level of abstraction, deep learning models have an advantage over conventional machine learning models. There is one more reason for this advantage that there is a direct learning from the data for all aspects of the model. With the increasing size of data and higher demand to find adequate insights from the data, conventional machine learning models see limitations due to the algorithm they work on. The growth in the size of data has triggered the growth of advance, faster and accurate learning algorithms. To remain ahead in the competition, every organization will definitely use such a model which makes the most accurate prediction. In this paper, we will present a review of popularly used deep learning techniques.

KEY WORDS: DEEP LEARNING, MACHINE LEARNING, NEURAL NETWORKS

INTRODUCTION

Deep learning, a family of machine learning algorithms, is inspired by the biological process of neural networks is dominating in many applications and proving its advantage over conventional machine learning algorithms (Goodfellow et al, 2016). It is only because of their capability in producing faster and more accurate results. It attempts to model high-level abstraction in data based on a set of algorithms (Deng et al, 2014). In deep learning techniques, there is a direct learning from the data for all aspects of the model. It starts with lowest level features that present a suitable representation of the data. It then provides higher-level abstractions for each of the specific problem in which it is applied. Deep learning becomes more useful when the amount of training data is increased. The development of deep learning models has increased with the increase in the software and hardware infrastructure (Aghdam et al., 2017, Nisbet et al, 2018).

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Deep learning models use multiple layers which are the composition of multiple linear and non-linear transformations. With the increase in the size of data, or with the developments in the field of big data, conventional machine learning techniques have shown their limitation in analysis with the size of data (Chen, 2014). Deep learning techniques have been giving better results in this task of analysis. This technique has been introduced worldwide as breakthrough technology because has differentiated machine learning techniques working on old and traditional algorithms by exploiting more human brain capabilities.It is useful in modeling the complex relationship among data. Instead of working on task-specific algorithms it is based on learning data representations. This learning can be supervised, unsupervised or semi-supervised, (Hoff, 2018).

In deep learning models, multiple layers composed of non-linear processing units perform the task of feature extraction transformation. Every layer takes the input as the output of its corresponding previous layer. It is applied in classification problems in a supervised manner and in pattern analysis problems in an unsupervised manner. The multiple layers which provide the high-level abstraction, form a hierarchy of concepts. There are deep learning models which are mostly based on artificial neural networks which are organized layer-wise in deep generative models. The concept behind this distributed representation is the generation of observed data through the interaction of layered factors. The high-level abstraction is achieved by these layered factors. A different degree of abstraction is achieved by varying the number of layers and the size of layer (Najafabadi et al, 2015).

The abstraction is achieved through learning from the lower level by exploiting the hierarchical exploratory factors. By converting the data into compact immediate representations of principal components and removing redundancies in representation through derived layered structures, the deep learning methods avoid feature engineering in supervised learning applications. In unsupervised learning where unlabeled data is more abundant than labeled data, deep learning algorithms can be applied to such kind of problems. The deep belief networks are the example of deep learning model which are applied to such unsupervised problems, (Auer et al., 2018).

Deep learning algorithms exploit the abstract representation of data which is because of the fact that more abstract repetitions are based on less abstraction. Due to this fact, these models are invariant to the local changes in the input data. This has the advantage in many pattern recognition problems. This invariance helps the deep learning models feature extraction in the data. This abstraction in representation provides these models the ability to separate the different sources of variations in data. The deep learning models outperform old machine learning models by manually defining the learning features. This is because of the fact that it relies on human domain knowledge rather than relying on available data and the design of models are independent of the system's training.

There are many deep learning models developed by the researchers which give a better learning from the representation oflarge-scaleunlabeled data. Some popular deep learning architectures like Convolutional Neural Networks (CNN), Deep Neural Networks (DNN), Deep Belief Network (DBN) and Recurrent Neural Networks (RNN) are applied as predictive models in the domains of computer vision and predictive analytics in order to find the insights from data. With an increase in the size of data and necessity of producing a fast and accurate result, deep learning models are proving their capabilities in the task of predictive analytics to address the data analysis and learning problems.

Since, there are various deep learning techniques are in existence and each of these has a specific application due to their working model. So, it is necessary to review these models based on their working and applications. In this paper, we now present a review of popular deep learning models focused on artificial neural networks. We will discuss ANNs, CNNs, DNNs, DBNs,and RNNs with their working and application.

ARTIFICIAL NEURAL NETWORK

Artificial Neural Network is a computational model inspired by the biological neural networks. Billions of neurons are connected together in the biological neural network which receives electrochemical signals from it neighboring neurons. They process these signals and either store them or forward to the next neighboring neurons in the network (Yegnarayana, 2018, Garven et al, 2018).

It is represented in figure 1 given below.

Every biological neuron connected to the neighboring neurons and communicate to eachother. The axons in the network carry the input-output signals. Theyreceive the inputs from the environment which create the impulse in form of electrochemical signals which travel quickly in the network.A neuron may store the information or it may forward it to the network. Theytransfer the information to the neighbors through theirdendrites.

Artificial neural networks work similarly to the working of biological neural networks. An ANN is an interconnection of artificial neurons. Every neuron in the layer is connected to all the neurons of previous and next layers. There is a weight given as the labels at each interconnection between neurons.Each neuron receives input which is the output of neurons of the previous layer. They process this input and generate an output which is then forwarded to the neurons of next layer. There is an activation function used by each neuron of

the network which collects the inputs, sums the inputs and generate the output (Hopfield, 1988). There are various types of activation functions which are chosen on the basis of required output.

A simple artificial neural network is composed of three layers. The input layer, the hidden layer and the output layer. The inputs in the form of input vector are applied to the input layer. The number of neural nodes in the input layer depends on the number of attributes in the input. The output of each neuron at input layer is forwarded to every neuron of hidden layer where they are received as inputs. The hidden layer is also referred as the processing layer. Because this is the main layer where the processing is performed on inputs. The number of nodes at hidden layer are decided randomly first and it may be adjusted during training. The outputs of each neural node at hidden layer is then forwarded to output layer where they are received as inputs. The output layer then generates the output which is collected as final output of the network. The number of nodes at output layer depends on the type of output(Abraham, 2005). In classification problems, the number of nodes are same as the number of classes the inputs are to be assigned. In regression problems, there may be only output node to produce an output value.

On the basis of layers, there are two types of feed-forward artificial neural networks. The first type is the single layer feed-forward ANN and the second type is the multilayer feed forward neural network. In a single layer, there is no any hidden layer in the network. It is the simplest kind of neural network. The network is composed of the input layer and the output layer only. The inputs applied to the input layers are directly forwarded to the output layer for generating the outputs.

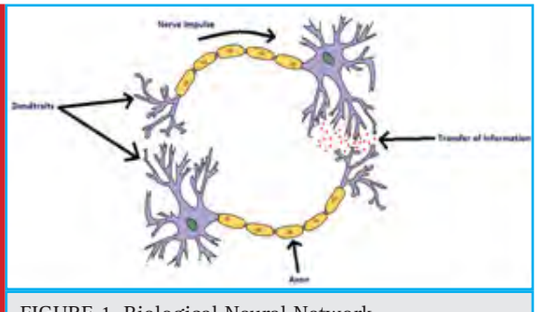

FIGURE 1. Biological Neural Network

FEED-FORWARD ARTIFICIAL NEURAL NETWORK

There are various types of artificial neural networks each has a specific property and can be applied in a different problem domain. Feed-forward structure of artificial neural networks have been used very popularly in many applications. A feed-forward network is one where the signals travel in one direction only that is the forward direction, means from the input layer to the output layer (Bebis et al, 1994). There are feed-backward or feedback neural networks which we will discuss later in this chapter. Every neural network works on some learning algorithm.

There are various types of learning algorithms which are selected depending on the problem to which the network is being used. The training of the networks is done by implementing the learning algorithm. Backpropagation learning algorithm is very popular and applied in many applications to train the neural network. It adjusts the weight of interconnections using error in output at a layer. This error is propagated in the backward direction to the previous layers. That is why it is called back-propagation algorithm (Buscema, 1998). There are many other algorithms for each supervised and unsupervised training of the network. The architecture of a feed-forward neural network is represented in figure 2 given below.

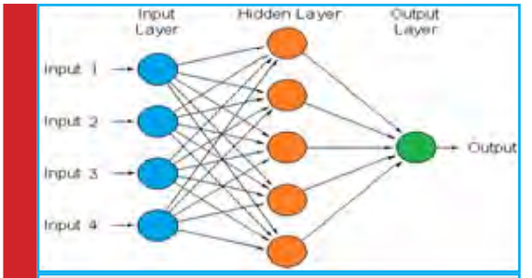

FIGURE 2. Feed-Forward Artificial Neural Network

In above figure, the architecture of a feed-forward neural network is represented. This network is a composition of artificial neurons where every neuron is connected to the neurons of its previous and next layers. During training of the network, the inputs in form of a vector are applied to the vector of neural nodes at input layer. In many learning algorithms, a bias input is also applied to the main input. This bias value is fixed during the training. The first input pattern is applied and it is transferred to the hidden layer. The activation function used at neurons generate the output which is collected at the output layer. In classification problems, the step functions are generally used and in regression problems, the logistic problems are used. The training of the network is performed on the dataset in many epochs. Some algorithms use gradient descent to stop the training process after reaching to certain error.

Let the inputs I_1, I_2... , I_n are applied to the input layer of the network, then the net input received at a single neuron ofhidden layer is:

$$y_{in_1} = I_1 * w_1 + I_2 * w_2 + \ldots + I_n * w_n + b \quad (1)$$

$$\text{or, } y_{in_1} = \sum_{i=1}^{n} I_i * w_i + b \quad (2)$$

Where w is the weight on interconnection and *b* is the bias value, and hence the net input received at the hidden layer, if there are *m* neurons, is represented in the form of a vector as:

$$Y_{in} = [y_{in_2}, \ldots, y_{in_m}] \quad (3)$$

Let the inputs are represented in the form of the vector as $I = (I_1, I_2 \ldots, I_n)$ and *W* is the matrix of weights associated with the interconnections between the input layer and the hidden layer then Y_{in} will be defined as the cross product of the input vector and the weight matrix, i.e.,

$$Y_{in} = I \times W \quad (4)$$

If *f* is the activation function used at this neuron, then the output of the neuron is obtained as:

$$y_{out_1} = f(y_{in_1}) \quad (5)$$

$$\text{or, } y_{out_1} = f\left(\sum_{i=1}^{n} I_i * w_i\right) \quad (6)$$

Similarly, the output generated by all the neurons of the hidden layer can be represented in the form of a vector as:

$$Y_{out} = [y_{out_1}, y_{out_2}, \ldots, y_{out_m}] \quad (7)$$

Now, this output Y_{out} is supplied as input to each neuron of the output layer. Let *V* is the matrix of weights associated with the interconnections between the hidden layer and the output layer then the input received at output layer will be the cross product of Y_{out} and *V*. Let Z_{in} is the net input received at output layer, then it can be represented as:

$$Z_{in} = Y_{out} \times V \quad (8)$$

Let is the output generated by each of the i^{th} neuron at output layer and there are *p* number of nodes are there at this layer, then the net output collected at output layer can be represented as the vector of outputs generated by each neuron. It can be given as:

$$Z_{out} = [Z_{out_1}, Z_{out_2}, \ldots, Z_{out_p}] \quad (9)$$

ACTIVATION FUNCTIONS

Every neuron in the neural network generates the output which is referred as the activation of the neuron. Initially, when the neurons are not generating any output are said to be not activated. When the applied input values reach toa certain threshold, the neurons start firing the output which is called that the neuron is activated. For this activation, a function is used which maps the net input received to the neuron with the output. This function is called the activation function. There are various types of activation functions used at neurons depending on the problem to which the neural network is being applied (Roy, Chakraborty, 2013). The popularly used activation functions are the step function and the sigmoid function. Here we will present a brief description of these functions.

STEP FUNCTION

There are two types of step functions, the binary step function,and the bipolar step function. The binary step function produces 0 as the output if the net input is less than the certain threshold value θ otherwise, it produces 1 as the output.It can be represented mathematically as given in equation 10 and graphically as given in figure 3.

$$f(x) = \begin{cases} 0, & x < \theta \\ 1, & otherwise \end{cases} \quad (10)$$

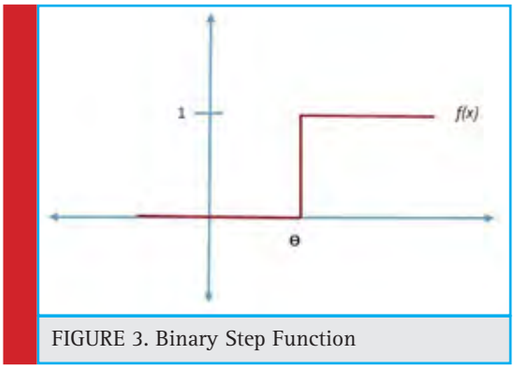

FIGURE 3. Binary Step Function

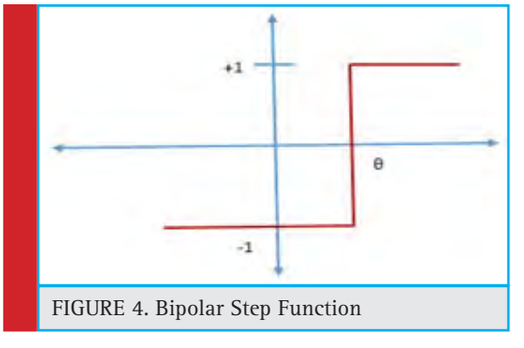

FIGURE 4. Bipolar Step Function

The bipolar step function is used when the neural network is to be applied to bipolar data instead of binary data. This function gives -1 and + 1 as the output in place of 0 and 1 depending on the threshold. This func-

tion can be represented mathematically as given in equation 11 and graphically as given in figure 4.

$$f(x) = \begin{cases} -1, & x < \theta \\ +1, & otherwise \end{cases} \quad (11)$$

SIGMOID FUNCTION

Since the step functions are not continuous, so they are not differentiable. There are some machine learning algorithms which require the continuous and differentiable activation functions and hence the step functions cannot be used in those problems. The sigmoid functions can be approximated with maintaining their property of differentiability. There are two types of sigmoid functions used in this type of problem domain, the binary sigmoid function,and the bipolar sigmoid function. They both have the continuous outputs.

The binary sigmoid function is also called the logistic sigmoid function. It can be represented mathematically as given in equation 12 and graphically as given in figure 5.

$$f(x) = \frac{1}{1+e^{-\alpha x}} \quad (12)$$

where α is called the steepness parameter.

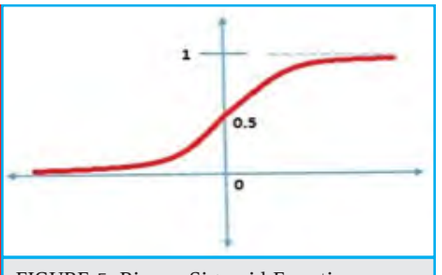

FIGURE 5. Binary Sigmoid Function

The binary sigmoid function has the limitation that it cannot be applied to bipolar data. In this case, the bipolar sigmoid function is used for continuous output. It can be represented mathematically as given in equation 13 and graphically as given in figure 6.

$$f(x) = \frac{1-e^{-\alpha x}}{1+e^{-\alpha x}} \quad (13)$$

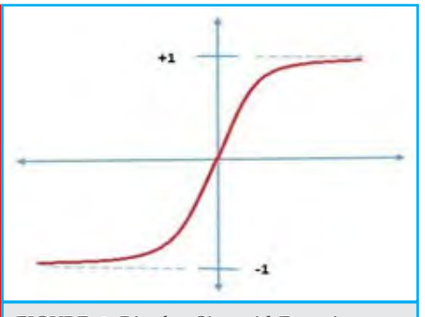

FIGURE 6. Bipolar Sigmoid Function

LEARNING BY ANNS

An artificial neural network has three important characteristics, the architecture, the activation function and the learning. The weights associated with the interconnection between neurons of the network are initialized randomly before training of the network. These weights are adjusted by following the learning algorithm and when finalized, the network is said to be a stable of the fitted network. A network fitted after training can be applied to a problem (Haykin, 1998). During training, the weights of the neural network are updated at each iteration of the training until some stopping condition is satisfied. Let $w(k)$ is the weight at k^{th} iteration of the training then the new weight at $(k+1)^{st}$ is obtained as given in the equation 14.

$$w(k+1) = w(k) + \Delta w(k) \quad (14)$$

where the $\Delta w(k)$ is the change in weight w at k^{th} iteration. Different learning methods give a different method to obtain the $\Delta w(k)$.

There are various learning methods used by neural networks which are categorized mainly into two categories, the supervised learning and the unsupervised learning (Jain et al, 1996).

SUPERVISED LEARNING

The supervised learning methods work with the labeled data. Labeled data means the data where there are input and output labels given in the data. The training data for a neural network is referred as the training pattern. Each training pattern consists of the input patterns and corresponding output patterns in case of supervised learning. The learning algorithms devise a function mapping between the input and output patterns of the data. Once the network is trained by following the learning algorithm, it can generate output for an unknown input pattern (Reed et al, 1999). Here we will present a very brief description of the supervised learning algorithms popularly used in the training of neural networks.

HEBB RULE

It is one of the earliest learning algorithms used by the artificial neural networks. According the Hebb rule or Hebbian learning, the change in weight w_i can be obtained as:

$$\Delta w_i = I_i \times t \quad (15)$$

where I_i is the corresponding input value and the t is the target value. The Hebb rule has the limitation that it cannot learn if the target is 0. This is because the change in weight Δw_i will become 0 when we put t=0. So it is

applied when the input and output both are in bipolar form (Kempter et al, 1999).

PERCEPTRON LEARNING RULE

According to the perceptron learning, if the output of the neuron is not equal to the target output, then only the weights associated with the interconnections should be adjusted otherwise they should not be altered (Ng et al, 1997). According to this rule, the change in weight w_i is obtained as:

$$\Delta w_i = \eta \times (t\text{-}y_{out}) \times I_i \quad (16)$$

where η is a constant and known as the learning rate.

DELTA RULE

The Delta rule is also known as the Least Mean Square (LMS) or Widrow-Hoff rule. It is a widely used learning method used in the training of neural networks. It produces the output in binary form by reducing the mean squared error between the activation and the target value (Auer et al, 2018). According to the Delta rule, the change in weight Δw_i *is* obtained as:

$$\Delta w_i = \eta \times (t\text{-}y_{in}) \times I_i \quad (16)$$

where the symbols used have their usual meaning.

THE BACKPROPAGATION ALGORITHM

The backpropagation is the most popular learning algorithm used for training the artificial neural network in case of supervised learning. In this algorithms, the neural net repeatedly adjusts the interconnection weights on the basis of error and deviation from the target output in response to the training patterns. The error in this method is calculated at the output layer of the network and propagated back through the network layers (Adeli et al, 1994). We will discuss this method in detail in chapter 6 while discussing the training of Hybrid Deep Neural Network.

UNSUPERVISED LEARNING

When the training data available for training the neural network does not has the input-output labels, the learning performed on this data is called the unsupervised learning. In this case, the algorithms learns to derive structure from the data. There are many machine learning problems like clustering and anomaly detection use unsupervised learning (Hastie et al, 2008). In clustering problems, during training of the neural network, the input vectors which are to be applied to the network are combined to form clusters. When the network is trained or stable, on applying a new input vector, the neural network gives the output response indicating the cluster to which the input vector belongs.The popular unsupervised learning algorithm used for clustering using neural network is Winner-Takes-All method. It is a competitive learning rule which chooses the neuron with the greatest total input as a winner (Kaski et al, 1994).

DEEP LEARNING MODELS

There are various deep learning models developed by the researchers and they are applied in a different problem domain. In all the models, the common characteristic is the multiple layers of learning. Here in this section, we will present a short survey of popularly used deep learning models.

DEEP NEURAL NETWORK

Deep neural network is a variant of multilayer feed-forward artificial neural network. It has more than one hidden layers between the input layer and the output layer (Bengio, 2009). The number of neurons are similar in each of the hidden layer. Initially, the number of neurons are fixed randomly and it is adjusted manually during training of the network. Larger the number of nodes at hidden layer may result in an increase in the complexity and hence the decrease in the training performance. So, the selection of number of nodes at this layer is carefully considered. This architecture devises a compositional model in which the object is referred as the layered composition of primitives. It has the capability to model complex non-linear relationships in the training data. The benefit of using extra hidden layers in the network enables the composition of features from lower layers. These features potentially model complex data with fewer units (Ngiam et al, 2011).

There are two issues also associated with the deep neural networks. First, the issue of overfitting which is common in many neural network models and second, the issue of computation time. The problem of overfitting has more chances to arise in deep neural network due to the use of extra layers. Due to this issue, it models the rare dependencies in the training data. The network gives better result on training data and degraded in accuracy on validation data. To avoid the issue of overfitting in deep neural networks, regularization methods like weight decay or sparsity can be used during training which excludes the modeling of rare dependencies. With the increase in smaller training sets can also overcome the problem of overfitting. The computation time of the learning model depends on many parameters like such as the layer size, the learning rate, and the initially chosen weights (Szegedy et al 2013). The number of nodes in the hidden layers increase the complexity of the system and it requires more computational time. It

should be carefully considered while selecting all these parameters.

A typical architecture of the deep neural network is represented in figure 7.

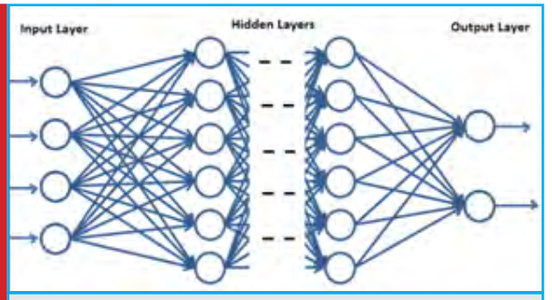

FIGURE 7. Deep Neural Network

All the processing in the deep neural network is very much similar to the multilayer feed-forward artificial neural networks. For training of the network, the back-propagation learning method is used widely to find the matching between the actual output and the desired output. The change in weight in this process is calculated as:

$$w_{ij}(t+1) = w_{ij}(t) + \eta \frac{\partial C}{\partial w_{ij}} + \xi(t) \qquad (18)$$

where η is the learning rate, C is the cost function and ξ is the stochastic term. w_{ij} is the weight associated with the interconnection between i^{th} node of one layer and j^{th} node of next layer.

The deep neural networks have a wide range of applications. They are applied in automatic speech recognition, image recognition, visual art processing, and natural language processing, drug discovery, customer relationship management, mobile advertising and many more fields.

CONVOLUTIONAL NEURAL NETWORK

The convolutional neural network is a variant of a multilayer perceptron. They are inspired by the biological process of visualization. This model is a composition of neurons, learnable weights, and bias values. It consists of an input layer, an output layer and multiple hidden layers between the input layer and the output layer.The hidden layers of the network are the composition of the convolution layers, pooling layers, fully connected layers and the normalization layers. They are designed in such a manner that they require a minimal amount of preprocessing (Aghdam, 2017, Krizhevsky et al 2012). The architecture of a convolutional neural network is represented in figure 8 given below.

The architecture of convolutional neural network comprises neurons in three dimensions, the depth, the

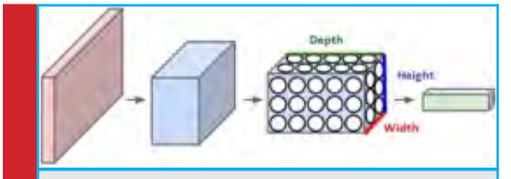

FIGURE 8. Convolutional Neural Network

height and the width which are presented in one of the layers. The 3D input volume is transformed to 3D output volume of neuron activations by every layer of the network. A typical architecture of convolutional neural networks consist of the following:-

(i). Convolutional Layer: The convolutional layer of the network is termed as the core building block which comprises a set of learnable filters. It is said that these filters are convolved around the layer. It applies a convolution operation to the input which is to be passed to the next layer as a result of this operation. The network learns from the filters as they are activated after detecting certain specific type of features at certain spatial input position.

(ii). Pooling Layer: Pooling layer helps the convolutional neural network in avoiding the issue of overfitting which is a common issue in artificial neural networks. Pooling, which is a form of non-linear down-sampling, combines the outputs of neurons of one layer into a single neuron of next layer. The max-pooling partitions the input data into a set of non-overlapping slices and produces the maximum output for each set.

(iii). Local Connectivity: In convolutional neural networks, neurons of one layer are connected only to the neighboring neurons of adjacent layers. When dealing with the input of high volume, this features avoids the problem of connectivity and hence reduces the complexity of the network.

(iv). Parameter Sharing: There is a feature of parameter sharing in convolutional neural networks which helps in controlling the free parameters. Weight vectors and bias values are shared among the neurons of the network which helps in less parameter optimization and faster convergence during training.

In the field of natural language processing, convolutional neural networks are applied to text analytics and sentence classification problems (Kalchbrenner et al, 2014). Itis also used in the time-series analysis which is helpful in predicting stock prices, heights of ocean tides and weather (LeCun et al, 1998). The architecture of convolutional neural network has been used in predicting the DNA sequence binding (Zeng et al, 2016). These architectures are also used in drug discovery by

predicting the interactions between biological proteins and molecules (Strigl et al, 2010).

However convolutional neural networks have been applied very usefully in many fields and they have given better results, some limitations are also associated with this model. It requires a large data set and hence needs a long training time. There is the issue of performance and scalability also associated as this architecture is GPU based (Hinton, 2009).

DEEP BELIEF NETWORKS

A deep belief network is a variant of the deep neural network. It is a graphical model which is composed of multiple layers of hidden units. The hidden units are called the latent variables. There is an interconnection between layers of the network but there is no connectivity among units of the network. This graphical model learns to extract the deep hierarchical representation of the training data (Hinton et al, 2006). The graphical model has both directed and undirected edges. The training of the network is performed in two successive steps, the unsupervised training and then thesupervised training. During the unsupervised training, the network learns to probabilistically reconstruct its inputs when trained on a set of example. As a result of this training step, the layers act as feature detectors. After this step, the supervised training is performed on the network to perform the task of classification (Salakhutdinov et al, 2007).

The deep belief network can be described by separating its architecture in two parts, the belief network,and the Restricted Boltzmann Machine. The belief network is a directed acyclic graphical model comprises the stochastic variables. These variables have states either 0 or 1 where the probability of becoming 1 is obtained by a bias and weighted inputs from other units. The belief network solves two types of problems, the inference problem,and the learning problem. The inference problem infers the state of the unobserved variables and the learning problem adjusts the interconnection between learning variables. This helps the network in generating the observed data. The Restricted Boltzmann Machines are the generative models of the artificial neural network which learns from the probability distribution of a set of inputs (Larochelle et al, 2008).

A typical architecture of deep belief network is represented in figure 9.

The deep belief network is composed of Restricted Boltzmann Machine (RBM) and a feedforward multilayer perceptron. The RBM is used at pre-training phase and the multilayer perceptron is used at the fine tune phase. The hidden units of the network are the neurons which cannot be observed directly but they can be inferred

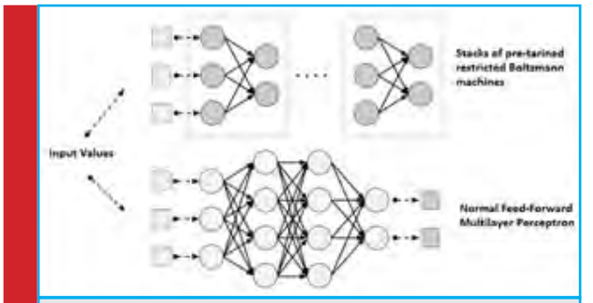

FIGURE 9. Deep Belief Network

from the other observable variables. In deep belief networks, the distribution between observed input vector X and n^{th} hidden layer h_n is modeled as:

$$P(X, h_1, h_2 \ldots h_n) = \left(\prod_{i=1}^{n-2} P\left(h_i | h_{i+1}\right)\right) P\left(h_{n-1}, h_n\right) \quad (19)$$

where, X= h_0, P (h_{i-1}, h_i) is a conditional distribution of visible units and P (h_{n-1}, h_n) is the joint distribution for visible units. During the first step of the training, the network learns a layer of features from the visible units. Then, in the next step, it treats the activation of previously trained feature as visible unit and learns features in a second hidden layer. After following successive steps in such manner, the whole network is said to be trained when the learning for the final hidden layer is achieved.

Deep Belief Networks have been used in financial business predictions in order to empower the financial industries. These networks have also been used in time series prediction which then leads to financial market, signal processing,and weather information prediction. Draught has also been predicted by this model. It is also used in predicting the quality of sound vehicle interior noise (Medsker et al, 2001).

However, Deep Belief Networks have a wide range of application, some limitations are also associated with this model. Since deep belief networks are formed with Boltzmann Machines, they have the limitation that when the size of the machine is increased, the training time of the model exponentially increased.

RECURRENT NEURAL NETWORKS

Recurrent neural network belongs to the class of artificial neural network. In these networks, there is a directed cyclic connection between its internal nodes along a sequence. It exhibits the dynamic temporal behavior of a time sequence. These networks use internal memory states to process in the input sequences (Li et al 2015). In conventional artificial neural networks, input values in an input vector are independent of eachother and hence processed independently. But there are many tasks

where the output is dependent on previous calculation in a sequential process. The recurrent neural networks are applied to such type of tasks where there is sequential process on inputs. This network is called recurrent because it performs the same task for every element in the sequence. The memory used by the network stores the information about previous calculations. Practically, these networks recall calculations of few previous steps only (Schuster et al, 1997).

The working of the recurrent neural network can be explained by the architecture represented in figure 10 given below.

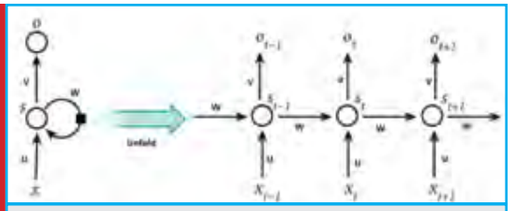

FIGURE 10. Recurrent Neural Network

The above representation shows a recurrent neural network being unfolded into a full network to process a sequence of inputs. Here, one layer works for each input value in the sequence. If folded or combined all the layers together as a single hidden layer, the weights and bias remain same because of only one hidden layer is used in the network. Let x_t is an input to the network at timestamp t and $s_t$is the hidden state or memory at timestamp t. This s_t is calculated on the basis of hidden state at previous timestamp and the input at current timestamp as

$$s_t = f(U.x_t + W.\ s_{t-1}) \quad (20)$$

where f is a nonlinear function which may be *tanh* or *ReLU*. s_{t-1} is initialized to zero at first timestamp. o_t is the output at t timestamp which is calculated as

$$o_t = f(V.\ s_t) \quad (21)$$

in the above equation 4.21, f is a logistic function which may be *softmax* or a normalized exponential function.

Unlike the other deep neural networks where different parameters like weights and bias are used at different hidden layers, in the recurrent neural network these parameters are shared across all the timestamps. This helps in reducing the number of parameters during learning. In some applications, there is input required at each timestamp and there is an output produced at each timestamp. But it is not necessary in every application of recurrent neural network. This because of the use of hidden state which captures information about some sequences.

There are multiple extensions of the recurrent neural network. They are discussed briefly as given below.

- **Bidirectional Recurrent Neural Networks:** This network is based on the concept that the output at t timestamp is not only dependent on the previous elements in the sequence but it also depends on the future elements. It architecture is such as two recurrent neural networks are stacked on top of eachother. Its output is calculated based on the hidden state of both networks (Irsoy et al, 2014).
- **Deep Bidirectional Recurrent Neural Networks:** These networks are similar to the bidirectional recurrent neural networks with an addition that they have multiple layers per timestamp. It gives the benefit of higher learning capacity but it needs a large size of training data (Hochreiter et al, 1997).
- **Long Short-Term Memory (LSTM) Networks:** This variant of the recurrent neural network is applied to avoid the vanishing gradient problem in backpropagation learning. In these networks, the memory units are called as cells which are very efficient to capture long-term dependencies. It takes the previous state s_{t-1} and current input $x_t$as input to the cells and these cells decide internally that which information will be stored and which information will be erased (Saad et al, 1998).

Recurrent Neural Networks have a large number of applications in predictive analytics. It has been widely used in stock market predictions for a long period of time (Connor et al 1994). Its application in time series prediction has given the generalization of performance than other models (Hu et al, 2007). These networks, after combining dynamic weights, have been used to predict the reliability of the software (Barbouniset al 2006). With the addition of spatial correlation features, the recurrent neural network is used to predict the speed of wind (Levin, 1990).

Apart from the above important applications, RNNs have some limitations. There is a slow training time of these networks. In RNNs, number of hidden neurons must be fixed before training. While processing a vocabulary, size of context must be small (Sundermeyer et al, 2013).

CONCLUSION AND FUTURE SCOPE

In this paper, we have discussed the various techniques used in deep learning applications. All these models have an outstanding record in the area of machine learning. There is a scope to create new features in these models so that they can be applied in many domains with better performance. The new techniques may be integrated to exploit the opportunities of the model in prediction. Parameter tuning can also help to improve the perfor-

mance of these models. Parameter tuning can also help to improve the performance of these models. So it can be said that there is a very wide opportunity and open scope for this model.

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Extraction of bacitracin from *Bacillus subtilis* BSG and its optimization using response surface methodology

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ABSTRACT

Bacillus sp. was isolated from the soil sample of Gunupur, Odisha, India and screened to produce bacitracin at the laboratory. The bacitracin from the bacterium was extracted using butanol-ether solution as a solvent and its antibacterial properties against five bacteria namely *Staphylococcus aureus* (MTCC 98), *Escherichia coli* (MTCC 739), *Micrococcus luteus* (MTCC 106), *Salmonella typhimurium* (MTCC 1254), and *Pseudomonas aeruginosa* (MTCC 2453) have been studied in vitro by agar well diffusion method. Simultaneously, the individual, square and combined effect of incubation time, pH and temperature on the bacitracin production was studied using Response Surface Methodology (RSP). Analysis of variance (ANOVA) has shown a coefficient of correlation value of 0.9611) and a quadratic correlation for bacitracin production was derived with 95% confidence level. Incubation time of 26.5 h; pH of 5.67; and temperature of 30.65°C have been found to be as optimum operating conditions for a maximum bacitracin production from *Bacillus sp*. BSG of 8.72µg/100 ml.

KEY WORDS: SOIL MICROORGANISM; BACITRACIN; ANTIMICROBIAL ACTIVITY; ANOVA; RESPONSE SURFACE METHODOLOGY

INTRODUCTION

The use of antibiotics in clinical biology is a common phenomenon and their uses are rapidly increasing day by day. Most of the antibiotics have been obtained from microorganisms. Antibiotics which have been used today are either natural or semi synthetic or synthetic by nature (Trookman *et al*., 2011). Semi synthetic antibiotics are prepared by modifying the natural antibiotic, either chemically or enzymatically with an aim to enhance the efficiency of original antibiotics. Antibiotics used in medical practices have been obtained from few groups of microorganisms only. The two genera of bacteria *Streptomyces* and *Bacillus* are the maxi-

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mum contributor of antibiotics (Pavithra *et al*., 2009). The antibiotics produced by *Bacillus* species are being recommended for infection caused by gram positive bacteria. *Bacillus* species produces about 167 antibiotics which are polypeptide by nature (Arias *et al*., 1999). The two species of *Bacillus* such as *B. subtilis* and *B. brevis* produce 66 and23 polypeptide respectively. The most important peptide antibiotics produced by *Bacillus* species is Bacitracin. It is commonly used in medical preparations, either alone or with other antibiotics, against all kinds of gram positive bacteria and to some extent gram negative bacteria. The antibiotic interferes with protein synthesis and disrupts the cell of parasitic bacteria. The bacitracin production by *Bacillus licheniformis* can be enhanced by acetoin reductase activity and the transcription factors *Spo0A* and *AbrB* regulate the bacitracin synthesis, (Kasetty *et al*., 2015 Lagzian *et al*., 2018).

Many investigations have been conducted on bacitracin, but the optimization ofits production remains unexplored. The present work aims at the isolation of bacitracin producing the soil inhabitant *Bacillus* bacterium obtained from the local soil sample with the purpose of establishing its antibacterial activity against five bacteria such as *Staphylococcus aureus* (MTCC 98), *Escherichia coli* (MTCC 739), *Micrococcus luteus* (MTCC 106), *Salmonella typhimurium* (MTCC 1254), and *Pseudomonas aeruginosa* (MTCC 2453). Simultaneously, central composite design was used to investigate the effect of incubation time, pH and temperature on the production of bacitracin. The production parameters are optimized in order to maximize the production of bacitracin.

MATERIALS ANDMETHODS

EXPERIMENTAL PROCEDURE

Isolation of Bacillus species

Soil samples were collected by sterilized plastic bags from different parts of Gunupur area. The soil inhabitant microorganism was isolated by following serial dilution (10^{-4} folds) using nutrient agar plate (Coppuccino *et al*., 1996) and CFU of the soil microbes' were recorded.

Sub culture and characterization of Bacterium

The isolated bacterium was sub cultured in the laboratory using the same nutrient by following agar slant technique, and preserved at the low temperature (4°C) for further use. The characterization of the bacterium was made at the laboratory by morphological observation and biochemical tests. The suspected *Bacillus* species were identified by molecular techniques. The growth curve of the bacterium was determined by spectrophotometric techniques (Goodfellow *et al*., 1980).

Extraction, purification and identification of Bacitracin

The batch culture technique was used for the extraction of bacitracin from isolated bacterium. The bacterium was inoculated in the nutrient broth at 37° C at slow agitation condition for 72 hrs and at log phase of growth the extraction was obtained. The purification of the bacitracin in the culture medium was done using butanol-ether solution (pH=4) as a solvent (Murphy *et al*., 2007). At acidic condition, the bacitracin remained in lower aqueous layer was collected carefully followed by neutralizing with sodium bicarbonate and was lyophilized to obtain the purified bacitracin. Thin Layer Chromatography (TLC) and GC-MS had been used to identify the obtained bacitracin . The silica phase acts as the solid phase of the TLC and the mobile phase was prepared with chloroform and methanol in the ratio of 9:1. The Rf value is considered for identification of bacitracin (Phillips, 1999). Confirmation of bacitracin was made by GC-MS (MSGC- 11) instrument with the capillary column of HP-3 (50 mm × 0.521mm, film thickness 0.25µm). 1µL of extract was carefully injected into GC-MS for analysis. The chemical compositions were identified by comparing their retention indices (RI) and mass fragmentation pattern.

Standardization of Production of Bacitracin

The production of bacitracin was carried out with solid state fermentation. The fermentation was made in an Erlenmeyer flask (capacity 1 liter) by using wheat bran as substrate. The substrate (100 gm) was added to 100 ml of phosphate buffer. Then the flask along with *the* substrate had been sterilized in autoclave at 15 psi for 20 minutes. Isolated microorganisms were inoculated carefully into the flask and incubated at 37° C for 24 hrs. The antibiotic was extracted by following standard procedure with little modifications (Rajan *et al*., 2014), followed by filtration of aliquot and then centrifuging at 10,000 rpm at 4°C for 20 minutes.

Study of Antibacterial activity

Agar well diffusion method was used to investigate the antimicrobial properties of the bacitracin obtained from solid state fermentation. The Muller-Hinton agar (MHA) plates were prepared by using 20ml of the medium and left overnight at room temperature to check for any contamination to the plates. The test bacteria was grown in a nutrient broth and diluted (OD 620 nm = 0.1) to obtain a bacterial suspension of 1×10^{8} CFU/ml before applying onto the agar plate. Agar wells of 5mm diameter were prepared using sterilized *steel* gel puncher and each well received 10µL of the extracts. The agar plates were incubated in a BOD chamber at 37oC for 48 h. The antibacterial activity of the isolated compound against each

test organisms was quantified by measuring the zone of inhibition. The average diameter of three replicates for each organism was determined and expressed in millimeter (mm).

STATISTICAL ANALYSIS

The CCD comprises of a full fractional factorial design with center points amplified by a group of 'star points'. Statistical methodologies such as RSM based on CCD can also be used to maximize the production of a substance by optimization of operational factors. In compare to conventional methods, apart from the individual contribution of process variables, the contributions of interaction among process variables can be investigated using RSM (Dora *et al.*, 2013). Thus, Response Surface Methodology (RSM) based on Central Composite Design (CCD) was used to explore the effect of the different production parameters viz. temperature, pH and time on the production of bacitracin.

RESULTS ANDDISCUSSION

CHARACTERIZATION OF BACILLUS SUBTILIS

The isolated soil microorganisms obtained from different soil samples of the Gunupur area was characterized morphologically and biochemically (Table 1). The cultured bacteria showed irregular colonies in culture media and shape of the bacteria was rod shaped. The gram staining test had shown gram positive. Similarly, glucose fermentation test and VP tests of the isolated bacterium were positive whereas catalase and lactose fermentation test found to be negative. The isolated microorganism was identified by following Bergay's manual of systematic bacteriology (Sharma *et al.*, 2010) and found as *Bacillus subtilis* as shown in fig 1. The batch culture of the isolated bacterium retained lag phase up to 4 hrs fol-

Table 1. Morphological and biochemical characterstics of microorganism isolated from soil sample of Gunupur area, Odisha

Characters	Observation
Shape	Irregular
Margin	Lobate
Color of the Colony	White
Structure	Filamentous
Size	Rod Shaped
Gram Strian	+ve
Carbohydrate fermentation	-ve
Mannitol Fermentation	-ve
Glucose Fermentation	+ve
Catalase fermentation	-ve
Nitrate reduction	-ve
Methyl	-ve
Indole	-ve
Litmus	-ve
Oxidose	-ve
Urase	-ve
Lactose Fermentation	-ve
Voges proskauer	+ve

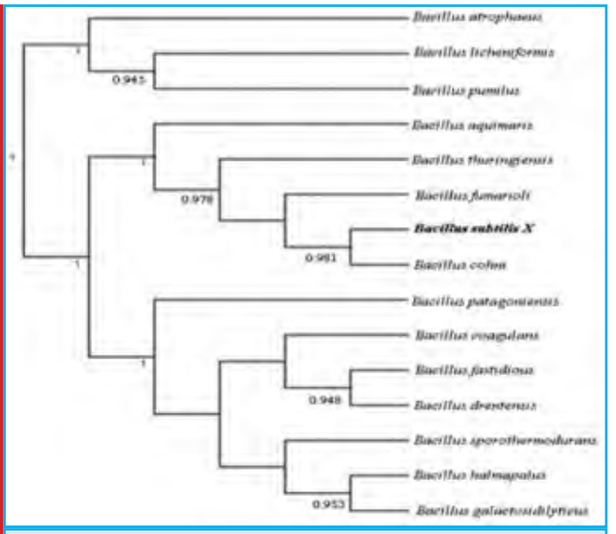

FIGURE 1. Molecular characterization and phylogenic analysis of the isolated *Bacillus subtilis*

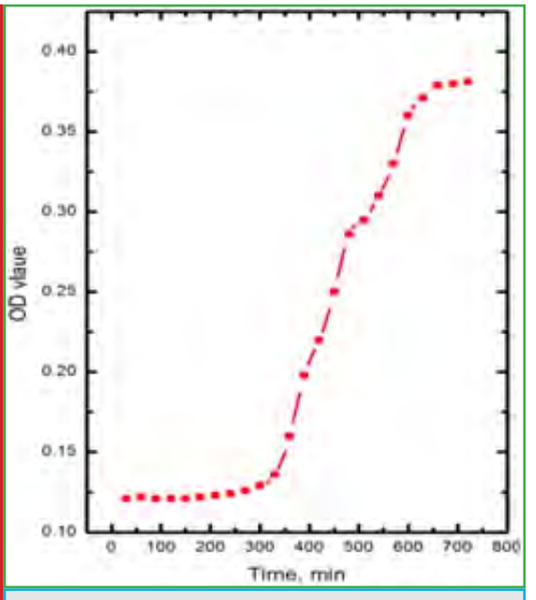

FIGURE 2. Growth curve of the isolated *Bacillus subtilis* in batch culture with nutrient medium

lowed by log phase (Fig. 2). The morphological features of isolated microorganisms presenting the gram + ve, rod shaped, and having endospore confirms the presence of *Bacillus subtilis* (Das *et al.*, 2012).

Effect of carbon and nitrogen sources on the production of bacitracin

The effect of carbon sources on production of bacitracin is represented in table 2. The extreme production of bacitracin was obtained when maltose is used as the carbon source. Further, minimum production was obtained when fructose is used as the carbon source. To standardize the effect of nitrogen sources on bacitracin production, it was observed that methionine found to be the best nitrogen source among the studied nitrogen compounds (table 3). Earlier report shows that methionine can be used as suitable source of nitrogen for growth

Table 2. Effect of different carbon sources for production of bacitraction by the isolated microorganism

Carbon Sources	Production of bacitracin (µ/100ml of culture)
Glucose	6.0 ± 1.2
Fructose	3.0 ± 1.6
Sucrose	6.5 ± 1.4
Mannitol	8.6 ± 1.2
Sorbitol	4.8 ± 1.8
Starch	4.5 ± 2.0

Table 3. Effect of different nitrogen sources for production of bacitracin by the isolated microorganism

Nitrogen Sources	Production of bacitracin (µ/100ml of culture)
Asparagine	7.0 ± 1.5
Tyrosine	7.5 ± 1.9
Methionine	8.0 ± 1.2
NH4Cl	3.0 ± 2.0
NaNO3	4.0 ± 1.8
Urea	4.5 ± 1.6

of *Bacillus* species (Kasetty *et al.*, 2015). In the study, least production was observed when NH_4Cl is used as the nitrogen source.

Individual effect of incubation time, pH and temperature on bacitracin production

The production of bacitracin by the isolated bacterium was high at log phase of growth. The initiation of production of bacitracin was observed after 8 hours of inoculation and maximum amount, after 30 hours of inoculation. The activity continued up to 60 hours of inoculation and then found to decline as depicted in fig. 3. The effect of pH on bacitracin production is shown in fig. 4. The maximum production of bacitracin was obtained in acidic condition (pH 5.5). The environmental conditions play an important role for the growth and production of metabolites in Bacteria (Kang *et al.*, 2017). There was no production of antibiotic at 20° C by the isolated microorganism. The maximum production of bacitracin was obtained at 35°C. Beyond 350 C, the

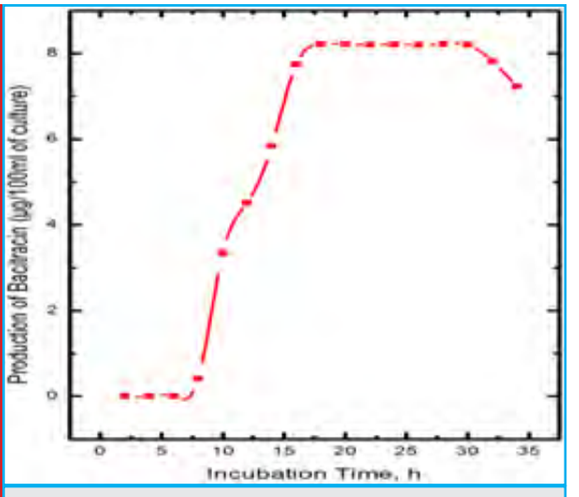

FIGURE 3. Production of bacitracin by isolated Bacillus subtilis in batch culture with respect to incubation time

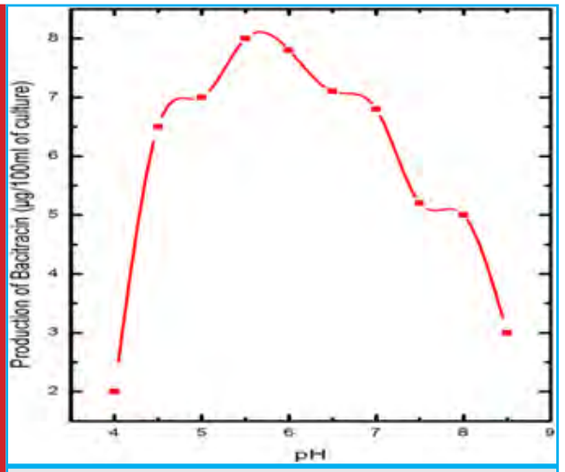

FIGURE 4. Effect of pH on production of bacitracin by isolated *Bacillus subtilis*

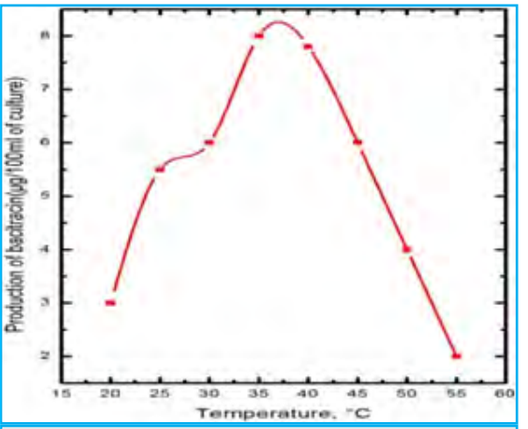

FIGURE 5. Effect of temperature on production of bacitracin by isolated Bacillus subtilis

production of antibiotic decreases as observed in fig. 5. Because *Bacillus* subtilis is a mesophillic bacterium, the growth shall be optimum at 250C - 350C. Therefore, the production of bacitracin is optimum at this temperature (Deslouches, *et al*., 2015).

The chromatogram of GC-MS of extract is represented as fig. 6. It is observed that, there are two major peaks having the retention time of 10.478 and 14.617 and the area occupied are 69.27% and 2.51% respectively. The molecular weight of the bacitracin was measured as 1.6 k Da. The lyophilized antibiotic is faint yellow in color, amorphous and water soluble. The zone of inhibition of isolated bacitracin against five pathogens namely *Staphylococcus aureus*, *Escherichia coli, Micrococcus luteus, Salmonella typhimurium* and *Pseudomonas aeruginosa* is represented in the table 4. The maximum zone of inhibition was observed against *Staphylococcus aureus* (23 mm) and the minimum zone of inhibition was observed against *Salmonella typhimurium* (12 mm).

Table 4. Zone of Inhibition (in mm) against five pathogens by the bacitracin extracted from the isolated microorganism.

Test Organism	Zone of Inhibition (in mm)	
	After 24 hours	After 48 hours
Staphylococcus aureus	16.6 ± 1.4	23.0 ± 1.2
Escherichia coli	12.4 ± 1.3	15.6 ± 1.6
Micrococcus luteus	14.5 ± 1.2	18.2 ± 1.4
Salmonella typhimureum	8.3 ± 1.6	12.0 ± 1.4
Pseudomonas aureginosa	11.4 ± 1.7	17.6 ± 1.5

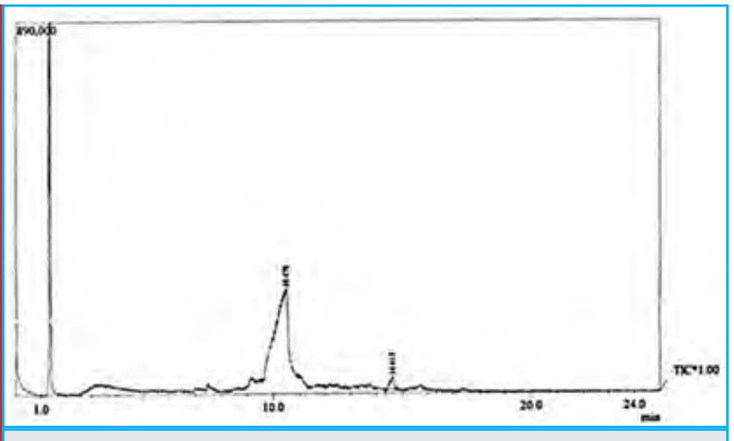

FIGURE 6. Chromatogram of GC-MS analysis of Bacitracin by the isolated Bacillus subtilis

Table 5. Level of independent variables.

Variables	Symbol	-α	-1	0	+1	+α
Incubation Time, h	A	2	8	18	28	34
pH	B	4	5	6.5	7.5	8.5
Temperature, °C	C	20	28	37.5	48	55

Table 6. Experimental design matrix and response

Run	Coded variables			Actual variables			Response
	A	B	C	A	B	C	(Y)
1	-1	-1	-1	8	5	28	1.2
2	+1	-1	-1	28	5	28	7.5
3	-1	+1	-1	8	7.5	28	2
4	+1	+1	-1	28	7.5	28	6.5
5	-1	-1	+1	8	5	48	2.5
6	+1	-1	+1	28	5	48	6.2
7	-1	+1	+1	8	7.5	48	3.3
8	+1	+1	+1	28	7.5	48	4.5
9	-α	0	0	2	6.5	37.5	1.1
10	+α	0	0	34	6.5	37.5	7.8
11	0	-α	0	18	4	37.5	3.7
12	0	+α	0	18	8.5	37.5	5.1
13	0	0	-α	18	6.5	20	5.8
14	0	0	+α	18	6.5	55	4.5
15	0	0	0	18	6.5	37.5	8.22
16	0	0	0	18	6.5	37.5	8.36
17	0	0	0	18	6.5	37.5	8.3
18	0	0	0	18	6.5	37.5	8.42
19	0	0	0	18	6.5	37.5	8.33
20	0	0	0	18	6.5	37.5	8.34

Statistical analysis

From the analysis of experimental data, it was found that the production of bacitracin is influenced by three process parameters viz. incubation time, pH and temperature. Thus the RSM based on CCD is employed in order to estimate the combined effect of the operating parameters on the bacitracin production. The range of the process parameters and the complete design of the matrix along with the experimental data are presented as tables 5 and 6 respectively. Table 6 is planned to obtain a quadratic correlation for the production of bacitracin consisting 2^3 numbers of axial runs, 6 numbers of star configurations and 5 numbers of center points. The runs 15-19 around the center points in table 6 are repeated in order to determine experimental errors. The correlation obtained for the production of bacitracin in terms of coded factor is presented as eq. 1.

$$Y=8.33+1.97A+0.092B-0.211C-0.53AB-0.73AC-0.087BC -1.42A^2-1.43B^2-1.172C^2 \quad (1)$$

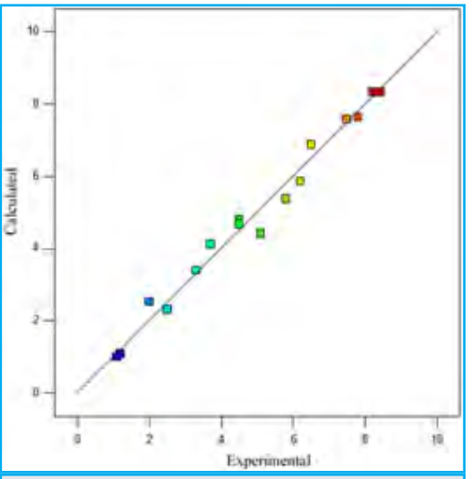

FIGURE 7. Comparison of calculated values of bacitracin production from eq. 1 with the experimental ones

Table 7. ANOVA for bacitracin production

Source	Sum of Squares	Degree of freedom	Mean Square	F Value	p-value Prob > F	Remarks
Model	118.5209	9	13.16898	75.4718	2.13265E-07	significant
A	53.25338	1	53.25338	305.1967	2.98275E-08	significant
B	0.115238	1	0.11523	0.6604	0.037374471	significant
C	0.610016	1	0.61001	3.4960	0.034333927	significant
AB	2.31125	1	2.31125	13.2458	0.005405229	significant
AC	4.35125	1	4.35125	24.9371	0.000745341	significant
BC	0.06125	1	0.06125	0.3510	0.568118214	
A^2	27.5197	1	27.51969	157.7162	5.22146E-07	significant
B^2	28.20921	1	28.20920	161.6678	4.69653E-07	significant
C^2	18.76233	1	18.76232	107.5274	2.64328E-06	significant
Residual	1.570399	9	0.17448			
Lack of fit	1.548479	5	0.30969	56.5138	0.000840525	
Pure Error	0.02192	4	0.00548			
Cor Total	120.0913	18				

The positive and negative signs against each factor in eq. 1 show the synergistic and antagonistic effect of those respective terms on bacitracin production. The calculated values of bacitracin production from eq. (1) are compared with the experimental ones and represented as fig. 7. The values of coefficient of correlation (R^2), adjusted R^2 (R_{adj}^2), predicted R^2 (R_{pred}^2) are found to be 0.986, 0.973 and 0.901 respectively, which shows that the calculated values are analogous with the experimental ones. The adequate precision, which measures the signal to noise ratio, is found to be 24.21, indicates that the correlation developed can be used to navigate the design space. The Analysis of variance (ANOVA) for the production of bacitracin is represented as table 7. The F-value" of 75.47 indicates that the model is significant and there is only 0.01% chance of failure of this correlation. The significance of the model terms can be estimated through the "P-value". The terms A, B, C, AB, AC, A^2, B^2 and C^2 are significant, because the "P-value" for these terms are less than 0.05.

Combined effect of process parameters on the bacitracin production

Fig. 8 shows the combined effect of pH and incubation time on the bacitracin production at a temperature of 37.5°C. Bacitracin production increases with the increase in pH up to 6.5, beyond which it decreases, which may be due to the effect on the rate of nutrient transport and consumption from the medium to bacterial body. Again, the bacitracin production increases exponentially with the increase in incubation time up to 22.75 h, then start decreasing. This may be due to decrease in essential nutrients in the medium. A maximum bacitracin production of 8.3 µg/100ml is found as depicted in fig.7. Similarly, the combined effect of temperature and incubation time on the bacitracin production at pH of 6.25 is presented as fig. 9. It was observed that the bacitracin production increases up to a temperature of 38°C and after that the production decreases. The increase may be due to proper functions of enzymes involved in the bacitracin synthesis pathway of microorganisms. Beyond 38°C what happens so that it decreases, it may be due to the inhibitory effect of enzymes involved in bacitracin synthesis pathway also write down. Fig. 10 shows the combined effect of temperature and pH on the bacitracin

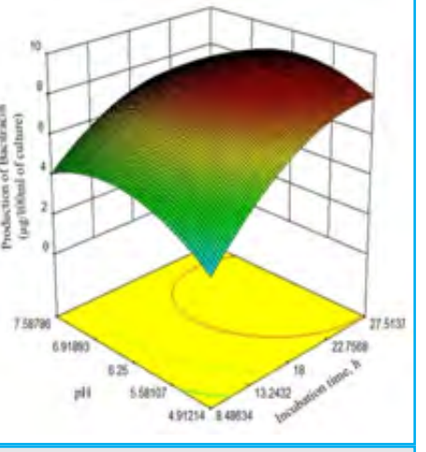

FIGURE 8. Combined effect of pH and incubation time on the production of bacitracin at a temperature of 37.5°C

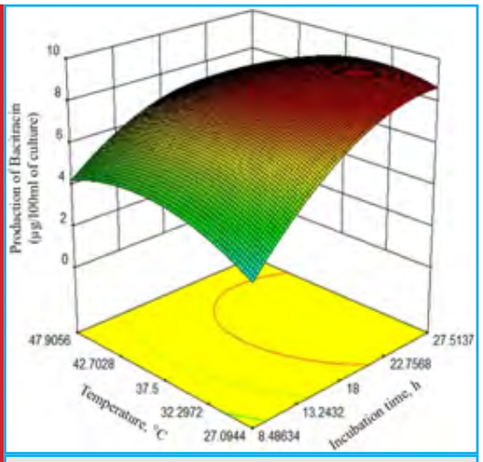

FIGURE 9. Combined effect of temperature and incubation time on the bacitracin production at a pH of 6.25

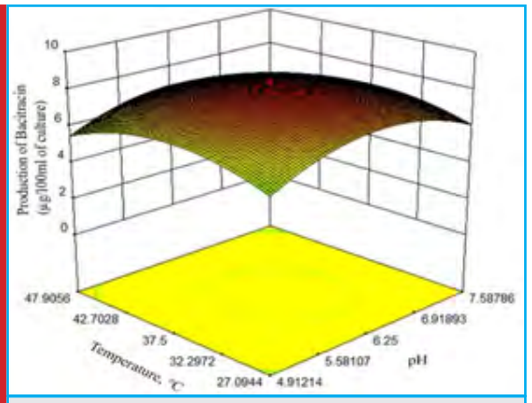

FIGURE 10. Combined effect of temperature and pH on the bacitracin production at 18h of incubation time.

production is 18h of incubation time. A maximum bacitracin of production of 7.2 µg/100mlis found (fig. 11).

Optimization of the production of bacitracin

The focal objective of this study is to optimize the process parameters for the production of bacitracin using the developed correlation (eq 1). The quadratic correlations are optimized using quadratic programing to maximize the production of bacitracin (Bodroth *et al.*,

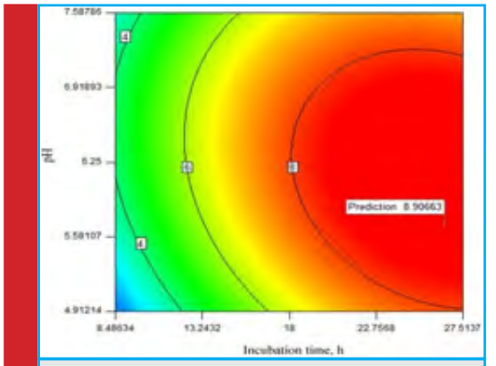

FIGURE 11. Optimization region of pH and incubation time for maximum production of bacitracin.

2012). The optimum region of pH and time for the maximum production of bacitracin is presented as fig. 11.The optimized conditions are found to be incubation time of 26.5 h; pH of 5.67 and temperature of 30.65°C for a maximum production of bacitracin of 8.9 µg/100ml as shown in table 8.

CONCLUSION

The *Bacillus subtilis* BSG isolated from Gunupur soil is a gram +ve, rod shaped bacteria has the capabilities to produce bacitracin, a peptide antibiotic. The bacitracin production was optimized in different parameter and found the suitable production point. The bacitracin production will be affected by imbalanced carbon and nitrogen sources. The present work will give a milestone for the production of antibiotics in pilot plant scale. Bacitracin also has potential antimicrobial properties with maximum inhibition for pathogenic bacteria. Simultaneously, RSM based CCD was used to estimate the individual, combined and the square effect of all incubation time, pH and temperature on the bacitracin production. Using ANOVA results a quadratic correlation was developed with a coefficient of correlation of 0.986. Again, the process parameters are optimized so as to maximize the bacitracin production. The optimized conditions is found of incubation time of 26.5h; pH of 5.67 and temperature of 30.65°C for a maximum production of bacitracin of 8.9 µg/100ml. Therefore, the correlation developed and

Table 8. Optimized conditions for bacitracin production

Incubation time, h (A)	pH (B)	Temperature, °C (C)	Production of bacitracin (µg/100ml of culture) (Y)	
			observed	predicted
26.5	5.67	30.65	8.72	8.90663

the data reported may provide convenient information for the economic production of bacitracin within ranges of the operating parameters investigated.

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Exploring teachers professional identity: Role of teacher emotions in developing professional identity

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ABSTRACT

The purpose of this study is to develop an understanding of the connections between teacher's emotional development and professional identity. Twenty teachers in their beginning year of profession were interviewed to reflect on emotional events and how these emotions help them in developing their professional identity. The research question for this study is; how do teachers' reflections of emotional events contribute in the development of their professional identity? Findings of the research yielded a model of professional identity that reflected the teachers understanding of themselves in relation to different emotional events. This model includes four key indicators: a) Identity beliefs, b) emotional events and identity negotiation, c) Teachers' attributes and d) adjustment. All participants exhibited the role of emotional events in development of professional identity. Some teachers elaborated that pleasant emotional events confirmed their identities and others elaborated the unpleasant emotional events which caused them to confront and adjust their emergent identities.

KEY WORDS: PROFESSIONAL IDENTITY, TEACHER EMOTIONS

INTRODUCTION

Teachers' professional identity has emerged as a separate area of research (Bullough, 1997; Claudia et al , 2013; Connelyly and Clandinin, 1999; Kompf, Bond, Dworet & Boak, 1996). The concept of identity is defined in various ways in research literature and the concept of professional identity is used in different ways in the domain of teaching and teacher education. In some studies, the concept of professional identity was related to teachers' concepts or images of self (i.e., Claudia et al, 2013; Nias, 1989) and it was argued that these concepts or images of self strongly determine the way teachers teach, the way they develop as teachers, and their attitudes towards educational changes. Professional identity formation is a method involving many knowledge sources, such

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as knowledge of affect, teaching human relations and subject matter (Antonek et al., 1997). Professional identity formation is often presented as a struggle because teachers have to make sense of varying and sometimes competing perspectives, expectations and roles that they have to confront and adapt to (Samuel & Stephens, 2000; Volkmann & Anderson, 1998; see also Bullough, Knowles & Crow, 1992; Mawhinney & Xu, 1997; Roberts, 2000). Research proved the crucial role of teaching practice and reflective activities in the identity formation of teachers (Ilze & Rita, 2016). Researching professional identity at early career stages can help educators to emphasize the multidimensionality and complexity of the teaching profession (Predrag, Biljana & Dusan, 2018)

Professional identity is an ongoing process of interpretations and reinterpretation of experiences (Kerby, 1991), a notion that corresponds with the idea that teacher development never stops and can be best seen as a process of lifelong learning (i.e., Day, 1999; Graham & Young, 1998). From a professional development perspective, therefore, professional identity formation is, in our view, not only an answer to the question "Who am I at this moment?", but also an answer to the question "Who do I want to become?" Professional Identity implies both person and context. A teacher's professional identity is not entirely unique. Teachers are expected to think and behave professionally, but not simply by adopting professional characteristics, including knowledge and attitudes that are prescribed but also by including personal characteristics. Chang-Kredl & Kingsley (2014) also emphasise the dynamic nature of professional identity; it is a continuous process in which identity is formed, built, and shaped. Teachers differ in the way they deal with these characteristics depending on the value they personally attach to them.

Generally, it is stated that professional identity is dynamic (e.g. Beijaard et al. 2004). Sugrue (2005) elaborates on this and argues that identity is not distinctly individual and unalterable. People may strive to maintain their habits and routines, but are not immune to outside influences (Sugrue, 2005). Teachers obtain more and more experience, and more and different influences affect teachers as teachers have worked longer in their profession. Ibarra (1999) poses that people use trial versions of their professional identity before assuming a fully elaborated professional identity.A teacher's professional identity consists of sub-identities that more or less harmonize. The notion of sub-identities relates to teachers' different contexts and relationships. Some of these identities may be broadly linked and can be seen as the core of teachers' professional identity, while others may be more peripheral. It seems to be essential for a teacher that these sub-identities do not conflict, for example, that they are well balanced. During initial teacher training student teachers often experience such conflict (i.e. Volkmann & Anderson, 1998).

TEACHER EMOTIONS

Teacher emotions have been regarded as an important field of research over the past two decades (Frenzel etal., 2009; Fried et al., 2015). Teacher professional development research has been continuously focused towards investigating the so-called 'rational' factors (e.g., teacher knowledge, skills, and capacities). However important these rational and fundamental aspects are, teacher emotions have often been ignored or underplayed (Crawford, 2011; Day, 2011; Hargreaves, 2001; Sutton & Wheatley, 2003) in teacher improvement and identity formation initiatives.Emotion is a mysterious human phenomenon that has puzzled many for centuries. Schutz, Hong, Cross, and Osbon (2006) define emotions as "socially constructed, personally enacted ways of being that emerge from conscious and/or unconscious judgments regarding perceived successes at attaining goals or maintaining standards or beliefs during transactions as part of social-historical contexts".

Research on teacher emotions in education has warranted attention since the late 1990s (Hargreaves, 1998; Marshak, 1996) and has attracted increased attention in recent years. This is motivated by the realization that teacher emotions influence teacher behaviour (Becker, Goetz, Morger, & Ranellucci, 2014; Hagenauer & Volet, 2014; Sutton & Wheatley, 2003), teaching (Gong, Chai, Duan, Zhong, & Jiao, 2013; Saunders, 2013; Trigweel, 2012), professional identity (Lee, Huang, Law, & Wang, 2013), teachers' lives (Hargreaves, 2005; Schutz, 2014; Schutz & Zembylas, 2009; Taxer & Frenzel, 2015), student behaviour and learning (Brackett, Floman, Ashton-James, Cherkasskiy, & Salovey, 2013; Chang, 2013; Jennings & Greenberg, 2009), and educational change (Day, 2011; Leithwood & Beatty, 2007). Although previous research on teacher emotions has made substantial progress, it has most frequently used semi-structured interviews (Sutton & Wheatley, 2003).

Farouk (2012) states that teacher emotions comprise individual teacher's dynamic mental state level, ability of emotional self-regulation and response to exterior stimuli, and an approach of synthesis. Teacher emotions are not "internalized sensations that remain inert within the confines of their bodies but are integral to the ways in which they relate to and interact with their students, colleagues and parents" (Farouk, 2012). Therefore, teacher emotions are relational with the environment, which means teacher emotions do not exist within an individual or environment independently, rather they involve person-environment transactions (Schutz et al., 2006). Understanding emotions triggered by vulnerabil-

ity may constitute an opportunity for teachers to educate in a way that really makes a difference to students' and teachers' lives but also teacher effectiveness (Day et al., 2007; Kelchtermans, 2005, 2011). Emotions have great potential to strengthen not only interpersonal relationships experienced in the classroom and broader contexts, but also create opportunities for learning and teaching in various situations (Bahia et al., 2013). A lack of negative emotions also limited teachers' learning processes (Klara et al; 2017).

TEACHER IDENTITIES AND TEACHER EMOTIONS

Although there is a great variation in how scholars define and measure teacher identities (e.g. Beijaard, Meijer, & Verloop, 2004), growing literature suggests three salient features. First, identities are not fixed, but fluid processes involving 'interpretation and reinterpretation of experience' (Sutherland, Howard, & Markauskaite, 2010) i.e. it is not about what someone *is*, but rather identity is about what someone is *becoming*. Second, it involves a negotiation between the person and an understanding of the contexts in which he/she works. And third, it involves human agency.

Yoo and Carter (2017) carried out an ethnographic study in the form of a professional development program focused on creative writing and writing practices. They identified four different types of emotions that the participants experienced: (1) energy, excitement, and passion; (2) inner conflict, frustration, and discouragement; (3) vulnerability, engagement, and hope; and (4) generosity, gratitude, and inspiration. This line of research shows that every change, reform, or development in teaching is accompanied by various emotions. Related to that emerging teacher identity, Nias (1996) indicated that teaching is not just a technical job; rather, teachers invest their 'selves' into their work. This investment involves emotional experiences that provide salient information regarding one's evolving identity commitments. Importantly, this relationship is reciprocal and teachers' emerging identities not only influence their actions and emotions, but their actions and emotions also influence their professional identity formation. Teacher identity and emotion are not linear or unidirectional; rather, they are inextricably related to each other through an ongoing, multidirectional, transactional process. For example, when teachers experience particular unpleasant emotions, those emotions may indicate or signal a threat to their identities by challenging existing identities related to their beliefs about teaching. In contrast, pleasant emotional episodes may indicate a confirmation of an emerging identity (Cross & Hong, 2009). Similarly, incoming identities (e.g. expectations about a teacher's role) may influence how subsequent emotional reactions emerge and/or are interpreted.

RESEARCH AIMS

Previous research provides a little knowledge about the importance of emotions in teaching and the emerging identities of beginning career teachers; this research is an attempt to understand how these two connect. Therefore, the goal of this inquiry was to ask beginning teachers to reflect on pleasant and unpleasant emotional episodes early in their first year in the classroom and to discuss the ways in which these emotional experiences help them in development of professional identities. The purpose of the research is to draw on teachers' reflections about emotional events and identity constructs to begin to theorize on their connections. Through open-ended semi-structured interviews, teachers were asked to reflect on emotional events as a way to begin to understand the ways emotions may contribute in emergent identity processes. These emotional events are seen as 'beacons of our true selves' and 'become the fulcrums through which we begin to deconstruct and construct our sense of self' (Zembylas, 2003).

METHODS

This paper encompassed a pilot qualitative study to respond to the research question that how teachers in their beginning years develop an understanding of emotional events in their classroom and how these emotional events relate to their emerging teacher identities.

PARTICIPANTS

Participants of the study were twenty teachers in their beginning year of profession teaching in eight different high schools across the state of Punjab of India. They ranged in age from 25 to 31 (average 28), included six males and fourteen females. Our participants taught science (n = 12) and math (n = 8) in schools. Objective of the study was to listen to how beginning year teachers who confront emotional events more frequently than more experienced teachers, talk about the ways in which those events update their beliefs about teaching and their identities as an emergent teaching professional. Data was collected from interviews of teachers in their first school year. All interviews were lasted between 60 and 90 min. In this interview, teachers were asked to reflect upon pleasant and unpleasant emotional episodes they had experienced. For example, 'Describe a recent occasion when you were aware of your emotions in the classroom,' and 'how do you think your emotional experiences affect how you think about yourself as a teacher?' This interview structure was designed to allow the participants to reflect on past experiences with emotions in the classroom and to discuss their thoughts

regarding how they feel themselves as teachers and the role emotional events may have played in this.

DATA ANALYSIS

Participants' responses to each question in the interviews were compiled and themes were examined from two perspectives: within and across participants. The semi-structured format of interview helps to compare the responses of participants. Each participant was asked to discuss his/her pleasant and unpleasant emotional events in the classroom and their reactions to these events. Themes emerged when considering responses across each participant. In the second step data was compared to the relevant literature on professional identities and emotional labor. Multiple readings of the transcripts ensured that our emergent themes reflected the overall context (Groenewald, 2004; LeCompte & Preissle, 1993; Thompson, Locander, & Pollio, 1989). Collectively, these processes fulfilled our goal to capture participants' most salient experiences related to understanding how emotional events signal and influence their emerging teacher identities.

RESULTS

Findings of the research yielded a model of professional identity that reflected the teachers understanding and development of themselves in relation to different emotional events. This model includes four key indicators: 1) Identity beliefs, 2) Emotional events and identity negotiation 3) Teachers' attributes and 4) Adjustment

1) Identity beliefs

Identity beliefs represent reference points teachers used to determine where they are as compared to where they want to be. Within the interviews with participants, a variety of statements were identified that reflected some divergent expectation. For example, Mr Sunil Kumar (a 28-year-old science teacher) exclaimed that: 'I never realized that how difficult it is to be a teacher,' suggesting that what he was experiencing was basically not what he expected, suggesting he is experiencing emotional problem. Ms Rashmi (a 30-year-oldmaths teacher), stated that: 'I know math but, what is coming out is not matching with what I need to do for them.'

By contrast, a few of teachers expressed incoming identity beliefs that were often not too far from what they experienced or how they expected to be in the classroom. For example, Ms Shweta (a 27-year-old science teacher), seemed very clear in asserting that she was not a 'traditional' teacher but saw herself as 'very open minded and enthusiastic.' Her openness led her to observe that her students think of her as 'their mom' because she's 'so caring.' In this case, her expectations played out in ways that may not lead to identity struggles in the classroom. Ms Meena (a 30-year-old Math teacher) also seemed to know more clearly who she was as a teacher. Ms Meena saw herself as someone who is kind, not a dictator, and someone who tries to 'take their (students) feelings into consideration.'

These examples illustrate how participants' incoming identity beliefs were sometimes challenged as they continued the process of becoming a teacher. Among most of the participants, there was a clear struggle present as they searched for ways to reconcile incoming beliefs and expectations with the living experiences of teaching. These matches and mismatches resulted in emotional events that associate with teachers' identity and the emotional efforts associated with that identity belief.

2) Emotional Events and Identity Negotiation

An emotional event refers to those emotions that are triggered by some social interaction or experience with students, teachers, or administration. Although not all emotional events have the potential to influence a teacher's emerging identities, there are examples where the critical emotional event seemed directly tied to some ongoing identity negotiation. Participants' experiences suggested that these emotional events could be either pleasant or unpleasant. For example, Ms Kiran (a 26-year-old beginning science teacher) talked about the 'stress' she experienced as she attempted to get 'all' of her students to 'love science.' For Ms Kiran, it was an overwhelming feeling to handle her 'challenging' class.

By contrast, there were also a number of pleasant emotional events that seemed to affirm some of these beginning teachers' identities about themselves as teachers. For example, students of Ms Rashmi's other classes were 'really engaged' and 'interested.' Mr Rajneesh (a 28- year- old math teacher) talked about the 'joy of just seeing the excitement in the students.' Mr Shubham (a 31-year-oldscience teacher), talked about the joy he experienced 'when the class is really attentive and enthusiastic, it feels really good.'

In general, these emotional events either tended to call into question or affirmed participants' perceptions of themselves as teachers.

3) Teachers' attributes

Participants of the study varied in the attributes and argued that pleasant and unpleasant emotional events influenced their attributes like job satisfaction, flexibility, understanding, awareness, encouragement, motivation That is, teachers at times reported that emotionally challenging/satisfying events were either due to reasons under their personal control (they are a bad/good teacher), or not under their control (I have no power

over these students).External control seemed more prevalent when it came to unpleasant emotional events. For example, Mr Sunil accepts that he could not motivate his students was because science was not 'relevant' to them. Likewise, Ms Kiran indicated that her students 'don't care' and Ms Meena talked about struggles she was having while she attempted to teach students who are 'not motivated'. In these examples, teachers make sense of frustrating emotional events in terms of factors that are perceived to be beyond their personal control (i.e. the problems rest within the students, not within them) and influence their attributes.

However, there were also examples of internal control. For example, Mr Suraj talked about feeling helpless because 'you don't really know what to do to fix something.' He also talked about 'doing a bad job,' suggesting that at the end of the day, when he is feeling frustrated, he feels like he is 'doing a bad job. Ms Manjeet (29-year-old science teacher) talked at length about her relative 'inexperience' in dealing with students who were very close to her and who persistently challenged her ability to understand students nature and attitude and what it means to be a teacher. For example, Ms Manjeet believed that a teacher should be 'understanding and flexible.' However, by enacting these dispositions with her students, it invited experiences that challenged these beliefs leading her to question whether or not she should be 'harder' or stricter with students. She attributed failure to handle students due to lack of experience. These teachers were actively trying to better understand themselves, their control, and their role in teaching their students.

Some participants approached emotional events related to classroom management in a more useful manner. Ms Sharda (25 year-old math teacher) said, 'I feel I am doing something good, even if it is bad, I can learn from it.' Similarly, Mr Shubham offers a more balanced attribution analysis of his role in students' lives. When students didn't do well, he experienced frustration and often wondered what he could have done differently to get them to remember what he is teaching. But he also expressed his awareness that the responsibility lies with both him and his students – it is a shared effort and therefore shared responsibilities. But he still seems to be working through this idea that if students are struggling, he understands and is patient as teacher that they are trying and therefore, he must try not to be overly frustrated with them – or at least shouldn't let them know he is frustrated.

4) Adjustment

Some of participants also talked about how emotional episodes were often times presented as valuable opportunities to learn what kind of adjustments they needed to make to the way they were approaching situations and thinking about teaching. Many of these experiences cantered on classroom management.

Ms Seema (a 26-year-old math teacher) was a particularly interesting case as a new teacher who was dipped in questions about how to adjust his teaching efforts and identity. This was revealed when she shared how she felt about being a teacher and feeling emotionally invested in her students. During her interview, Ms Seema broke out in tears and said, I love them, they are like my kids. I never thought it would be something that I am experiencing at all. I mean...when they start telling me their problems (crying) I wish I could do more for them and I want to be sympathetic but I can't be, Ms Seema revealed an emergent emotional struggle that forces her to reflect on her identity as a teacher. Her interactions with these students prompted a deep sense of empathy for their problems and a desire to 'save' them, but at the same time, caused her to struggle because it conflicted with what it meant to be a teacher to these students. For Ms Seema, emotional events with her students signalled a deep conflict between her 'fantasy' teacher identity (I can teach them all and save them all) and her 'survival' teacher identity (I just have to find a way to cope) (Bullough, 2009).

For Ms Kiran, it was about figuring out how to handle ongoing frustration with students who she felt were chronically not engaged. Initially she felt so 'overwhelmed' and 'frustrated 'that maybe she wasn't 'cut out for teaching' But over the time, she made adjustments that were learned through ongoing experiences with her students who she saw as not always so unengaged. For Ms Kiran, her ideas of teaching and specifically her beliefs about her capacity to be a good teacher were shaped over time by positive and negative experiences in engaging students in science.

DISCUSSION AND IMPLICATIONS

Participants could identify emotional events that were signals for 'teacher identity' –the active process of constructing and deconstructing understanding of what it means to be a teacher as one reflectively confronts the process and outcome of some emotionally engaging classroom-based events. Frustration originating from a perceived lack of control, or experiences that conflicted with incoming expectations often led to some type of identity work (i.e. teachers wondered what kinds of shifts they needed to make to cope or make things different in their classroom), as we see in the cases of teachers such as Mrs Kiran and Ms Seema, respectively. Satisfaction coming from successful teaching events often confirmed teaching identities. For most of our participants, feelings of happiness, joy, or satisfaction often accompanied successful teaching events and days defined as events in

which students were engaged or demonstrably learned something. For these moments, the positive emotions emanating from how students behaved often confirmed what it means to be a 'successful' teacher – sometimes independent of whether they had control over the situation or not. Teacher attributes and adjustments were also identifiable in the identity processes. Reflections on emotional events often led to sense making exercises in the form of attributes and potential adjustments.

Although these reflections seem to coincide with directional hypothesis of this study about the process of 'identity formation' for new teachers (i.e. beliefs, emotions, attributes, adjustment), not all teachers' reflections lined up this way, suggesting that the connections between these four features are multi-layered and multidirectional. It may well be the case that evolving emotions play a larger role in triggering later identity processes. It is also true that teachers' identity development includes a much wider swath of experiences than just what happens in the classroom. Although relationships with students and relevant instructional experiences play a significant role in a teacher's life, this narrow view neglects the broader contexts of teaching that include relationships with colleagues, administrators, and the press to fulfil many externally imposed demands and policies. These may be the limitations of this study.

This research will add to existing literature examining how teachers come to understand what it means to be a teacher. From this study, one can learn the importance of emotional events in shaping the nature of teachers' identities. As an example, this study suggests that emotional experiences that conflict with new teachers' expectations of what it means to be a teacher have great potential to trigger 'identity formation.' In fact, it seems as if these gaps between expectations and experiences have great potential for triggering important opportunities for teachers to engage in active explorations about what it means to be a teacher. This study yielded a model of 'identity work' that involves (1) some beginning orientation of teacher identity beliefs, (2) the experience of pleasant/unpleasant emotional events, and (3) teacher attributes and (4) identity adjustment (or change in beliefs about self as teacher). This model provides an entry point for scholars to understand on how emotions and identity processes converge and emerge. As a starting point, the model helps generate lines of future inquiry. For example, are emotional events the only source of identity adjustments? What is the significance of pleasant vs. Unpleasant emotional events for influencing identity-based attributes and adjustments?

This study has the potential to provide avenues for enhancing teacher education programs, particularly the pre service teacher experience when teachers are beginning to get their 'feet wet' in actual classroom settings. It is only through direct teaching experiences that teachers can be made aware of the range of emotional struggles they will encounter in their future classrooms. In this regard, teacher education programs should provide training in mindfulness exercises including emotional responses to students and events. Teachers should regularly record the range of emotional experience they observe and undergo as student teachers. This could assist these trainees with how to think and act through the many conflicting situations and emotional events of teaching.

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Protective effects of *Aloe vera* extract on aluminium sulphate induced alterations in serum lipid profile of male albino rats, *Rattus norvegicus*

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ABSTRACT

The present study suggests that treatment with *Aloe vera* a medicinal plant belonging to the family - *Liliaceae*, used in traditional Indian medicine system and its active constituent Aloin has a positive and therapeutic effect in lowering the lipid profile level in aluminium sulphate exposed rats for a period of 60 to 90 days. Lipid profile (total cholesterol, triglyceride, HDL and LDL) levels were found to significantly increased (P<0.05) after treatment of $Al_2(So_4)_3$ in Group II compared to normal control Group I treated with normal diet. Group III and Group IV animals treated with $Al_2(So_4)_3$ and Aloe vera extract and $Al_2(So_4)_3$ and Aloin respectively, showed significant decrease in lipid profile at (P<0.05). The present study also validates that *A.vera* extract and pure aloin was effective in reducing Al toxicity in lipid profile (Total Cholesterol, Triglyceride, HDL and LDL) of treatment in the long term 60 and 90 days of aluminium exposed rats.

KEY WORDS: *ALOE VERA*, ALUMINIUM TOXICITY, TOTAL CHOLESTEROL, TRIGLYCERIDE, HDL AND LDL

INTRODUCTION

Aluminium (Al) is the third most abundant metal present naturally in the Earth's crust. It is also present in soil, air, water, several eatables, and commercial products such as food storage material, cookware, and medicinal products including drugs. Exposure to humans occurs through different routes. The common routes of exposure include inhalation, oral, and skin. Exposure is more common among people working in Al industries. The extensive use of Al cookware leads to ingestion of small quantities of Al every day. Al is found to be a component of commonly used medications such as anti-ulcer drugs such as sucralfate, antacids containing Al, hae-

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modialysis fluid, phosphate binders and vaccines. Al is also found in anticaking agents, preservatives, fillers, coloring agents, emulsifiers and baking powders. Such extensive use of Al in consumable and non consumable products will certainly lead to Al entry and deposition in human body, (Denise *et al*., 2007; Verstraeten *et al*., 2008; Gura, 2010; Thirunavukkarasu *et al*., 2013; Kalaisevi *et al*., 2015; Sakr *et al*., 2017; Konda *et al*., 2017; Ahmed *et al*., 2018).

Al does not have any physiological role in the body but it gets stored mainly in the blood, lungs, liver, bones, brain, spleen, kidney and muscles. It may act as a competitive inhibitor for elements such as magnesium, iron and calcium because of its atomic size and electric charge and may results in anaemia and bone damage. Al-induced neurotoxicity and changes in serum lipid profile and vitamins. High level of exposure can cause toxicity such as nephrotoxicity and hepatotoxicity. It was already been reported in patients with chronic kidney disease who were on dialysis with Al-containing dialysis fluid. Al toxicity has been associated with Alzheimer's disease, dialysis, Parkinson's dementia. It is due to oxidative stress and lipid peroxidation in tissues, Protein and DNA (Tchounwou *et al*., 2012; Thomford *et al*., 2017; Azza *et al*., 2017).

Lipid is an important component of human body because it is a main constituent of cell membrane, several hormones and also performs many other cellular functions (Esther *et al*., 2013). Lipids being insoluble in the blood so it is transported from the cells by low density and high density lipoproteins (Brown *et al*., 2007; Kaji *et al*., 2013). High density lipoproteins (HDL) tend to carry cholesterol away from arteries back to the liver (Van der Veen *et al*., 2009). Therefore, high serum cholesterol level can be due to hepatic dysfunction. Although several factors, such as life style, a diet rich in cholesterol, age and hypertension, have been reported to cause heart failure (Kumar *et al*., 2011). High levels of cholesterol, particularly LDL cholesterol, are mainly responsible for hypercholesterolemia provoked cardio toxicity (Azad *et al*., 2001).

Several anti-hyperlipidemic agents are currently available; however most of them have associated with various unwanted effects. Hence, people are switching towards safer alternatives, specially derived from plants with limited side effects. The World Health Organization (WHO) has given its estimation that more than 2/3rd of the global population in recent times depends on alternative sources of treatment to fulfil the basic health care requirements and this most importantly embroils the usage of plant products. This means that nearby two-thirds of the people globally trust on plants as a reliable way of their medication. Nowadays, vigorous research is ongoing to discover nontoxic and beneficial herbs. Herbal Medicine or herbalism is the practice or art of employing herbs and herbal preparations in order to remain healthy and also for the treatment and improvement in prognosis of diseases. *A.vera* is a medicinal plant belongs to the family *Liliaceae* its active constituent aloin have antioxidant properties, protective against heavy metal toxicity. Its therapeutic applications include wound healing, diabetes, burns for easing intestinal, curing ulcers and arthritic swelling (Kumar *et al*., 2010; Sai *et al*., 2011; Jakkala *et al*., 2015; Mahor *et al*., 2016; Gupta *et al*., 2017). The aim of the present study was to investigate the protective role of *A.vera* on Al induced changes in lipid profile (cholesterol, triglyceride, HDL and LDL) of experimental rats.

MATERIALS AND METHODS

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 Peer Gate, Bhopal, (M.P.) India and the voucher specimen (403/Saifia/Bot/16) has been deposited at the Herbarium of the Saifia Science College, Peer Gate, 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 & 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.

Drugs and chemicals: In this study, Al-sulphate $(Al_2So_4)_3$ was purchased from Aldrich chemical Company (St. Lousis mo, USA) and Standard Aloin (C21H22O9) 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 98mg $(Al_2So_4)_3$ /L (1/25 LD50). The dose of A.vera extract and Aloin were 100 mg/kg BW. These doses were selected based on basis of pilot experiments.

Maintenance of animals and approval of protocol: Healthy adult male albino rats (*Rattus norvegicus*) weighing 120-150g were used for the present investiga-

tion. 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 (Lorke, 1983).

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 (Laxman *et al*., 2016) for 60 and 90 days. Group III: - This group animals were fed with normal diet and received aluminium sulphate (98 mg/kg body weight) and *Aloe vera* extract (100 mg/kg body weight) for 60 and 90 days. Group IV: - This group animals were fed with normal diet and received aluminium sulphate (98 mg/kg body weight) and Aloin (100 mg/kg body weight) for 60 and 90 days.

Animal Grouping and Treatment Schedule: Four groups of rats, six rats in each, received the following treatment schedule: Group I rats received normal diet and water *ad libitum*, as control group. Group–II rats administered twice with Aluminium sulphate (98 mg/kg/day) dissolved in (1ml/kg b.wt) water were injected dose orally for 60 and 90 days. Group III, will be administered with Aluminium sulphate (98 mg/kg/b.w.) with *Aloe vera* extract (100 mg/kg/b.w.) dose orally for 60 and 90 days and last Group-IV rats were administrated Aluminium sulphate (98 mg/kg/b.w.) with Aloin (100 mg/kg/b.w.) dose orally for 60 and 90 days.

Collection of Blood Sample and Estimation of Serum Lipid profile Investigations: 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. Serum was collected carefully and kept at-20°C until analysis of Total cholesterol, High Density Lipoprotein (HDL) cholesterol and triglycerides by using kits supplied by Span Diagnostic Ltd. Plasma concentrations of total cholesterol, triglycerides, HDL & LDL fractions were measured by using standard methods with commercially available kits. LDL cholesterol was calculated with the Friedewald formula as follows: LDL cholesterol = total cholesterol – HDL cholesterol – (triglycerides/5) (Friedewald *et al*., 1972).

STATISTICAL ANALYSIS OF DATA: All parameters were presented as mean ± SEM. One-way analysis of variance followed by Bonferroni multiple comparisons using a computer-based fitting program (Prism, Graph Pad) were performed. Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

It was obsevered that all four groups of rats received the following treatment schedule: shows the significant change in all parameters discussed here. After 60 days (Group II) showed a significant ($P<0.05$) increase in the level TC, TG, HDL and LDL due to Al toxicity compared to group I. whereas significant ($P<0.05$) decrease in TC, TG, HDL and LDL level was reported in group III and group IV (Table:1), (Fig: 1).Experimental results shows. After 60 days group III and group IV showed a significant ($P < 0.05$) decrease in the level TC, TG, HDL and LDL which is induced due to Al toxicity group II compared to group I (Table: I), (Fig: I).

After 90 day study it was observed that Al toxicity enhances compared to 60 or 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 toxicity in various parameters studied after 90 days. After 90 days group III and group IV showed a significant ($P<0.05$) decrease in the level of TC, TG, HDL and LDL which is induced due to Al toxicity group II nearest about to group I. (Table:II) and (Fig: II),

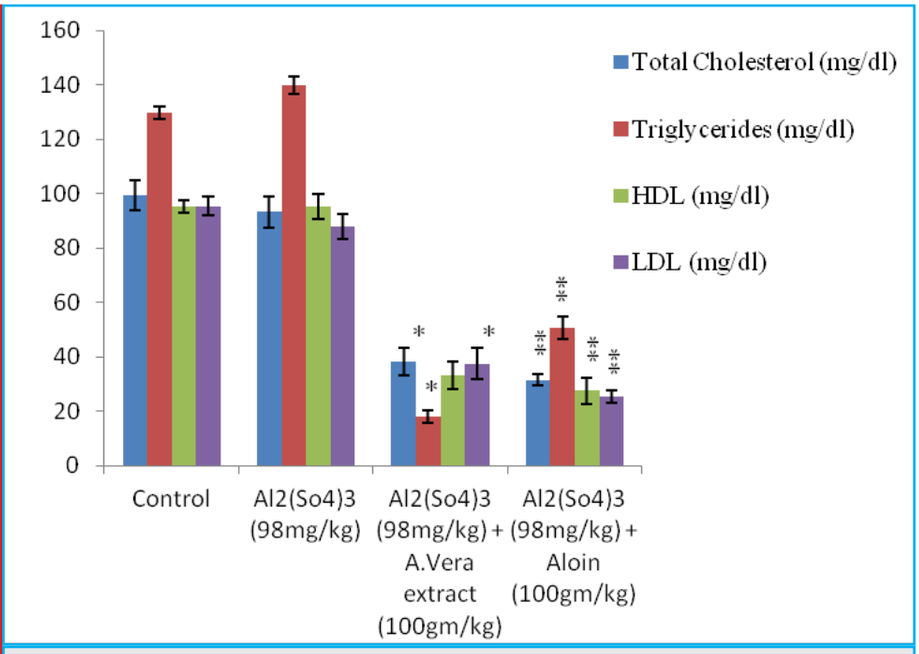

FIGURE 1. Graph showing variation in different levels of Total Cholesterol: Triglycerides, HDL and LDL against Aluminium sulphate (98mg/kg/bw) induced toxicity after 60 days.

Table 1. Effects of orally administrated A. vera extract and aloin on Total Cholesterol, Triglycerides, High density lipoproteins, Low density lipoprotein intoxicated with Aluminium sulphate after 60 days.

Group	Treatment	Total Cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
I	Normal Control	99.33±5.391	93.33±5.755	38.17±5.115	31.50±1.871
II	Al2(So4)3 (98mg/kg)	129.83±2.316	139.83±3.312	17.83±2.317	50.67±4.179
III	Al2(So4)3 (98mg/kg) + A.vera extract (100gm/kg)	95.16±2.136 *	95.17±4.446*	33.17±5.037*	27.50±4.848 *
IV	Al2(So4)3 (98mg/kg) + Aloin (100gm/kg)	95.33±3.444**	87.83±4.401**	37.33±5.715 **	25.17±2.317 **

* & **= indicates significant values, significantly different at P ≤ 0.05.

Table 2. Effects of orally administrated *A. vera* extract and aloin on Total Cholesterol, Triglycerides, High density lipoproteins, Low density lipoprotein intoxicated with Aluminium sulphate after 90 days.

Group	Treatment	Total Cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL (mg dl)	LDL (mg/dl)
I	Normal Control	101.83±4.622	94.67±5.785	40.00±3.950	33.17±2.927
II	Al2(So4)3 (98mg/kg)	133.17±4.070	141.17±4.834	15.67±1.633	53.67±4.633
III	Al2(So4)3 (98mg/kg) + A.Vera extract (100gm/kg)	92.83±3.189*	92.50±5.925*	35.50±3.507 *	29.50±4.593 *
IV	Al2(So4)3 (98mg/kg) + Aloin (100gm/kg)	92.67±8.238 **	85.50±9.731 **	38.67±4.885 **	23.67±3.983 **

* & ** = indicates significant values, significantly different at P ≤ 0.05.

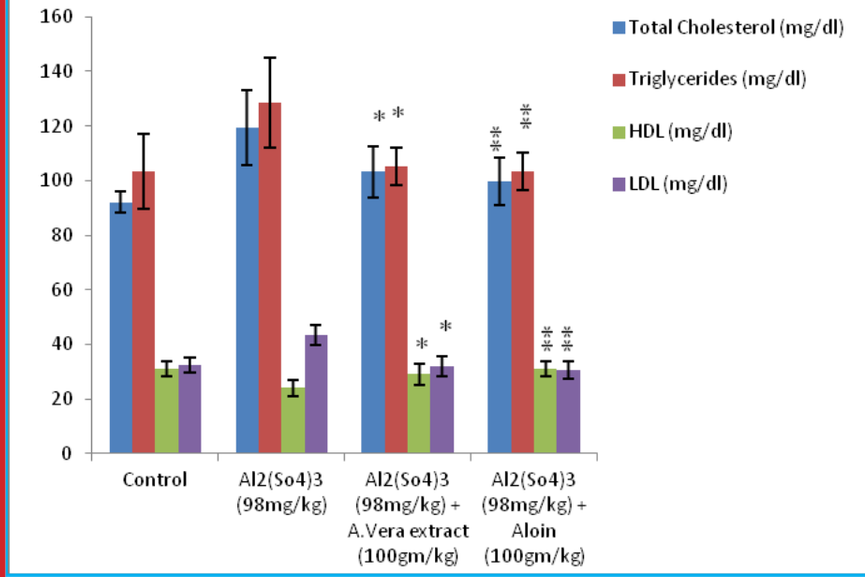

FIGURE 2. Graph showing variation in different parameter levels of Total Cholesterol: Triglycerides, HDL and LDL against Aluminium sulphate (98mg/kg/bw) induced toxicity after 90 days.

DISCUSSION

The hypertriglyceridemia and lipid oxidation were main features of this altered metabolism. Hyperlipidemia is a condition where there is an elevation of the serum levels of total cholesterol (TC) and triglycerides (TG) due to the lipid metabolism alteration, with an increase in the liver lipogenesis and lipolysis in the adipocytes. Low-density lipoprotein (LDL) is the compound containing both lipid and protein, which transport cholesterol to tissues other than the liver. High-density lipoprotein (HDL) is the compound containing both lipid and protein, which transport cholesterol to the liver for excretion in the bile. (Kalaiselvi *et al.*, 2015; Gouda *et al.*, 2018).

Aluminium (Al) is toxic to humans and animals. Its toxicity results to generation of reactive oxygen species in lipids which leads to oxidative damage of biomolecules in an organism. The present study investigated the effects of Al-sulphate in toxicity induction and beneficial effect of *A.vera*, aloin against the induced toxicity in rats. The findings of this study were that Al perturbed the metabolism of lipids (cholesterol, triglyceride, LDL and HDL) in rat. It may up-/down-regulation the levels of these lipids due to up- or down-regulated of enzyme. These perturbations were presented in the plasma as hypertriglyceridemia, hypercholesterolemia and hypophospholipidemia. the increase in plasma cholesterol as a result of ingestion of Al. Due to Al ingestion caused a preferential activation of receptor sites on the cells which favoured the synthesis of cholesterol in these organs by up-regulating hydroxymethylglutaryl coenzyme A reductase (a rate-limiting enzyme in cholesterol synthesis pathway) since virtually all cells can synthesize cholesterol or Al changes the integrity of the cell membrane thereby causing a constipation of cholesterol in the organs by modification of the composition, structure and stability of the cell membranes. The liver has been shown to be one of the target organs of Al toxicity- induced injury, liver damage is likely to cause some membrane lipids to be released into circulation; metabolism with oxidative stress and lipid peroxidation and reactive oxygen species as hydroxyl and superoxide radicals in liver alter the lipid level in serum (Kolomiytseva, 2011; Ugbaja *et al.*, 2015; Younes *et al.*, 2018).

Al causes toxic effect on biochemical parameters i.e. Cholesterol, Triglycerides It shows an increasing trend because prolonged metallic stress in the experimental animals makes adaptation difficult and creates weakness, anemia. In the field of environmental bio monitoring these parameters have been effectively used as potential biomarkers of Al toxicity in animals and human. This present study was carried out to investigate the effect of A.vera, a well-known medicinal plant with antioxidant properties, on Al-induced alterations in lipid metabolism. In comparison to controls, rats with Al toxicity displayed higher cholesterol, triglyceride, HDL and LDL concentrations in serum (Joshi *et al.*, 2013).

A.vera extract has a wide range of therapeutic applications. *A.vera* gel contains anthroquinones (aloin, aloe-emodin) which may have a variety of properties of antioxidant agent, including the protective role for heavy metal toxicity. Previous studies have also shown that as an antioxidant, plant extracts may improve the pro-oxidant effects of Al (Nada *et al.*, 2013; Jakkala *et al.*, 2016; Mahor *et al.*, 2016).

In this study, the *A.vera* extract proved to be quite effective in lowering the lipid profile (total cholesterol, triglycerides, HDL and LDL) Al toxicity. Al in the blood was significantly reduced due to administration of the *A.vera* extract since it possesses chelating properties. Administration of the *A.vera* extract for 60 and 90 days lead to decreases in cholesterol, triglycerides, HDL and LDL levels in the Al-sulphate exposed animals. This implies an exacerbating effect of *A.vera* on Al toxicity.

CONCLUSION

The present study also validates that *A.vera* extract and aloin was effective in reducing Al toxicity in lipid profile (Total Cholesterol, Triglyceride, HDL and LDL) of treatment 60 and 90 days.

CONFLICTS OF INTEREST

The authors have no conflict of interest to declare.

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A review on the pharmagnostic evaluation of Meswak, *Salvadora persica*

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ABSTRACT

Due to broad spectrum in physiologic diversity and their wide range of pharmacological activities, plants are playing an important factor for the pharmaceutical industry. Meswak tree is shrub and botanically known as *Salvadora persica* L. It has been used since ancient times as a chewing stick for oral hygiene. Many unique phytochemicals are naturally present in Miswak, which are described by traditional medicine as a remedy for various disease symptoms with beneficial properties. The biological active compounds that are present in plants are referred as phytochemicals. These phytochemicals are derived from different parts of plants such as leaves, barks, seed, seed coat, flowers, roots and pulps and thereby used as sources of direct medicinal agents. Phytochemistry describes the large number of secondary metabolic compounds present in the plants. The plants are the reservoirs of naturally occurring chemical compounds and of structurally diverse bioactive molecules. The extraction of bioactive compounds from the plants and their quantitative and qualitative estimation is important for exploration of new biomolecules to be used by pharmaceutical and agrochemical industry directly or can be used as a lead molecule to synthesize more potent molecules. This review includes the analytical methodologies in which extraction methods and the process of analysis for bioactive compounds present in the plant extracts through the different Aaalytical techniques like HPLC, GC, GC, OPLC etc. and the detection of compound by mean of FTIR, NMR, and MS.

KEY WORDS: *SALVADORA*, PHYTOCHEMISTRY, ANALYTICAL TECHNIQUES, MESWAK

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INTRODUCTION

Medicinal plants have been the keystone of traditional herbal medicine amongst occupant of rural area worldwide since old time. The therapeutic use of plants certainly goes back to the Sumerian and the Akkadian civilizations in about the third millennium BC. Hippocrates (ca. 460–377 BC), one of the ancient authors who described medicinal natural products of plant and animal origins, listed approximately 400 different plant species for medicinal purposes. According to the World Health Organization, a medicinal plant is the plant in which one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical compounds. Medicinal plant will have chemical components that are medically active in its parts including leaves, roots, rhizomes, stems, barks, flowers, fruits, grains or seeds, which are employed in the control or treatment of a disease condition. These chemical compounds or bioactive components (non-nutritional) are often referred to as phytochemicals ('phyto-'from Greek - phyto meaning 'plant') or phytoconstituents and are responsible for protecting the plant against microbial infections or infestations by pests (Doughari, *et al.* 2009) In present medical world oral hygiene is one of the most important daily routine practices for keeping the mouth and teeth clean and prevents many health problems, (Halawany *et al.* 2012).

Recently, there have been considerable interest in exploring the medicinal properties of *S. persica*. Methanol, ethyl acetate, and diluted acetone extracts of *S. persica* were screened for in vitro activity against some *Candida* species with the extract of *J. regia* L. (Naumi *et al.* 2009) The *S. persica* plant contain different ingredient which are helpful in the treatment of osteoporosis, (Fouda *et al.* 2017). The aqueous extract of *S. persica* leaves possesses analgesic activity and decreases carrageenan-induced inflammation in rat paw, (Ramadan *et al.*2016). Another study has revealed that there are 5-O-caffeoylquinic acid and 4,5-O-Dcaffeoylquinic acid present as the major phenolic compounds in the root of *S. persica* while the stem is rich in 5-O-caffeoylquinic acid, 3,5-O-Dcaffeoylquinic acid, catechin, and epicatechin, (Aumeeruddya *et al.* 2017). A high content of 5-O-caffeoylquinic acid, naringenine, and some alkaloids, including caffeine, theobromine, and trigonelline was also reported from the bark. A new sulphur-containing imidazoline alkaloid, persicaline, along with five known compounds was identified in *S. persica* which have different phytochemical activities, (Mohamed Farag *et al.* 2018).

Several studies have probed into the biological profile of this plant and a wealth of literature has emerged and published. In this direction we aimed to explore the up to date data review regarding *S. persica*. On the basis of this background, therefore, the purpose of this piece of study is to provide baseline information of the effectiveness of *S. persica* stick extract in different aspects. The phytochemical bio-application of *S. persica* in various fields have also been systematically reviewed. Lastly, possible future directions of research and priority are also discussed.

HISTORY

According to ancient Greek and Roman literatures from the 3500 BC, the evolution of the toothbrush may be traced from chewing sticks that were used by Babylonians and to toothpicks that were chewed to help clean the teeth and mouth, (Wu *et al.* 2001). During the old days, the laws of Manu of ancient Vedic India stipulated that the teeth be cleaned as part of the daily hygienic rituals, (Hyson *et al.* 2003). This review includes the history and the use of "Meswak" as an oral tool, as well as the biological effects of *S. persica* extracts. Chewing sticks are considered the most popular among all of the dental care tools for their simplicity, availability, low cost and their traditional and/or religious value (Halawany *et al.* 2012, Riggs *el at.* 2012).

Medical books of ancient India, Susruta Samhita and Charaka Samhita, have also stressed on oral hygiene using herbal sticks, (Dahiya *et al.* 2012). There are various biological properties, including significant antibacterial, (Al-sieni *et al.* 2013, Rasouli *et al.* 2014) antifungal, (Almas et al 1999) and anti-plasmodial effects in the extract of miswak. During the 2nd century BC, the Greek sophist, Alciphron, recommended a toothpick to clean the ''fibrous residue'' that remained between the teeth after meals. The Greek word, karphos, Alciphron used to describe the toothpick, is roughly translated to 'blade of straw'. The Romans had also used toothpicks from the mastic tree (*Pistacia lentiscus*). The Gospel of Buddhism mentions Buddha receiving a ''tooth stick from the god, Sakka''. The Talmud mentions ''quesem'', a splinter or wooden chip that was ''divided at one end by chewing and biting'' and used like a toothbrush.

COMMON NAME

Salvadora persica is commonly known as tooth brush tree but it does also have various names in different languages, in Arabic it is used to be called "Miswak" whereas in Hindi it is known a "Meswak" or "Pillu". The name of any plant varies as per the geographic areas. *S. persica* is well known plant in India and has many local names which include "Gudphala" in Sanskrit, "Uka" or "Ukaay" or "Oamai" in Tamil, "Gonimara", "Kankhira"

or "Genumar" in Kanada and "Gunnangi" in Telegu. In the western-northern area of India *S. persica* grows in large number. In Gujarat people know it as "Pilludi" or Pilu" or "Kharijal" whereas Rajasthani language has a name "Jaal" for it. In other languages it has venerable names, "Khabbar" in Sindhi, "Arak" in Assamese, "Peelu" in Punjabi, "Khakan" in Marathi. In Dutch it is known as "Zahnbürstenbaum", "Misvak ağacı" in Turkish, "Kerriebos" in Namibia, "Asawaki, kighir" in Nigeria and "Chigombo" or "Iremito" or "Mkayo" in Tanzania.

DESCRIPTION OF THE PLANT

Traditionally rheumatism, leprosy, gonorrhoea, ulcers, scurvy, tumours and dental diseases can be treated from *S. persica* (Miswak) (Almas *et al.* 1995, Jindal *et al.* 1996). Dr. Laurent Garcin proposed the term *Salvadora*, (Juan Salvadory Bosca, 1598–1681) while as *persica* was oriented from Persia and L. is used to indicate Carl Linnaeus (1707–1778), the father of modern taxonomy. As a shrub the miswak pant has long branches, often pendulous or semiscandent, glabrous or pubescent and the leaves are sub succulent; blades coriaceous, landeolate to elliptic, occasionally orbicular, 1–3–10 cm long, 1–2–3 cm wide, rounded to acute at apex, cuneate to subcordate at base. Flowers are small, greenish–white with lateral and terminal panicles up to 10 cm long and petals up-to 3 mm long. Drupes red or dark red purple when ripe. (Malik S *et al.* 1987) Besides its medicinal potentialities, it is also suitable in agroforestry systems as a wind break and helps in land reclamation (Gururaja GR *et al.* 2004, Bhatia B *et al.* 2000). The ripe fruits of this tree are sweet and edible (locally called as Piloo) and consumed by rural/tribal population. The seeds of *Salvadora* yield a pale-yellow solid fat, rich in lauric and myristic acid content which is used in making soaps, illuminants, varnishes, paints as well as in food industry. It is recognized as nonconventional oil seed tree crop.

TAXONOMIC POSITION

The genus Salvadora belongs to family 'salvadoraeceae'. It comprises three genera (i.e. Azima, Dobera and Salvadora) and 10 species distributed mainly in the tropical and subtropical region of Africa and Asia. It belongs to 'Magnoliophyta' division which is further classified in different classes in which *Salvadora* belong to 'Magnoliopsida'. The order of plant is 'Brassicales'.

USE AND PHYTOCHEMICALS

In Middle Eastern, some Asian and African cultures chewing sticks are prepared from the roots and twigs of *S. persica*. To prepare this type of sticks the stings or roots are cut into pieces of 10-to 25-cm long. The sticks of Miswak can usually be used 3–10 times daily considered as an inexpensive and an efficient oral hygiene. (AI-Bagieh NH *et al.* 1988) The Primary Health Care Approach (PHCA) principles entirely consider the use of Miswak. (Hyson JM el at. 2003) The use of chewing sticks as an oral hygiene tool like Miswak, where it is traditionally grown is encouraged and recommended by the World Health Organization (WHO). (WHO *et al.* 1987) In addition Miswak is also recommended for the teeth whitening, the memory improving tool, the breath freshener, calming the bile, drying up the phlegm, the gums strengthening, sharpening the vision and increasing the appetite, (Almas *et al.* 2001). Antimicrobial substances such as sulfur can be extracted from its roots and stems, moreover Trimethylamine, benzyl isothiocyanate, Salvadorine, beta cholesterol, tannins, saponines, sodium chloride, potassium chloride, vitamin C, flavonoids and sterols are associated with anti-bacterial effects. Besides this, the significant amounts of added silica can help to remove plaque mechanically, (Almas *et al.*1995). In this plant fluoride is also found in measurable quantities, (Darout *et al.* 2000) which is easily dissolved and released in water.

MATERIALS AND METHODS

PROCEDURE FOR SEARCHING INFORMATION

Relevant literature survey was done by scientific websites and tabs i.e Google Scholar, Scientific journal. Information was also obtained from books and e-articles. The scientific name of the plant was validated using The Plant List. Published review papers on *S. persica* were used as guidelines to design the present study and also to add missing data to ensure a more comprehensive and up-to-date review is obtained. The reference lists of review and research papers were searched for further relevant information. Regarding the search methodology, the following keywords were searched: "*Salvadora persica* plant extraction", (Google = 68,300 search results, Articles = 4,640).

PLANNING, DESIGN AND DESCRIPTION OF SECTIONS

This review consists of different nine Sections covering the traditional uses, phytochemistry, pharmacological properties, and bio-applications of *S. persica*. The third subsection of seventh section highlights about the extraction and separation of bioactive compounds of Miswak plant. Section 8 and 9 include the separation of phytochemicals in *S. persica* plant through different extraction methods. The sections reviewed about the phytochemicals and bioactivity of different compounds

from the plant extract. The detailed antimicrobial activity of *S. persica* has been displayed in the section 10, lastly, section 11 provides an overview of the potential applications of *S. persica* in various fields.

PLANT MATERIAL

First the fresh plant/plant parts can be collected randomly from the semi-arid or xerophytic region. The sticks of plant are dried at 55^{0}C by use of an oven for three to four days and then cut into slices then ground into a fine powder using a mixture grinder. The extract of plant Miswak can be prepared by adding 40 g of the Miswak powder to 200 ml of solvent in which water, ethanol and hexane are preferred, in a closed container and stored at room temperature for 48 h. The solvents are then filtrated through a Whatman No. 1 filter paper and allowed to evaporate at 40^{0}C in an oven for 72 h. The dried extracts are considered 100% pure and used to prepare different concentrations by adding the same solvents in an amount of 100, 250 and 500 g/ml. different commercial toothpaste brands can be used to control for all of the antimicrobial tests by making the concentration of 100 g/ml by allowing to dry and ground. 250 mg of dried extract is dissolved to prepare the Miswak mouth wash by using of 1 L of distilled water. (Mohammad Abhary *et al.* 2016)

As scrutinizing the aimed review article, it is observed that after the collection of plant extraction is carried out by different methods according to the nature of phytochemical which are present in plant. As the review on Miswak, some common method of extraction includes cold extraction and solvent extraction using Soxhlet apparatus. At present a common Universal Extraction System (Buchi) is used for the purpose of extraction.

PLANT EXTRACTION

Cold extraction method

It is reviewed that several of extraction is done by this method because of low costing and high productivity with efficiency. During the process of cold extraction, Measurable weight of dried powder is taken and respective solvents is added into conical flask then allow at room temperature for thirty-minute then after it is kept for seven days and during this period shaking is allowed after each twenty-four hours for seven days. Finally filter the extract through Whatman filter paper under vacuum and dry it at room temperature in watch glass dish. Note down the weight of each dish prior to drying of the extracts and after drying too. The difference was calculated by the weight of the extract. (Harborne *et al.* 1973).

Solvent extraction method

Recently the Universal Extraction System (Buchi) is used for solvent extraction. First the dried plant powder taken various parts placed in glass thimble for extraction purpose with the use of various solvents. For each extract the procedures are carried out for 10 cycles, and the temperature is adjusted just below the boiling point of the respective solvents. The resulting solvent extract is filtered, concentrated in vacuum concentrator and used to determine the presence of phyto constituents (Harborne *et al.* 1973)

Supercritical fluid extraction (SFE)

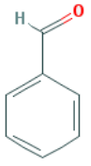
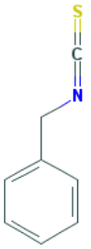
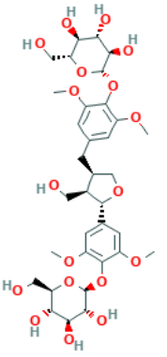
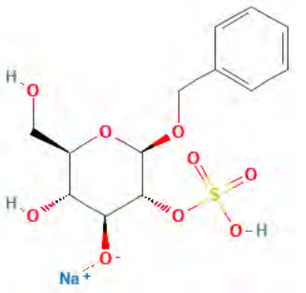
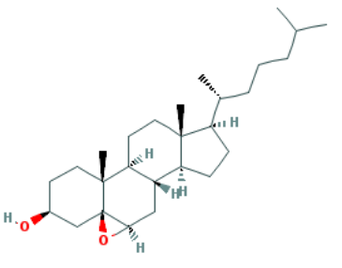
Supercritical fluid chromatography (SFC) provides a useful alternative to gas chromatography and liquid chromatography for some plant samples which involves use of gases as mobile phase at a temperature and pressure exceeding its critical point, usually CO_2 is used and compressing them into a dense liquid under these conditions the mobile phase is neither a gas nor a liquid. The turbid liquid is then pumped through a cylinder containing the material which to be extracted. From there, the extract- hampered liquid is pumped into a separation chamber where the extract is separated from the gas and the gas is recovered for re-use. CO_2 is commonly used because its low critical temperature, 31 °C, and critical pressure, 72.9 atm, are relatively easy to achieve and maintain. Solvent properties of CO_2 can be manipulated and adjusted by varying the pressure and temperature. The advantages of SFE are, no solvent residues left in it as CO_2 evaporates completely, (Patil *et al.* 2010).

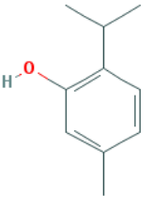
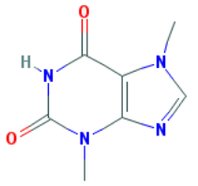
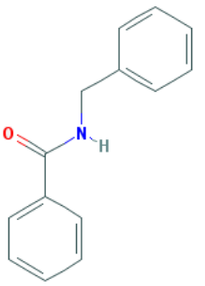
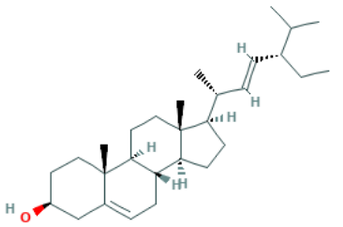
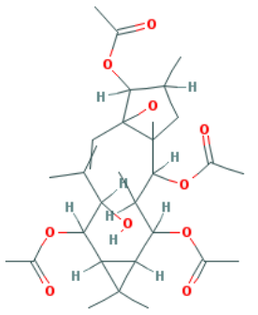
Microwave-assisted extraction (MAE)

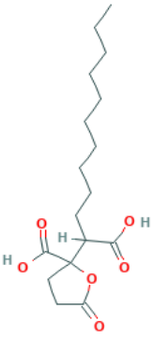
The combination of microwave and traditional solvent extraction is simply termed as microwave extraction. Microwave-assisted extraction is considered as the heating of solvents and plant tissue using microwave which increases the kinetic of extraction (Delazar et al 2012). To remove the minute microscopic traces of moisture present in plant cell, the extraction is heated in dried plant material. As a result of the heating up of this moisture inside the plant cell due the evaporation of moisture occurs and generates tremendous pressure on the cell wall. Due to the pressure the cell wall is pushed from inside and the cell wall ruptures. Thus, from the ruptured cells the exudation of active constituents occurs, hence increasing the yield of phytoconstituents, (Gordy *et al.* 1953 and Goldman *et al.* 1963).

IDENTIFICATION OF PHYTOCHEMICALS

The separation of bioactive compound which are present in the plant extract with different polarities is a challenging task for the process of identification and charac-

Name of Compound	Chemical Structure	Molecular Weight g/mol	Molecular Formula	PubChem CID
Benzaldehyde		106.124	C_7H_6O	240
Trimethyl amine		59.112	C_3H_9N	1146
Benzyl isothiocyanate		149.211	C_8H_7NS	2346
Salvadoraside		744.74	$C_{34}H_{48}O_{18}$	101630443
Salvadoside		372.32	$C_{13}H_{17}NaO_9S$	23664985
Cholesterol beta-epoxide		402.663	$C_{27}H_{46}O_2$	108109

Thymol		150.221	$C_{10}H_{14}O$	6989
Theobromine		180.167	$C_7H_8N_4O_2$	5429
N-Benzyl benzamide		211.264	$C_{14}H_{13}NO$	73878
Decane		142.2	$C_{10}H_{22}$	15600
Stigmasterol		412.702	$C_{29}H_{48}O$	5280794
9-Desoxo-9-x-acetoxy-3,8,12-tri-O-acetylingol		536.618	$C_{28}H_{40}O_{10}$	537583

Spiculisporic Acid		328.405	$C_{17}H_{28}O_6$	316426

terization of bioactive compounds. It is a common practice to use TLC, HPTLC, paper chromatography, column chromatography, Gas chromatography, OPLC and HPLC, should be used to obtain pure compounds in isolation of bioactive compounds. The pure compounds are then used for the determination of structure and biological activity, (Sasidharan *et al.* 2011).

Physicochemical and Phytochemical Studies

Phytochemical studies include extractive values, total ash, acid insoluble ash, total sugar, starch, tannin, and phenols can be calculated from the shade-dried and powdered (60 mesh) plant material. (Peach K *et al.* 1955, Ayurvedic Pharmacopeoeia of India. Part I *et al.* 2001, 2004). The Antioxidant Activity of the plant extracts and standard was assessed on the basis of the radical scavenging effect of the stable DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical. (Verma et al 2012, Gupta *et al.* 2015).

Methanol was used to make working solutions of the test extracts. Ascorbic acid was used as the standard in solutions ranging from 1 to 50 µg/ml. In methanol 0.002% DPPH solution is prepared. Then 2 ml of this solution was mixed with 2 ml of sample solutions (ranging from 25 µg/ml to 500 µg/ml) and the standard solution to be tested separately. These solution mixtures were kept in the dark for 30 min and optical density was measured at 517 nm using a Shimadzu spectrophotometer against methanol. The blank used was 2 ml of methanol with 2 ml of DPPH solution (0.002%). The optical density was recorded and percentage of inhibition was calculated using the equation: % of inhibition of DPPH activity = (A–B) /A × 100; where A is optical density of the blank and B is optical density of the sample.

HPTLC STUDIES

Air dried (45-55°C) powdered stem and twig of *S. persica* (2.0 g) in triplicate were extracted separately with 3 X 20 ml methanol. Extracts were concentrated under vacuum and re dissolved in methanol, filtered and finally made up to 100 ml with methanol prior to HPTLC analysis. Reagents used were from Merck (Germany) and standard ferulic acid was procured from Sigma-Aldrich (Steinheim), (Verma et al 2012, Gupta A *et al.* 2015).

Antimicrobial effects

Different antimicrobial activity was performed which can An in vitro study showed that the aqueous extract of *S. persica* miswak had an inhibitory effect on the growth of *Candida albicans* that may be attributed to its high sulfate content (al-Bagieh, N. *et al.* 1995). Some studies investigated the derivatives of *S. persica* miswak using three different laboratory methods, and demonstrated strong antimicrobial effects on the growth of Streptococcus sp. and *Staphylococcus aureus.* (Al Lafi, *et al.* 1995) In addition, some showed that Enterococcus faecalis is the most sensitive microorganism affected by the use of *S. persica* miswak, and noted no significant difference in the antimicrobial effects of freshly cut and 1-month-old miswak. A comparison of the alcoholic and aqueous extracts of *S. persica* miswak revealed that the alcoholic extract had more potent antimicrobial activity than did the aqueous extract (Al-Bagieh, *et al.* 1997).

Aqueous extract of plant inhibited all the microorganisms, showing greater activity on Streptococcus species. Methanolic extract was resisted by *L. acidophilus* and *P. aeruginosa.* At highest concentration tested (200 mg/ml), the aqueous extract was more efficient than the methanolic extract but were less efficient than the positive control *streptomycin* and *amphotericin B.* (Al-Bayati *et al.* 2008) Miswak extract displayed greater reduction in both *S. mutans* and *Lactobacillus* cariogenic bacteria counts. Reduction of microbial count in females was more for both microorganisms as compared to males, (Bhat *et al.* 2012). Ethanol extract was more effective than the aqueous extract in inhibiting the *S. mutans, L. acidophilus, E. coli, S. aureus, and P. aeruginosa* microorganisms. The aqueous extract did not display any inhibitory effect on *P. aeruginosa.* (Mohammed, *et al.*

2013). The miswak extracts showed comparable or slightly stronger activity compared to some toothpastes. The aqueous extract exhibit antibacterial activity on *M. bovis* (Fallah *et al*., 2015).

All solvent-extracts inhibited the *S. aureus, S. mutans, S. sanguinis, S. sobrinus, S. salivarius, L. acidophilus* microorganisms. Methanol extract was more effective than the other extracts. (Kumar *et al*., 2016) *S. persica* aqueous extract showed higher activity than methanol extract against *S. aureus* while the opposite effect was observed against *E. coli*. Compared to *C. mopane* and *D. cinerea, S. persica* was most effective against *S. aureus* but was least effective against *E. coli*, (Mudzengi et al., 2017).

CONCLUSION

Our literature review concludes that the use of *S. persica* miswak as an oral hygiene aid is effective. Descriptive and experimental studies have provided considerable evidence that the *S. persica* plant and its extracts exert beneficial effects on the oral tissues and help to maintain good oral hygiene. It is encouraging to note the large number of studies and clinical trials that have examined the effects of *S. persica* miswak and the value that people have attached to it since ancient times. The use of *S. persica* miswak alone or in combination with conventional toothbrushes, when performed judiciously, will result in superior oral health and hygiene. It is to be noted that there had been earlier attempts to summaries the medicinal potential of *S. persica*, even though with a different or a less broad ethnopharmacological focus. However, this review can be considered as the first attempt to broaden and critically assess scientific evidence on the ethnopharmacology of *S. persica*. It is obvious from this review that *S. persica* can be regarded as an important traditionally used medicinal plant harboring a panoply of bioactive compounds, pharmacological properties, and modern applications in emerging fields of interest. It is anticipated that this review article will open new avenues for research and stimulate further studies that will fill research gaps highlighted above.

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Cellulase production in *Lysinibacillus* sp isolated from the estuaries of Odisha

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ABSTRACT

Microbes are rich sources of natural products like secondary metabolites, enzymes and proteins. In this context the primary objective of the present work is to isolate microbes from natural habitats and characterize them on the basis of their ability to produce metabolites. Since estuaries are the junction of marine and coastal habitats and harbour a plethora of microbes therefore in this study estuaries along the coastal district of Balasore, Odisha were screened for cellulase secreting bacteria. Cellulase forms an important component of enzyme cocktail used for degradation of lignocellulosic biomass for production of biofuel. Several rounds of sampling, pure culture, morphological, biochemical and phylogenetic screening led to the identification of *Lysinibacillus* sp. having the ability to secrete cellulase. Physical as well as nutritional characterization like optimization of media, temperature, carbon and nitrogen requirements was performed to enhance the biomass formation. The isolated strain of *Lysinibacillus* sp. showed higher biomass and growth rate at 37°C, in Terrific Broth media supplemented with 0.5% Glucose and 0.5% Sodium Nitrite. Submerged fermentation under anaerobic condition at 37°C for 5 days led to release of 9.85µmole of glucose/ml of enzyme.

KEY WORDS: *LYSINIBACILLUS*, ESTUARY, CELLULASE, PHYLOGENETIC ANALYSIS

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INTRODUCTION

Several microorganisms are considered to be extremophiles as they are adapted to extreme environmental conditions such as high or low temperature, alkaline or acidic water, high pressure and substrate limitations. These characters make them potential industrial organisms as they produce several interesting metabolites to cope these extreme conditions. Various literatures are available where microorganisms isolated from extreme geographical locations have been used for production of hormones, proteins, enzymes and other primary and secondary metabolites (Coker, 2016; L Bergquist et al., 2014; Littlechild, 2015; Poli et al., 2017; Stierle & Stierle, 2014; Yin & Chen, 2015). One such extreme environment is the estuary. Estuaries have rich biodiversity due to variation in temperature, pH, salinity and availability of salts and other minerals (Campbell & Kirchman, 2013; Lallias et al., 2015). Several *Bacillus* species are known to be cellulase producers (Irfan et al., 2017; Sanjeev et al., 2017), but very few reports are available on cellulase production by *Lysinibacillus* strains

These brackish water bodies are an amalgamation of both fresh water from rivers and saline water from tidal waves of sea. This character makes it a very productive habitat for various flora and fauna including microorganisms (Moyle et al., 2010). Odisha is one of the coastal states situated on the eastern part of India having a coast line of 480 km. The coastline of the Balasore district is in the shape of a strip with a length of 81 km and 26 km wide. Several estuarine rivers like Budhabalanga, Subarnarekha flood the coastal areas. Many studies have been carried out on the macro flora and fauna of this area but very few documented information is available regarding the microbial biodiversity, (Subudhi & Patra, 2013; Sujana et al., 2015 Bomble et al., 2017).

Cellulases are a group of enzyme that degrade cellulose by hydrolyzing β-1,4 linkages in cellulose chains. Naturally, the cellulase is produced from widespread sources like fungi, bacteria, protozoans, plants, and animals (symbiotic bacteria in few ruminants and termites). The biotechnological application of lignocelluosic biomass in several industries like paper, textile, food, biofuel as well as agriculture has led to extensive research on production, biochemical as well as enzymatic characterization of cellulase. Microbes from many bacterial genera have proved to be a good source for cellulase production at industrial scale, Juturu & Wu, 2014; Kuhad, Gupta, & Singh, 2011 Kuhad et al., 2016 and Bomble et al., 2017). In the present study an attempt has been made to isolate cellulase producing bacteria from the estuaries around the Balasore district and optimize the culture conditions for the production of cellulase.

MATERIALS & METHODS

Sample collection and pure culture: Soil sediments up to a depth of 6-10 inch were collected from different spots of the Khandia estuary (21°19'1.65"N; 86°53'32.99"E) in Balasore district near the mouth of Khandia river. Soil samples were serially diluted in 1X PBS solution and plated on nutrient agar (NA) plate. The plates were incubated at 37°C for 24 hour and the colonies obtained were re-streaked on fresh NA plate and the mother plate was allowed to incubate further. The colonies that appeared after 48 hour and 72 hour of incubation were also re-streaked on fresh NA plates to obtain isolated colonies. Dilution streaking was performed for all the isolated colonies that were obtained from various samples. This process was repeated for several times till pure cultures were obtained. All pure cultures were labelled and stored at 4°C in NA stabs.

Screening for cellulase producing strains: The isolates were screened on the basis of their ability to secrete cellulase. NA plates were overlayed with 0.5% carboxymethyl cellulose (CMC). CMC acts as substrate for cellulase enzyme. The bacterial cultures were spread on the CMC supplemented plates. Post incubation the plates were stained with 0.1% Congo Red. The plates were destained with 1N NaCl solution and observed for clear zones (Meddeb-Mouelhi, Moisan, & Beauregard, 2014).

Morphological and physiological characterization of the isolates: The shape, size, elevation, margin and colour of the colony were observed and the morphology of the isolates was determined using Grams Staining method. Biochemical test like Citrate Utilization, Triple Sugar Iron, Mannitol Motility, Gelatin Hydrolysis, Oxidase, Indole production and antibiotic resistance tests were performed. Citrate Utilization was performed on Simmon's citrate agar medium (Himedia, M099), TSI was tested on Triple Sugar-Iron Agar (Himedia, MM021), Mannitol Motility was checked on Mannitol Motility Test Medium (Himedia, M770), Gelatin Hydrolysis was checked on Nutrient gelatin medium prepared in lab. The presence of oxidase enzyme was checked using Oxidase disc (Himedia, DD018) and Indole production was checked using Kovac's strip (Himedia, DD019). All experiments were performed using standard protocol as recommended by product manual. Resistances for ampicillin, kanamycin, tetracycline, penicillin and streptomycin were checked by disc diffusion method at 50µg/µl, 5µg/µl, 0.5µg/µl and 0.05µg/µl concentration for all the antibiotics.

Growth Characteristics of the Isolates: The isolates were grown in 4 different media (Nutrient Broth, Terrific Broth, Marine Broth and Artificial Sea Water) at 25 °C

and 37°C. The experiment was performed in a microbioreactor (m2p labs BioLector) where biomass was continuously monitored till the population reached at stationary phase. The growth was also monitored under various carbon sources (Glucose, Maltose, Lactose, Xylose, Cellobiose and starch) and nitrogen sources (Sodium nitrate, Di-ammonium hydrogen citrate, Ammonium nitrite, Tryptone, Yeast extract and Ammonium chloride). The concentration of carbon and nitrogen supplemented to the media were 0.5% each. Similarly salt tolerance was checked for the isolates in TB medium supplemented with various concentration of NaCl ranging from 0.5% to 12%.

Isolation of Genomic DNA: 5ml bacterial culture was inoculated and incubated overnight. After incubation the culture was transferred to a centrifuge tube and centrifuged at 10,000 rpm for 10 minutes until a compact pellet was formed. The supernatant was discarded and the pellet was resuspended in a mixture of 567µl TE buffer and 5µl RNAse A by repeated pipetting. 15µl 10% SDS and 4µl proteinase K (18 mg/mL) was added. It was mixed thoroughly and incubated 15-20 minutes at 65°C until all the cells are lysed.100µl of 5M NaCl was added and mixed thoroughly. 80µl of CTAB/NaCl (10% w/v; 0.7M) solution was added and mixed thoroughly and incubated for 10min at 65°C. Equal volume of chloroform/isoamyl alcohol (0.7-0.8ml) was added and mixed thoroughly and centrifuged for 5min at 10,000 rpm. 1 volume of isopropanol was added to the supernatant, shaken and centrifuged. The pellet obtained was washed with 70% ethanol, dried and dissolved in 50µl TE buffer.

PCR and sequencing: The 16s rRNA gene was amplified from the genomic DNA of all the isolates. The primers used in the study are as follows (Frank et al., 2008; Karakasidou et al., 2018): BAC27F AGAGTTTGATCCTGGCTCAG BAC1492RGGTTACCTTGTTACGACTT: The PCR was carried out at initial denaturation of95°C/5min, denaturation of 95°C/30sec, annealing at 42°C/1min, extension at 72°C/1min 30sec and final extension at 72°C/5min. This was repeated for 30 cycles.The sequencing of the purified PCR product was done using the BAC27F forward primer. The work was outsourced form SciGenom Labs Pvt. Ltd., Kerala, India.

Phylogenetic Analysis: The molecular characterization of all the isolates were done by 16s rRNA sequencing. The phylogenetic was prepared using MEGA 7 program (Sudhir Kumar, Stecher, & Tamura, 2016) with Neighbor Joining method. Sequence of *Lysinibacillus* sp. KEI3 was BLAST in EZ taxon (Chun et al., 2007) and only validly published sequences were taken as references in tree formation. Boot strap replication was performed 1000 times.

Submerged Fermentation: Bacterial isolates were cultured in Terrific broth medium under submerged fermentation conditions. 50 ml TB medium was prepared in 250 mL flask. The medium was supplemented with 0.5% Glucose and 0.5% Sodium Nitrite. Medium was sterilized by autoclaving. The flasks were incubated in a shaking incubator at 37 ± 2 °C for 5 days and then crude enzyme was extracted by centrifugation at 10,000 rpm for 20 min at 4 °C. The cell free culture filtrate (CFCF) was used as crude enzyme to test Cellulase activity. Cellulase activity was measured by DNS assay.

RESULTS

Isolation of Bacterial Colonies: After serial dilution, plating of soil samples and incubation, more than 50 colonies were obtained. Out of these, 11 pure cultures were obtained which were screened for their ability to degrade CMC by plating them on CMC agar plate and staining with Congo Red. Isolate 3 showed clear zones on CMC agar plates indicating their ability to secrete cellulase. Morphological and biochemical characterization was performed for Isolate 3 and the observations are presented in Table 1. The results indicate that the isolates belong to genus *Bacillus*.

Phylogenetic Analysis: Genomic DNA isolated from pure cultures were subjected to PCR amplification of the

Table.1. Biochemical characters of Lysinibacillus sp.KEI-3

TEST	Gram's staining	TSI	Mannitol Motility	Citrate utilization	Gelatin Hydrolysis	Oxidase	Indole	Ampicillin	Kanamycin	Tetracyclin	Penicillin	Streptomycin
KEI-3	+	K/A	-	-	+	+	-	+	+	+	+	+

(-) represents negative response to the test; (+) represents positive response or susceptibility to the test.

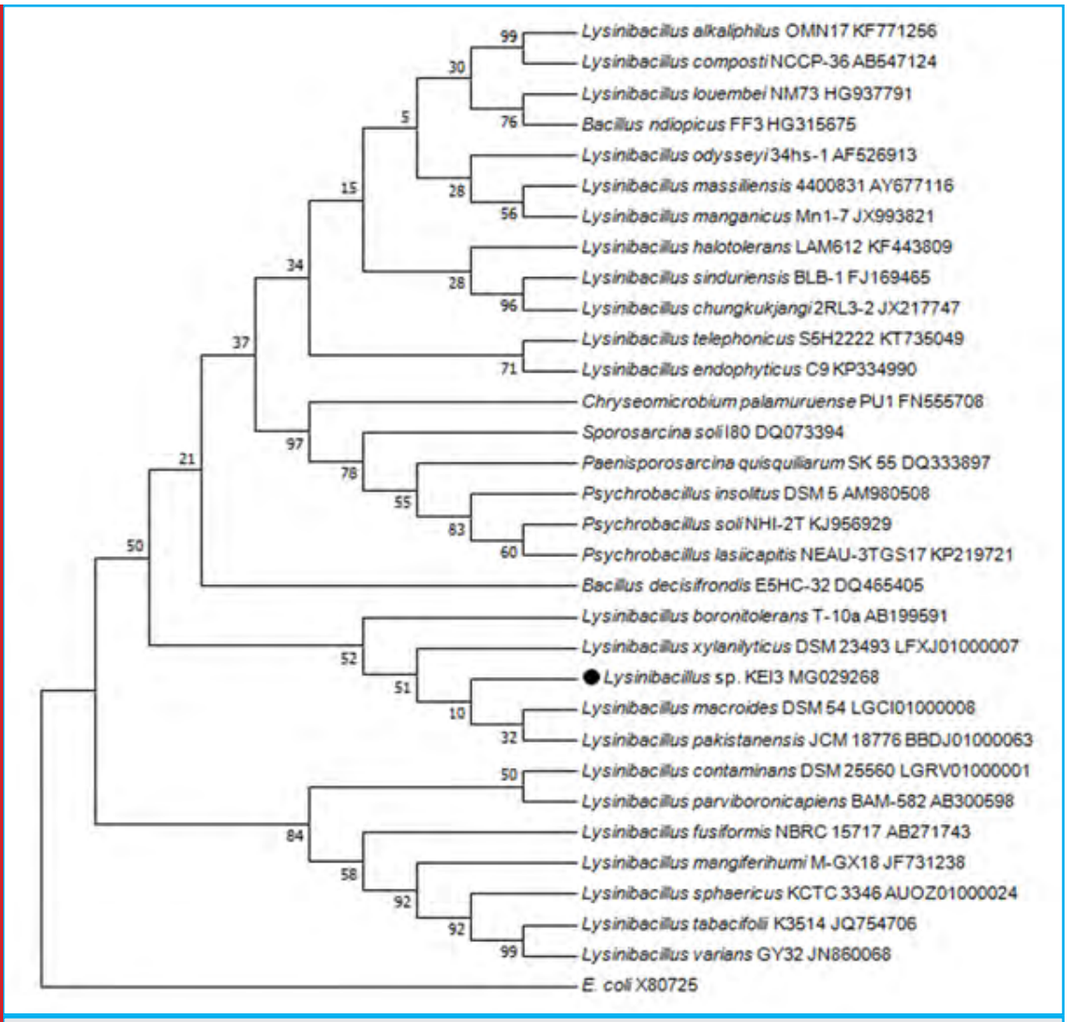

FIGURE 1. Phylogenetic tree prepared using MEGA 7 software with Neighbor Joining methodon the basis of 16sRNA sequencing.

16s RNA using BAC27F and BAC1498R primers which produced around 1450bp long 16S rRNA gene. Phylogenetic tree was prepared using MEGA 7 program and Isolate 3 was identified to be *Lysinibacillus* sp. which has maximum similarity to *Lysinibacillus fusiformis* strain 4 (KF916674) (Figure 1). The Isolate was named as *Lysinibacillus* sp.KEI-3.

Effect of different media on growth

Lysinibacillus sp. KEI-3 was inoculated in Nutrient Broth (NB), Terrific Broth (TB), Marine Broth (MB) and Artificial Sea Water supplemented with Glucose and Tryptone (SWGT) media and grown at 25°C and 37°C. The growth was monitored for 36 and 24 hour respectively and plotted to calculate specific growth rate. It was observed that when isolates were cultured at 25°C it had a very long lag phase and the specific growth rate was also slow in comparison to cells growing at 37°C (Figure 2). This phenomenon was observed for all media used for the study except TB medium, where irrespective of temperature the isolate *Lysinibacillus* sp.KEI-3 had a high specific growth rate. For TB medium the specific growth rate was $0.558h^{-1}$ at 37°C whereas at 25°C it was $0.451h^{-1}$ which is almost comparable. Interestingly in SWGT media the isolate had almost 20 hour long lag phase at 25°C after which there was increase in growth of the cells and the maximum specific growth rate achieved at 25°C was $0.338h^{-1}$ whereas at 37°C the it was $0.557h^{-1}$ (Figure 3).

It was interesting to observe that the isolate could not grow well in LB and MB. The reasons for LB are obvious that it is nutritionally less rich and complex than TB due to which the growth rate was slow. MB is high in salt

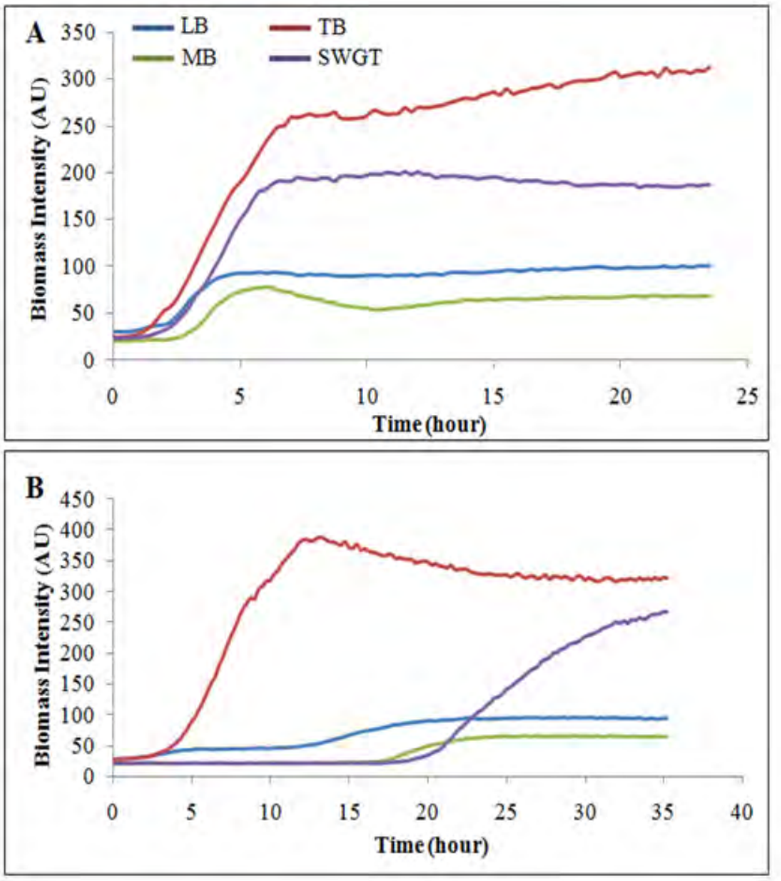

FIGURE 2. Growth profile of *Lysinibacillus* sp. KEI-3 strain in different media and different temperature. (A) growth curve at 37°C, (B) growth curve at 25°C.

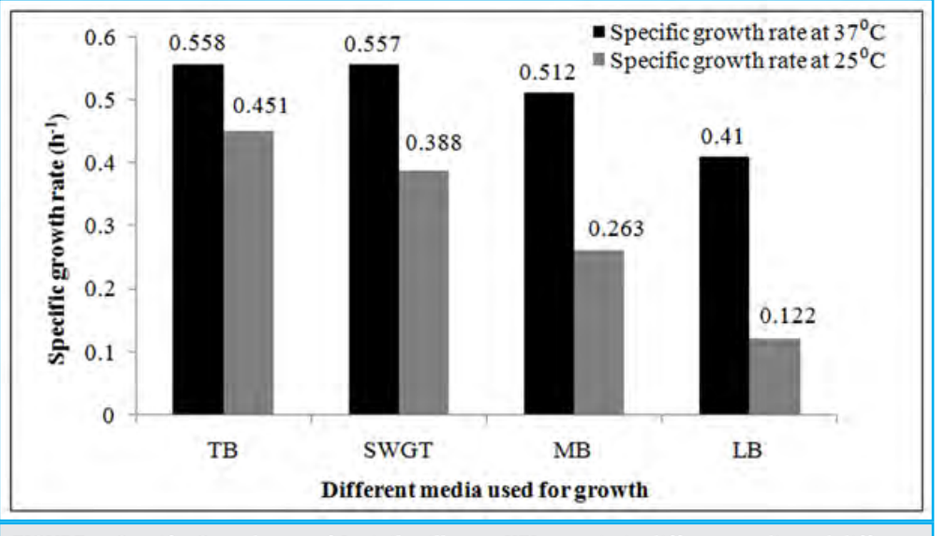

FIGURE 3. Specific Growth rate of *Lysinibacillus* sp. KEI-3 strain in different media and different temperature.

concentration and probably the isolate do not have high salt tolerance, which could be the possible reason for limited growth in MB medium. SWGT medium was formulated in lab where the composition of artificial seawater was almost similar to the original seawater but the salt concentration was less. Further it was enriched by addition of Glucose and Trypotone which are very good sources of carbon and complex nitrogen requirements.

Effect of different carbon source on growth: Six different carbon sources namely Glucose, Lactose, Starch, Xylose, Maltose and Cellobiose were selected in present study. These carbon sources were added at a final concentration of 0.5% separately in TB media. *Lysinibacillus* sp.KEI-3 was inoculated in these media and grown at 37°C. The growth was measured after 24 hour of incubation. It was observed that the isolate could effectively utilize both pentose and hexose sugars. Biomass profiling revealed that *Lysinibacillus* sp. KEI-3 effectively utilize monosaccharide (glucose and xylose), disaccharides (maltose, lactose and cellobiose) as well as polysaccharide (starch) (Figure 4).

Effect of different nitrogen source on growth: Different bacterial species utilize different nitrogen sources for their growth. The different nitrogen sources selected for this study are sodium nitrate, di-ammonium hydrogen citrate, ammonium nitrate, tryptophan type-1, yeast extract, and ammonium chloride. TB media was prepared to which different nitrogen source was added separately at a final concentration of 0.5%. The isolated strain was inoculated in these media and grown under 37°C. The growth was measured after 24 hour of incubation. It was observed that *Lysinibacillus* sp. KEI-3 can grow well in both organic as well as inorganic nitrogen sources and the maximum biomass was formed in media supplemented with yeast extract, tryptone and sodium nitrite (Figure 5).

Effect of different salt concentration on growth: To check the salt tolerance capacity, *Lysinibacillus* sp.KEI-3 was inoculated in TB medium supplemented with various concentration of NaCl. The salt tolerance was checked for concentration range of 0.5-12%. The strain was inoculated and incubated at 37°C for 24 hours. After incubation the biomass was measured spectrophotometrically by reading OD at 600nm.Since isolates could not grow well in Marine Broth (MB) due to its high salt content, therefore supplementing different concentrations of NaCl in the medium was used to check maximum salt tolerance of the *Lysinibacillus* sp.KEI-3which showed tolerance till 1% and beyond that there was decline in biomass formation. But it could grow till 12% with a lower growth rate (Figure 6).

Submerged fermentation: *Lysinibacillus* sp.KEI-3 was further subjected to submerged fermentation for production of cellulase. Terrific Broth was supplemented with optimized carbon and nitrogen sources (glucose/ sodium nitrite) that showed higher biomass for *Lysinibacillus sp*.KEI-3. Anaerobic fermentation was continued at 37°C for 4 days. Samples were collected and

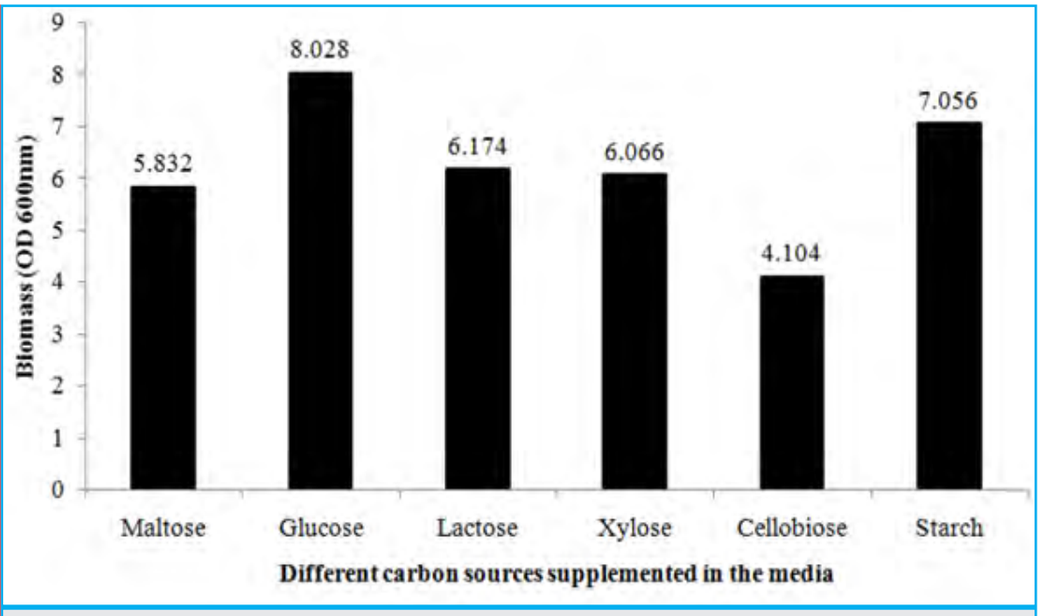

FIGURE 4. Growth profile of *Lysinibacillus* sp. KEI-3 strain with different carbon sources.

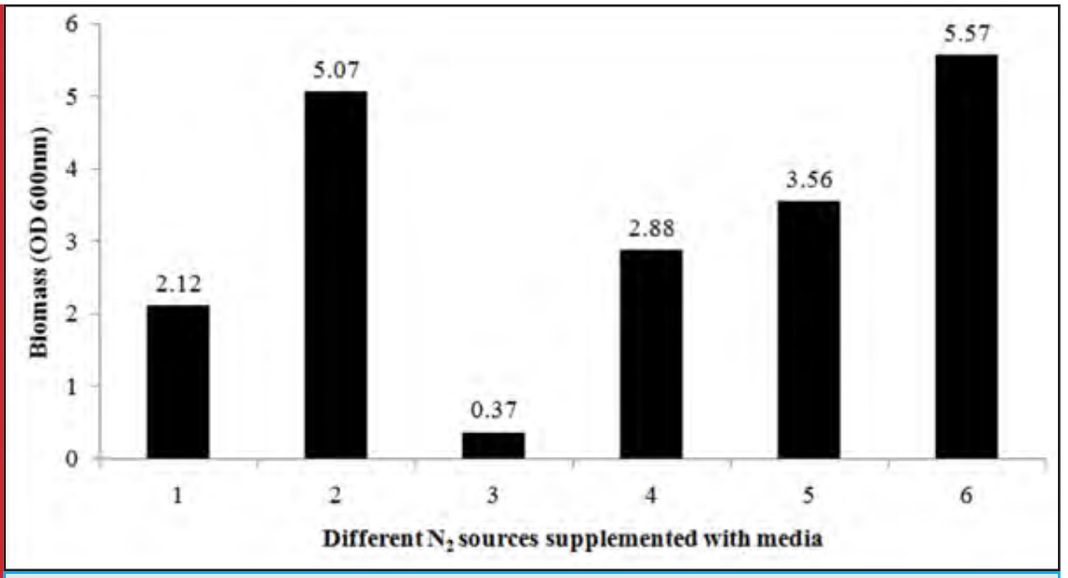

FIGURE 5. Growth profile of *Lysinibacillus* sp. KEI-3 strain with different nitrogen sources. The numbers on the horizontal axis represents various Nitrogen sources: 1.Ammonium chloride, 2.Sodium nitrite, 3.Di-ammonium hydrogen citrate, 4.Ammonium nitrate, 5.Tryptone Type-I, 6.Yeast extract.

cellulase produced was quantified by DNS assay. The results are represented in the form of µmole of glucose (reducing equivalent) released per ml of cellulase enzyme produced. Through the time course sampling, it was observed that till 48 hours there was no significant increase in cellulose production. Whereas, after 96 hours of incubation there was 2.5 times increase in cellulose production (Figure.7). This was a promising result and the production could be increased by further scaling up and optimization.

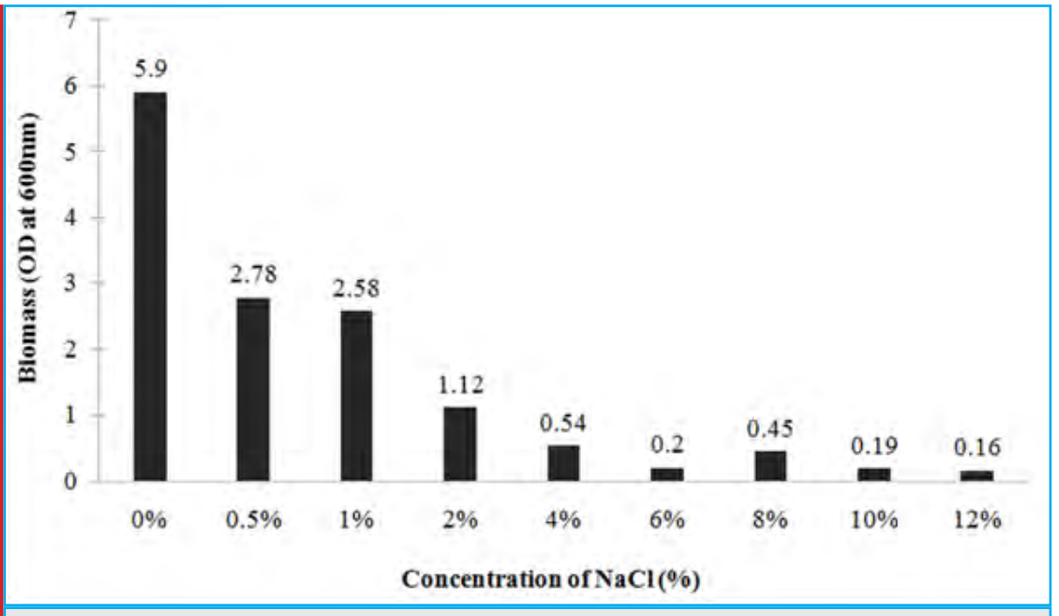

FIGURE 6. Growth profile of *Lysinibacillus* sp. KEI-3 strain at different salt (NaCl) concentration.

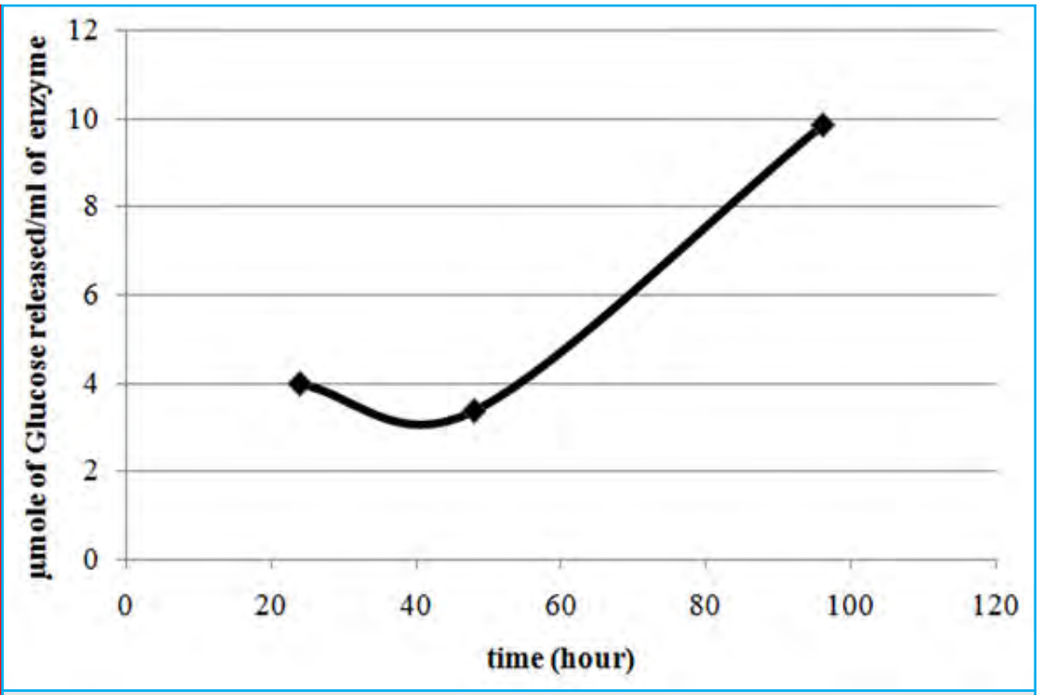

FIGURE 7. Cellulase production profile of *Lysinibacillus* sp. KEI-3 strain. Cellulase productivity is represented in the form of µmole of Glucose released per ml of cellulase produced.

DISCUSSION

70% of the planet's surface area is covered by oceans. The coastal environments support huge diversity of microbial life. But still only a small fraction of the species has been cultured and identified till date due to culture related problems. Heavy pollution has led to severe destruction of marine biological diversity (Abreo et al., 2015; Baum et al., 2015).Trawler fishing, pollution from industries and drainage system has led to increase in eutrophication which is further leading to change in aquatic ecosystem as well as destruction of habitats. This has led to decline in microbial biodiversity. So it has become imperative to identify and isolate the organisms and make a database, so that the information can be evaluated time to time to check for the loss of species in the marine world. Estuaries are dynamic in nature in terms of the nutritional content and the associated microbial population and this is mainly influenced by the convergence of fresh water and sea. The biochemical environment of the estuaries makes it ideal for availability of diverse microbial communities (Andersson et al., 2014; Zhang et al., 2014). In different parts of the world several studies have been undertaken to describe the microbial diversity along the estuaries and their physiochemical relation with the surrounding environment (Reed & Martiny, 2013; Sun et al., 2014 and Lallias et al., 2015).

Even though the eastern part of coastal India has several estuaries, no substantial studies have been undertaken to highlight the microbial diversity or the potentialities for bioprospecting. In this work both the aspects were covered, where pure cultures were isolated from soil samples collected from estuaries and screened on the basis of their ability to secrete cellulase enzyme. Morphological and biochemical characterization of the Isolate 3 showed similarity with *Bacillus* species. Phylogenetic analysis was done using 16s rRNA sequencing and the strain was identified to be *Lysinibacillus* sp.KEI-3.These soil bacteria are rod shaped and gram positive. *Lysinibacillus fusiformis, Lysinibacillus sphaericus, Lysinibacillus boronitolerans, Lysinibacillus macroides* and *Lysinibacillus xylanilyticus* are some of the strains that have been isolated and characterized previously. The strains belonging to this genus have several industrial importance such as xylan degradation (Lee et al., 2010), biodegradation of low-density polyethylene (Esmaeili et al., 2013), biotransformation of Indole to 3-Methylindole (Arora et al., 2015), biological pest control (Rojas-Pinzón & Dussán, 2017).

These characters make this genus an interesting target for microbiological studies.The biomass production

was optimized under several physical and chemical parameters such as temperature, media composition, carbon source, nitrogen source and salinity tolerance. The organisms showed high growth rate at 37°C in TB medium and SWGT medium. Bacterial species can utilize both pentose and hexose sugars (Cook et al., 1993; Kim et al., 2009; Liu et al., 2008). Each species has its own ability to breakdown and utilize several carbon sources. Also the preference of carbon source varies from species to species (Brückner & Titgemeyer, 2002; Görke & Stülke, 2008).Therefore the biomass yield was assessed with various carbon sources which showed that this species have a broad range of carbon preferences. They could well utilize both pentose as well as hexose sugars.

Organic as well as inorganic nitrogen sources are critical for growth of microorganisms (Wheeler & Kirchman, 1986). The carbon to nitrogen (C/N) ratio is important in a biological process (Cleveland & Liptzin, 2007). Microorganisms require a proper nitrogen supplement for metabolism during fermentation (Lin & Lay, 2004). Therefore the optimal nitrogen was characterized in order to obtain maximum biomass. It was observed that *Lysinibacillus* sp.KEI-3 could produce higher biomass when the media was supplemented with Sodium Nitrite. Since the Isolates could not grow well in Marine Broth (MB) due to its high salt content, therefore supplementing different concentrations of NaCl in the medium was used to check maximum salt tolerance. *Lysinibacillus* sp.KEI-3 had higher salt tolerance and it can grow even at 12 % NaCl.

Several *Bacillus* species are known to be cellulase producers (Irfan et al., 2017; Sanjeev et al., 2017), but very few reports are available on cellulase production by *Lysinibacillus* strains (Khianngam et al., 2014). Therefore submerged fermentation was done under the above-optimized conditions and it was observed that the cell could accumulate significant amounts of cellulase. Even though the total units of cellulase produced are low as compared to reported species, but it is a positive sign that the isolated strain is a cellulase producer. Further optimization of physical as well as bioprocess parameters could lead to accumulation of higher levels of cellulase at high cell density cultures.

In the present scenario, the carbohydrolytic bacteria or the lignocellulose degrading bacteria have a greater industrial demand given their application in saccharification of lignocellulose for biofuel production. In this context cellulase is the most common enzyme used in the cocktail for degradation of lignocellulosic biomass (Sindhu et al., 2016). Therefore there has been a continuous effort to screen and isolate efficient cellulase producing microbes. These strains are isolated from various geographical areas and also grown under various cheap and alternative substrates for production of enzymes. Apart from characterization of bacteria, process parameters as well as culture conditions are also being optimized to enhance bacterial biomass to increase the yield of cellulase. Optimization of media, carbon, nitrogen, salinity as well as temperature requirement led to increased production of cellulase in the *Lysinibacillus* sp.KEI-3.This isolated strain could further be screened for other enzymes like xylanase and pectinase, which would make it a potential strain for saccharification of lignocellulose biomass.

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Diversity analysis and characterization of antagonistic endophytic population from *Stevia rebaudiana*

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ABSTRACT

Antagonistic endophytic fungal community resident in medicinal plant *Stevia rebaudiana* Bertoni was studied at two sites within Bhopal, M.P. Among 339 recovered endophytic isolates from foliar tissues, 40 fungal isolates were found antagonistic against *Sclerotinia sclerotiorum*, casual agent of disease stem rot in stevia and soybean (Glycine max.). Antagonistic fungal population (40 isolates) consisted of 52.5% *Hyphomycetes* and 2.5% each of *Coleomycetes, Basidiomycetes, Ascomycetes* and *Sterile mycelia*. The percent colonization frequency of antagonistic endophytic community in foliar tissues ranged from 0.3%-5.3% whereas percent dominance was of the order, ranged from 2.31%-40.8%. Diversity analysis of the antagonistic endophytic population was determined in terms of Shanon index, Simpson index, Species eveness, Menhinick and Margalef richmess index .Antagonistic endophytic population was also evaluated for IAA production, siderophore and phosphorus solubilisation, considered as plant growth promotory attributes. Identification of the antagonistic endophytes was carried out by rDNAsequencing of the ITS region.

KEY WORDS: DIVERSITY INDEX, *S. SCLEROTIORUM*, ITS REGION, ANTAGONISTIC ENDOPHYTES, SEQUENCE PHYLOGENY

INTRODUCTION

Fungal endophytes possess huge diversity morphologically and biochemically (Strobel and Daisy, 2003). Endophytic fungi are known to reside in the tissues of plants above ground as well as below ground, parts of the plant (Zhang et al. 2006; Kusari et al. 2012). Endophytic fungi are an assemblage of microorganisms that chiefly belong to class *Ascomycetes* of kingdom fungi. A significant literature is available so far to show that these microorganisms, under laboratory culture conditions, produce numerous structurally diverse biologically active secondary metabolites that include antimicrobial substances. Different ecological factors such as seasonality, nearby vegetation and humidity influence the distribution of endophytic fungi in the host (Taylor

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et al; 1999; Toofanee and Dulymamode; 2002 Lumyong et al 2009, Dissanayake et al., 2016; Ratnaweera et al., 2017, Ratnaweera et al., 2018).

Stevia rebaudiana Bertoni is an herbaceous polyannual plant of the sunflower family (Fam. Asteraceae), generally known as candy leaf, sweet leaf, sweet leaf, or sugar leaf. The Stevia leaves also contain variety of glycosides compounds viz., flavonoid glycosides, Stevioside, Rebaudioside A, Rebaudioside C, coumarins, cinnamic acids, phenylpropanoids and some essential oils, (Midmore and Rank, 2002 Lavini et al., 2008).

Stevia rebaudiana Bert. Is a good source of sweeteners and is about 300 times sweeter than sucrose owing to presence of steviosides in its leaves. Previous studies have reported clearly the diversity of these fungal fungal endpphytes from *Stevia rebaudiana* which also indicates the presence of *Alternaria,, Aspergillus*, *Monodictys*, and *Curvularia* fungal genus from leaf of *Stevia rebaudiana* (Bert.). (Prakash et al., 2008; Madhumita and Chandra, 2013) Furthermore, these fungal isolates have been reported from almost all climatic regions of the globe viz., tropical, temperate and alpine (Arnold, 2007; Halmschlager et al 1993; Higgins et al 2007). The application of biocontrol agents has become one of the most promising tools for reducing the use of chemical pesticides in agriculture. The antagonism of biocontrol agent is based on different mechanisms i.e. nutrients, mycoparasitism, plant growth promotion and induction of the defense responses in plants (Howell, 2003, Sen et al, 2012; Hamzah et al, 2018).

In the present investigation the endophytes recovered from *Stevia rebaudiana* leaves have been tested for antagonistic abilities against *Sclerotinia sclerotiorum*, which is the major phytopathogen affecting varieties of crop plants in Central India (Prakash et al., 2008, Verma et al 2004).

Therefore, present investigation was carried out to understand the generic diversity of endophytic fungi in leaves of *Stevia rebaudiana* Bertoni and to compare the antagonistic endophytic assemblages in samples collected from two different sites in the same region. Thus a specific rationale for the selection of stevia plant for endophyte isolation and natural product discovery is used.

MATERIALS AND METHODS

Stevia rebaudiana **Bertoni:** *Stevia rebaudiana* Bertoni was selected as the target plant for isolation of fungal endophytes. Sampling was carried out from two sites. The first site was Misrod (23°16'N; 76°36'E), a village situated nearly 22 km away from the capital city Bhopal in the state of Madhya Pradesh. The second site of sampling was green house grown plants in the campus of (23°20'N; 77°45'E) Barkatullah University, Bhopal.

Samples Collection and Surface sterilization: Healthy and mature plants were carefully chosen for sampling and leaves were collected randomly. Plant material was brought to the laboratory in sterile bags. Surface sterilization of foliar tissues were done using reagents like 70% ethanol, 4% sodium hypochlorite and sterile distilled water for different time period for effective surface sterilization process. Leaves were thoroughly washed several times in sterile distilled water (SDW) for 5-10 sec then sterilized by exposing them to 70% ethanol for 2 min followed by treatment with 4% sodium hypochlorite for 2 min. The leaves were now immersed in sterile water (SDW) for 2-5 sec and allowed to dry on blotting paper.

Isolation of endophytic fungi from foliar tissues of plants: The surface sterilized leaf segments of 5mm size were placed on Potato dextrose Agar (Howksworth et al 1995), supplemented with chloramphenicol (0.2gl-1) to avoid bacterial contamination. Plates were incubated at 28±2 °C in for 3-5 days and were observed regularly for fungal growth.

Analysis of data: The colonization frequency (% CF) of endophytic fungi was calculated according to Hata and Futai (1995) and dominance as per Kumaresan and Suryanarayanan (2002). Utilizing the data of percentage colonization of fungal endophytes in leaves, for two sites. Simpson's Diversity indices and Shanon-Wiener indices were calculated. Species evenness and species richness was calculated according to Simpson (1949), Shanon and Weaver (1949), Ludwig & Reynolds (1988) and Margelef and Menhinick (1964)

In vitro antagonistic activity of fungal endophyte

Fungal isolates were screened for antagonism against *Sclerotinia sclerotiorum* by a dual culture technique on Potato Dextrose Medium (Szekeres et al, 2005).

Characterization of endophytes: The endophytic fungi were identified by their macroscopic & microscopic characteristics such as the morphology of the fruiting bodies and spore morphology. Morphological characterization was done on the basis of color, margin, reverse pigmentation & texture. (Rifai 1969).Antagonistic endophytic fungi are characterized functionally employing plate assays for amylase cellulase, Protease, pectinase, lipase and xylanase (Paterson & Bridge. 1994; Teather & Wood. 1982; Shakeri et al. 2007; Pointing. 1999; Sierra. 1957, Mishra et al, 2013, Aneja, 2003).

Plant growth promoting attributes: Plant growth promoting attributes of antagonistic endophyte were also studied. This included IAA (Brick et al. 1991) and siderophore production (Schwyn & Neilands. 1987) and phosphate solubilisation efficiency (Pikovskaya. 1948). Both qualitative and quantitative estimation were made.

Molecular identification of antagonistic endophyte: - Morphological identification of the organism was car-

ried out at National Fungal Culture Collection of India (NFCCI), Agharkhar Research Institute, Pune. For molecular identification, total genomic DNA of the endophytic fungus was isolated directely from actively growing mycelium growing in Potato dextrose broth (PDB), using CTAB method (Sambrook and Russel, 2001). DNA amplification was performed by PCR using primer pair ITS1: TCCGTAGGTGAACCTGCGG and ITS4: TCCTCCGCTTGATATGC (White et al. 1990).PCR was carried out according to the following protocol: initial denaturation at 95 °C for 5 min; denaturation at 95 °C for 1 min; annealing at 55°C for 45 sec; extension at 72 °C for 10 min; steps 2-4 were repeated 35 times. Sequencing of PCR product was carried at Xcelris Labs Ltd, Ahmedabad. The sequenced data was subjected to BLAST algorithm and submitted to Genebank for accession number. The potential antagonistic endophytes were submitted at National Agriculturally Important Culture Collection (NAIMCC), culture collection facility at ICAR-NBAIM, Maunath Bhanjhan (U.P).

Phylogenetic analysis: To know the phylogenetic relationship among the isolates and also to confirm their taxonomical status, certain ITS rDNA sequences were chosen from GenBank databases via BLAST search analysis. The sequences were chosen from the top 20 database hits obtained in the blast search by querying the obtained sequences individually. These sequences were aligned using CLUSTAL W 1.83 (Thompson et al., 1994). Phylogenetic trees were generated by neighbourhood joining method with 100 bootstrapping replicates using MEGA version 5.

RESULTS

Antagonistic Action and Diversity Analysis: A total of 339 recovered endophytic fungal isolates were screened for antagonistic ability against Sclerotinia sclerotiorum (culture obtained from Directorate of Soybean Research, Indore, M.P.) by using dual culture technique. The inhibition zone in dual plate assay averaged between 5 to 17 mm. Misrod field site harboured greater number of antagonists compared to the endophytes recovered from green house raised plants. Percent growth reduction of the pathogenic culture was recorded after 24 hrs. Isolate Aspergillus flavipes (NAIMCC-F-03153) strain 63 showed highest value of growth reduction i.e. 19% after 24 hrs followed by Alternaria alternata strain 99 and Aspergillus niger strain 89 (NAIMCC-F-03157) showed 17% and 18% growth reduction respectively (Fig 1); Least reduction of pathogen was recorded for isolate Alternaria brassicae strain 17 i.e. 6.9%. The percent growth reduction values ranged from 6.9% to 19%.

A total of 40 endophytic fungal isolates which showed antagonistic activity against *Sclerotinia sclerotiorum* consisted of 52.5% *Hyphomycetes* followed by 2.5% *Coleomycetes, Basidiomycetes, Ascomycetes* and Sterile mycelia each. The percent colonization in tissues samples ranged from 1% - 36.6% (site 1) whereas 1%-10.6% (site 2) (Fig 2) and percentage dominance of antagonistic endophytes ranged from 1.1%-40.6% (site 1) and 4.3%-45.3% at site2 (Fig 3). Diversity analysis of the antagonistic population was carried out which showed significant diversity index values at site 1 as compared to site 2, whereas Margalef & Menhinick's richness index value was maximum at site 2 as compared to site 1 (Table 1)

Characterization of antagonistic fungal endophytes

Morphological characterization of antagonistic fungal endophytes: Based on the morphology different antagonistic endophytic fungal isolates were recovered on PDA plate. Among 40 antagonistic endophytic isolates 62.5% showed reverse pigmentation, 15% showed velvety appearance on PDA plate while others appeared spory and cottony texture. Isolates *Aspergillus niger* strain 89

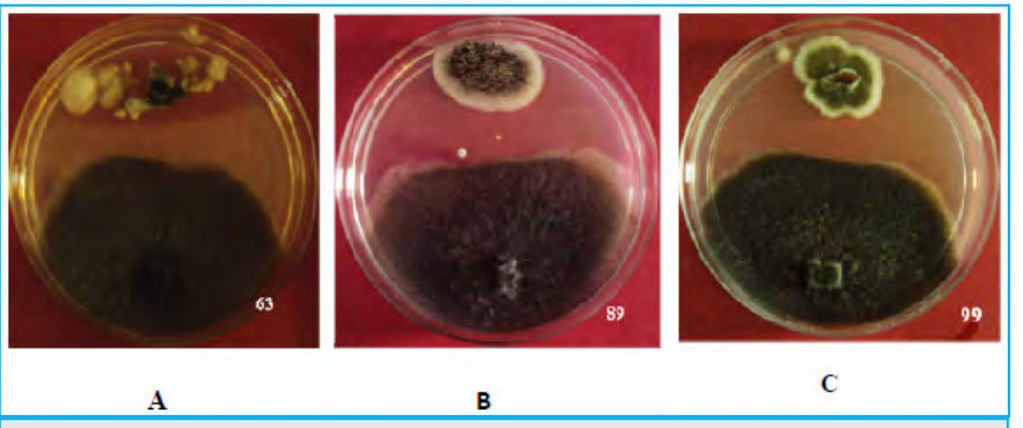

FIGURE 1. Dual culture assay shown by isolate A) Strain 63 B) Strain 89 C) Strain 99

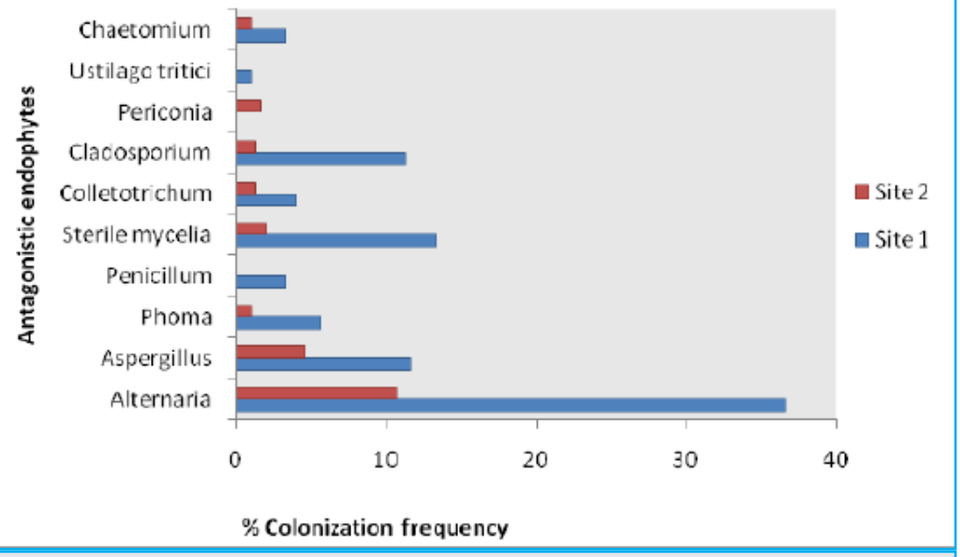

FIGURE 2. % Colonization frequency of the antagonistic endophytic population from both sites.

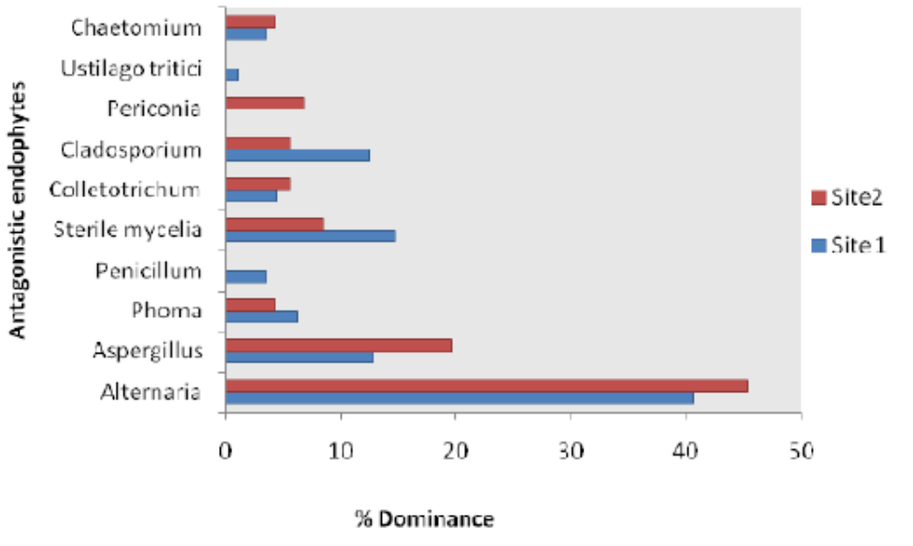

FIGURE 3. % Dominance of the antagonistic endophytic population from both sites.

(NAIMCC-F-03157), *Aspergillus niger* strain 88, *Aspergillus sp.* strain 37 (NAIMCC-F-03147), *Aspergillus flavipes* strain 63 (NAIMCC-F-03153) and *Aspergillus niger* strain 50 (NAIMCC-F-03151), showed dispersed growth on PDA medium.

Functional characterization of antagonistic fungal endophytes: The antagonistic fungal endophytes recovered from Stevia, were checked for their hydrolytic potential. They were screened for multiple enzyme activity on starch, pectin, lipid, carboxy methyl cellu-

Table 1. Diversity indices of two sites (Site 1 & Site 2)

Diversity indices	Site I	Site II
Simpson index (1-D)	0.821	0.7982
Shanon index (H')	1.856	1.781
Eveness (EH/s)	0.8001	0.8478
Menhinick S/√N	0.6305	1.121
Margalef S-1/ln(n)	1.378	1.638

lose, xylan and skim milk. Endophytes exhibited good amylolytic, cellulolytic, proteolytic, pectinolytic activities while xylanolytic and lipolytic activities were possesed by only few isolates. The confirmation of enzymatic activity was recorded by the presence of zone of clearance around the culture. *Alternaria alternata* strain 76 showed maximum zone of about 15 mm on starch agar plate while 11 mm zone was recorded for

Table 2. Plant growth promoting attributes of antagonistic endophytes

S.no	Isolates	Identity	IAA (µg/ml)	Siderophore (mg/ml)	% solubilising efficiency of Phosphate
1	SR/II/2	*Alternaria porri*	-	8.42±0.20	75
2	SR/II/4	*Alternaria alternata*	6.2±0.03	1.6±0.20	52
3	SR/II/5	*Alternaria brassicae*	-	3.79±0.05	71.4
4	SR/II/6	*Alternaria porri*	3±0.04	6.2±0.06	60
5	SR/II/42	*Alternaria sp.*	8±0.03	3.72±0.08	-
6	SR/II/45	*Penicillium mallochii*	6.3±0.05	4.2±0.04	32
7	SR/II/50	*Aspergillus niger*	4.8±0.01	-	6.25
8	SR/II/52	*Phoma sp.*	-	6.6±0.03	10
9	SR/II/54	*Phoma sp.*	4.6±0.02	8.7±0.03	20
10	SR/I/76	*Alternaria alternata*	-	3.2±0.02	5.4
11	SR/I/77	*Alternaria sp.*	5.5±0.03	6.9±0.02	22
12	SR/I/78	*Alternaria tenuissima*	6.5±0.02	8.02±0.03	11
13	SR/I/94	*Alternaria alternata*	3.8±0.01	2.8±0.03	8
14	SR/I/99	*Alternaria alternata*	5.3±0.3	-	36
15	SR/II/63	*Aspergillus flavipes*	4.6±0.04	11.12±0.16	22.2
16	SR/II/1	*Alternaria alternata*	-	17.09±0.1	-
17	SR/II/9	*Alternaria alternata*	9.8±0.6	7.45±0.12	17.3
18	SR/II/20	*Alternaria sp.*	6.3±0.06	8.03±0.11	70
19	SR/II/21	*Alternaria alternata*	-	8.03±0.11	50
20	SR/II/23	*Chaetomiumglobosum*	6.7±0.05	15.51±0.12	14
21	SR/II/24	*Alternaria alternata*	-	14.63±0.14	12
22	SR/II/26	*Alternaria alternata*	1.9±0.04	3.15±.10	10
23	SR/II/33	*Phoma sp.*	4.5±0.01	1.66±0.031	-
24	SR/II/36	*Alternaria brassicae*	8.2±0.7	27±20.22	65
25	SR/II/55	*Alternaria alternata*	-	4.42±0.14	14
26	SR/II/60	*Ustilago tritici*	5.5±0.04	9.51±0.17	-
27	SR/I/72	*Alternaria brassicae*	-	23±0.08	34
28	SR/II/81	*Alternaria alternata*	-	22±0.06	-
29	SR/I/85	*Alternaria alternata*	5.7±0.05	6.07±0.03	37
30	SR/I/95	*Alternaria alternata*	-	4.5±0.04	-
31	SR/II/100	*Alternaria alternata*	5.8±0.07	5.8±0.07	19
32	SR/I/83	*Alternaria alternata*	4.5±0.05	6.18±0.19	-
33	SR/II/18	*Phoma sp.*	-	1.3±0.02	85.7
34	SR/II/37	*Aspergillus sp.*	11±0.01	13.3±1.12	-
35	SR/II/46	*Aspergillus sp.*	6.8±0.06	8.06±0.07	-
36	SR/I/88	Aspergillus niger	8.1±0.2	-	-
37	SR/I/89	Aspergillus niger	11.2±0.4	6.72±1.4	-
38	SR/I/90	Alternaria alternata	-	-	35
39	SR/II/3	Sterile mycelia	-	8.42±0.20	85.7
40	SR/II/17	Alternaria brassicae	6.25±0.05	24.33±0.12	-

cellulolytic activity by isolate *Alternaria alternata* strain 26.Maximum pectinolytic activity was recorded by isolate *Alternaria alternata* strain 99, whereas isolate *Alternaria alternata* strain 4 (NAIMCC-F-03138), showed maximum zone (11 mm) of clearance on CMC plate. The isolates *Aspergillus sp*. strain 46, *Phoma sp*. strain18, *Phoma sp*. strain 33 and Alternaria alternata strain 94, *Aspergillus niger* strain 50, *Alternaria porri* strain 6 (NAIMCC-F-03139), and *Alternaria brassicae* strain 5 (NAIMCC-F-03140), showed minimum inhibition (2mm) on same substrate.

Plant growth promotiong attributes of antagonistic endophytes: The antagonistic endophytes were studied for plant growth promoting traits such as siderophore production, phosphate solubilisation, Indole acetic acid production. A comprehensive overview of the PGP traits of antagonistic endophytes is given in the Table 2. About 40% of the antagonistic endophytes were positive for siderophore production and the amount of siderophore produced ranged between 1.3-27 $mgml^{-1}$. On Pikovyaskya's agar, 31% of the antagonistic endophytes showed phosphate solubilisation; efficiency of P solubilisation ranged between 5.4%-85.7percent. Based on the results isolates *Phoma sp*. strain 18 and *Sterile mycelia* strain 3 were found to be most efficient P solubilizer. A total of 29% of the antagonistic endophytes were positive for IAA production which ranged between 1.9-11μgml^{-1} (Table 2).

Sequence analysis of ITS region of rDNA gene fragments

All the selected isolates produced a single PCR product with approximately 600 bp. Purification of the PCR product was performed using Banglore Genei purification kit and sequencing was performed by Xcelris Genomics, Ahemdabad using the same set of primers as mentioned earlier. The full length sequences of the isolates were compared with the related fungal sequences in the GenBank databases and sequence similarities were determined using BLAST sequence similarity search tool (Altschul et al., 1990). The sequences of ITS region of rDNA gene of the fungal endophytes were deposited in the GenBank and given accession numbers (Table 3).

Phylogenetic analysis

To know the phylogenetic relationship among the isolates and also to confirm their taxonomical status, certain ITS rDNA sequences were chosen from GenBank databases via BLAST search analysis. The sequences were chosen from the top 20 database hits obtained in the blast search by querying the obtained sequences individually. These sequences were aligned using CLUSTAL W 1.83 (Thompson et al., 1994). Phylogenetic trees were generated by neighbourhood joining method with 100 bootstrapping replicates using MEGA version 5.(Fig:4)

DISCUSSIONS

Characterization of fungal endophytes from *Stevia rebaudiana* Bertoni was considered important because only few attempts have been made earlier to characterize fungal endophytes from this useful plant, (Begum et al 2008; Kumari and Chandra 2013). Various fungal diseases have been reported to pose serious problems to *S.rebaudiana* Bertoni commonly known as Stevia, a popular non calorific sweetener. These include *Verticillium dahlia* on leaves (Farrar et al., 2000), *S. sclerotiorum* reported in Canada (Chang et al., 1997), *S. rolfsii* in India (Kamalakannan et al., 2007) and *Botryis cinerea* in Itlay (Garibaldi et al., 2009). Sclerotinia stem rot (white mold) of soybean was first reported in Hungary in 1924 and since has been reported in Argentina, Brazil, Canada, India, Nepal, South Africa and United States. A great economic loss to crop plants by different phytopathogens results in low yields. The species composition of the endophytic assemblage and frequency of infection varies according to host species and site characteristic such as elevation, exposure, associated vegetation, tissue type (Fisher et al., 1994) and tissue age (Fisher et al., 1986; Rodrigues, 1994).

In the present study, foliar endophytic microorganisms were studied using various diversity indices viz Simpson index (Simpson, 1949), Shannon index (Shannon and Weaver, 1949), evenness index (Ludwig and Reynolds, 1988) and richness index (Margalef, 1958; Menhinick, 1964). Higher value of Shannon index and evenness index with lower values of Simpson index indicated greater diversity. Bills et al. (2002) described a significant difference between tropical and temperate endophytes, in terms of their ability to produce number of bioactive natural compounds isolated from endophytes. This observation suggests the importance of the host plant in influencing the general metabolism of endophytic microbes.

Among 339 recovered endophytic fungal isolates, 40 isolates were screened out as potential antagonistic endophytes against broad spectrum plant pathogen *S. sclerotiorum* following using dual culture technique. It was found that maximum numbers of antagonistic endophytes were recovered from site1 as compared to site 2. This may be explained as site 1 is open agricultural field which was exposed to wide variety of phytopathogens so in order to overcome these pathogens several bioactive compounds are produced by them whereas site 2 is closed area which was exposed to limited number of phytopathogens. Sadrati et al. (2013) screened 20 endophytic fungi from wheat which showed antimicrobial activities against 12 pathogenic bacteria, yeast and two phytopathogenic fungi. Percentage growth inhibition ranged between 6.9%-19% after 24 hr of incubation.

Table 3. Genetic relatedness of GenBank to antagonistic fungal endophytes recovered from Stevia rebaudiana Bertoni using ITS rDNA gene sequence analysis

Isolate No.	Site	Accession no.	% Similarity	Organism
SR/II/2	1	KJ592050	99%	*Alternaria porri*
SR/II/4	1	KJ592051	100%	*Alternaria alternata*
SR/II/5	1	KJ592052	99%	*Alternaria brassicae*
SR/II/6	1	KJ603463	98%	*Alternaria porri*
SR/II/42	1	KJ713969	100%	*Alternaria sp.*
SR/II/45	1	KJ713970	99%	*Penicillium mallochii*
SR/II/50	1	KJ648618	100%	*Aspergillus niger*
SR/II/52	1	KJ713971	99%	*Phoma sp.*
SR/II/54	1	KJ648619	99%	*Phoma sp.*
SR/I/76	1	KJ713972	99%	*Alternaria alternate*
SR/I/77	2	KJ713973	100%	*Alternaria sp.*
SR/I/78	1	KJ728832	100%	*Alternaria tenuissima*
SR/I/94	1	KJ728833	100%	*Alternaria alternate*
SR/I/99	1	KJ728834	99%	*Alternaria alternate*
SR/II/63	1	KF671231	94%	*Aspergillus flavipes*
SR/II/1	1	KJ728835	100%	*Alternaria alternate*
SR/II/9	1	KJ735925	99%	*Alternaria alternate*
SR/II/20	1	KJ728836	98%	*Alternaria sp.*
SR/II/21	1	KJ728837	99%	*Alternaria alternata*
SR/II/23	1	KJ728838	100%	*Chaetomiumglobosum*
SR/II/24	1	KJ728839	99%	*Alternaria alternate*
SR/II/26	1	KJ728840	100%	*Alternaria alternate*
SR/II/33	1	KJ728841	100%	*Phoma sp.*
SR/II/36	1	KJ728842	99%	*Alternaria brassicae*
SR/II/55	1	KJ728843	99%	*Alternaria alternata*
SR/II/60	1	KJ735919	100%	*Ustilago tritici*
SR/I/72	1	KJ735920	99%	*Alternaria brassicae*
SR/II/81	1	KJ735921	100%	*Alternaria alternata*
SR/I/85	1	KJ735922	100%	*Alternaria alternata*
SR/I/95	1	KJ735923	100%	*Alternaria alternata*
SR/II/100	1	KJ735924	100%	*Alternaria alternata*
SR/I/83	2	KJ748009	100%	*Alternaria alternata*
SR/II/18	1	KJ748010	100%	*Phoma sp.*
SR/II/37	1	KJ767528	100%	*Aspergillus sp.*
SR/II/46	1	KJ767529	100%	*Aspergillus sp.*
SR/I/88	1	KJ767530	100%	*Aspergillus niger*
SR/I/89	1	KJ767531	100%	*Aspergillus niger*
SR/I/90	1	KJ767532	100%	*Alternaria alternata*
SR/II/3	1	NS	NS	*Sterile mycelia*
SR/II/17	1	KJ767533	100%	*Alternaria brassicae*

1= Misrod Agriculture Field 2= Green House NS= Not Sequenced

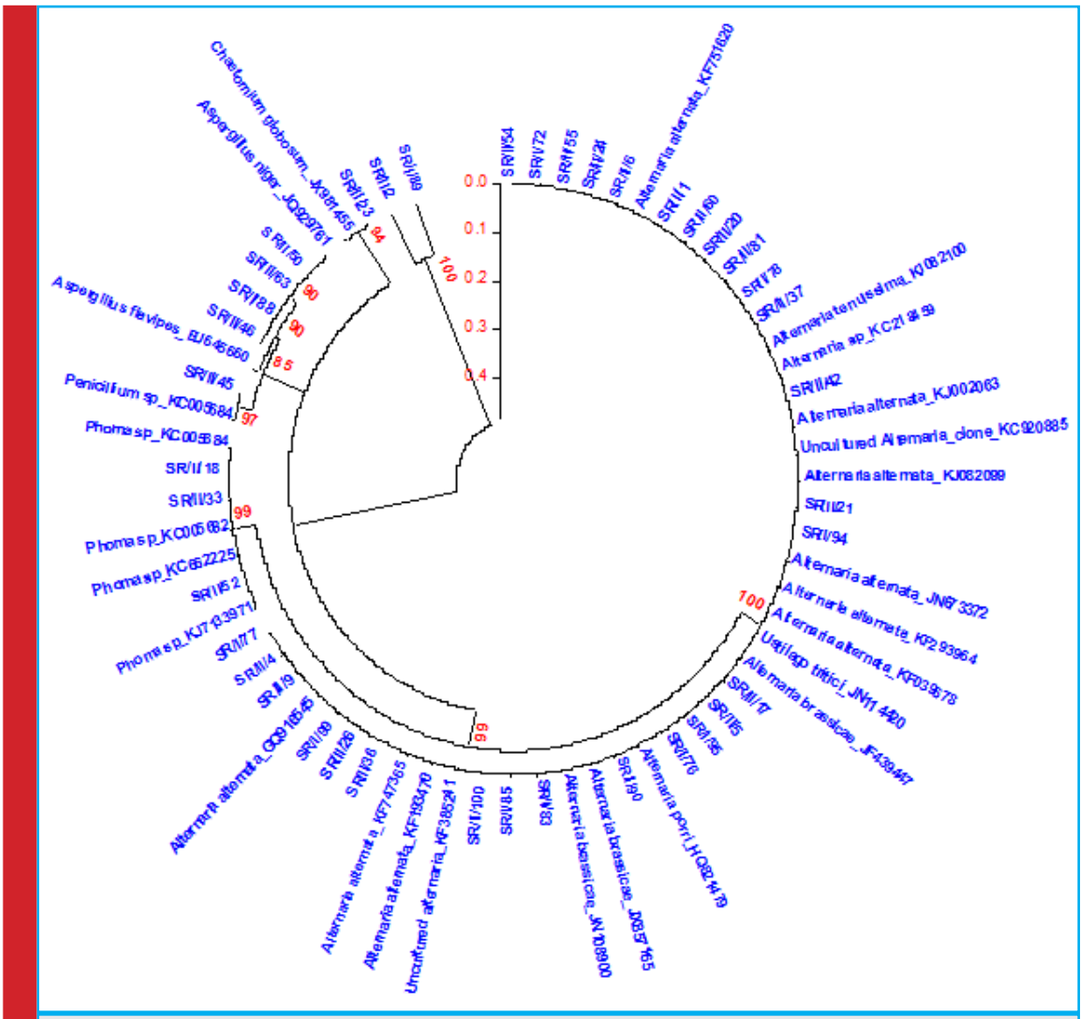

FIGURE 4. Phylogenetic tree generated on the basis of ITS rDNAsequences of antagonistic endophytic fungal isolates aligned with sequences in GenBank databases

Diversity analysis of the antagonistic endophytic population showed significant diversity index values. Maximum antifungal activity against *S. sclerotiorum* was recorded by *Aspergillus flavipes* strain 63 followed by strain *Aspergillus niger strain* 89 and *Alternaria alternata* strain 99 after 24 hrs of incubation.

For instance, site1 is open agricultural field with surrounding vegetation like wheat and soybean, which favours the establishment of endophytic colonization whereas site 2 is devoid of natural open conditions of environment which seems to be the major factor for tissue specific fluctuations in the recovery of endophytes The increased species richness in foliar tissues may be a result of super infection of the leaves overtime by airborne inocula (Carroll et al., 1977; Suryanarayanan and Vijaykrishna, 2001).

The 40 antagonistic endophytic fungal isolates were studied for morphological, functional and genotypic characterization. In the present study, significant functional diversity was observed among the antagonistic endophytes with respect to their hydrolytic potential viz. amylolytic, cellulolytic, lipolytic, proteolytic, pectinolytic and xylanolytic activities. 19% of the antagonistic endophytic fungal population showed amylase, cellulase, pectinase and protease production whereas 17% were xylanase producing and rest 7% were lipase producers. Fifty fungal strains isolated from medicinal plants (*Alpinia calcarata*, *Bixa orellana*, *Calophyllum inophyllum* and *Catharanthus roseus*) showed 64% were lipase producers, 62% were amylase and pectinase whereas 32% were cellulase and 30% were laccase producers (Sunitha et al 2013). Begum et al. (2008) reported

that majority of the endophytes from Stevia leaves were cellulolytic in nature. Suganthi et al. (2011) isolated and characterized *Aspergillus niger* (BAN 3E) out of five fungal isolates as the most potent α-amylase producer. Sidkey (2011) found endophytic strain F2Mbb to produce extracellular amylase.

These potential antagonistic endophytes were screened for plant growth promoting attributes like siderophore production, Indole acetic acid production and phosphate solubilisation. In present study, about 40% of the antagonistic endophytes were positive for siderophore production and amount of siderophore produced ranged between 1.3-27 mg ml^{-1}, 31% of the antagonistic endophytes showed positive results for phosphate solubilisation. Phosphate solubilisation efficiency ranged between 5.4-85.7% whereas 29% of the antagonistic endophytes were recorded positive for IAA production between the range 1.9-11 µg ml^{-1}.Certain endophytes were observed to improve the ecological adaptability of host enhancing their tolerance to environmental stress and resistance to phytopathogens (Kimmon et al., 1990; Struz et al., 1999). In a study performed on *Absidia corymbifera*, fungi isolated from rhizospheric soil, were found to produce siderophore in the range of 4-4.55 µg ml^{-1} (Holzberg and Artis, 1983). Maliha (2004) found *Aspergillus flavus, Aspergillus niger* and *P.canescens* as the most potent phosphate solubilizers, (Bilal et al, 2018).

Sequence analysis revealed that majority of fungal endophytes belonged to *Alternaria alternata* followed by *Aspergillus niger, Phoma, Chaetomium globosum,* and *Ustilago tritici*. Mandyam et al. (2010) employed sequencing of ITS region for studying Dark septate endophytes (DSE) in annually burned tallgrass prairie. In a nut shell, present investigation has shown that *Stevia rebaudiana* Bertoni harbours a good deal of antagonistic fungal endophytic community. These endophytes have exhibited various characteristics features which may pose better fitness to Stevia plant and reveal ecological significance of endophyte- host relationship.

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Genetic basis of poor scholastic performance among children: A review

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ABSTRACT

This paper presents concise review on selective pioneering works and recent progress on the topic genetic basis of poor scholastic performance among children. Formal school education plays a great role in everyone's life. Poor scholastic performance may be the result of Intellectual disability or mental retardation which manifests under age 18. Interaction of certain genes and environmental conditions can result in intellectual disabilities. This provides ample definitions of concepts, classification, causes and consequences, prevalence, and involvement of chromosomes in mental disability cases. This reveals that the magnitude of genetic variations in mental deficiency and scholastic performance of children suggests strong genetic component.

KEY WORDS: SCHOLASTIC PERFORMANCE SCHOOL CHILDREN GENETIC REASONS

INTRODUCTION

Formal school education plays a great role in everyone's life. Unsettled poor scholastic performance poses instant and lifelong unfavorable effects on a child and adolescent's growth and cognitive development. Optimum cognitive development of a child influences his/her learning behavior which is influenced by interaction of family, society, psychology, education, and economical atmosphere of the child. Poor scholastic performance is observed among some children (Carlson and Corcoran, 2001; Landry, 2014). Interactions of Gene and environment can result in different disease phenotypes and intellectual abilities (McKusick, 1983; Deary, 2012). All traits of an individual are products of heredity and environment interaction. Individuals with varied genotypes appear differently by exposure to the common environmental factors (Davies, 2016). Intelligence was one of the first human traits to be the target of genetic research even before psychology emerged as a scien-

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tific field. The correlation between DNA sequence and behavioral differences such as intelligence is considered causal because DNA variations can lead to behavioral differences but behavioral differences do not change DNA sequences (Deary et al., 2006; Sniekers et al., 2017).

Intellectual disability and Poor Scholastic Performance

The "poor scholastic performance" is a broad term and defined differently by scholars. Okoye (1982) defined poor scholastic performance as one in which a student is not successful in attaining standard performance in a specified evaluation exercises involving a test, an examination or a set of constant assessment. Poor scholastic performance may be the result of Intellectual disability or mental retardation which manifests under age 18. American Association on Mental Retardation (AAMR) and American Psychiatric Association (APA) define mental retardation on the basis of certain formulations developed by them. This refers to extensive limitations in functioning characterized by radically sub-average intellectual level, existing concurrently with limitations in two or more of the following adaptive skill areas: communication ability, self-care, social skills, self-direction, community use, health and safety, leisure, home living and work (Luckasson et al. 1992).

The American Psychiatric Association (APA) is responsible for naming, defining, and describing mental disorders. Fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), APA changed the term mental retardation and proposed the new term intellectual disability or intellectual developmental disorder.

Definition of Concepts

Defining mental retardation remains a challenge and a matter of controversy. Ever since people have been able to distinguish mental retardation from other forms of mental disability, a central theme of definitions has concerned the failure of mentally retarded persons to adapt adequately to their surroundings. Older definitions were couched in terms of adult behavior, and there was a tendency to avoid precise criteria for deciding in borderline instances.

Many writers have attempted to specify quantitative standards for deciding mental subnormal level. The most widely used objective criterion of this sort has been the score obtained on a standardized test of intelligence such as the Stanford-Binet Intelligence Scale or the Wechsler Intelligence Scale for Children (WISC). In 1916, Terman introduced a grouping of ability according to IQ's obtained on the Stanford-Binet (Terman and Merrill, 1937). This system became widely used and, in fact, became the standard classification system (Table 1). An IQ of 70 has gained considerable popularity as a cutoff score for the retarded group.

Doll (1953) provided more specific definition. In addition to the element of social adaptation, he emphasized the emergence of handicap in childhood, its constitutional nature and its incurability. He considered some criteria generally considered essential to an adequate definition and concept. These involve social incompetence, mental sub-normality, developmentally arrested, obtained at maturity, constitutional origin and essentially incurable. Tredgold (1956) defined mental deficiency as a state of incomplete mental development of such a kind and degree that the individual was incapable of adapting himself to the normal environment of his fellows in such a way as to maintain existence independently of supervision, control or external support.

The American Association on Mental Deficiency defines mental retardation as a significantly sub-average general intellectual function existing concurrently with deficits in adaptive behavior, and manifested during the developmental period. In this definition the retarded person is judged in terms of his success with the developmental tasks appropriate for his age: in the preschool period sensorimotor behaviors assume greatest importance, while during the school years academic ability is of first interest, and during adulthood economic independence and social recognition (Suess et al., 1983; APA, 2000). Furthermore, this definition makes it clear that a designation of mental status should be a description of present behavior and implicitly disowns the notion of potential intelligence.

There are a number of difficulties with these scoring criteria. Because an IQ is simply a score obtained on the basis of a restricted sample of behavior, there are significant limitations as to what can or should be expected of it, even if the tests are perfectly reliable and children are always able to put forth their best efforts. Furthermore, no cutoff score will ever be adequate to define mental retardation independent of the setting in which the individual finds himself. Different skills and abilities are required at different ages and in different environments. Retardation must therefore be gauged in large part against current environmental demands, (Reschly et al., 2002).

Apart from this, the cutoff scores for measures of intellectual function are better recognized than the cutoff scores for measures of adaptive behavior. There is open agreement in the major diagnostic systems that performance on the intellectual dimension must be approximately two or more standard deviations below the population mean, which translates into an IQ score of 70 or less on measures with a mean of 100 and a standard deviation of 15 (Reschly et al. 2002; Greene et al., 2004).

Classification systems

Mentally Retarded or intellectually disabled individuals comprise a very heterogeneous group both in their

Table 1. showing WHO List of Causal Factors of Mental Retardation

S. No.	Causal factors
1.	Infections and intoxications
2.	Trauma and physical agents
3.	Disorders of metabolism, growth or nutrition
4.	Gross brain damage (postnatal)
5.	Diseases or conditions due to unknown prenatal influences
6.	Chromosome abnormalities
7.	Prematurity
8.	Major psychiatric disorder
9.	Psycho-social (environmental) deprivation
10.	Other and unspecified

Source: WHO: Mental health: strengthening our response (www.who.int/news-room/fact-sheets/detail/mental-health-strengthening-our-response)

behavior and in the causes of their deficiency. Different classification systems have been proposed in the past to bring some order in this disarray. Most systems have approached the problem from one of three viewpoints: severity of the handicap, etiology of the symptoms, and the symptom collection.

Additional criteria for classification

Persons with mental retardation can also be grouped by age, an important criterion in education and longitudinal evaluation. Mainly for purposes of management, mental retardation can also be subdivided according to the biological syndrome. This classification offers advantages for special training and schooling in mentally retarded patients with associated deficits such as blindness, deafness, and spina bifida, (Halgin and Whiteborne, 2005).

Causes and Consequences

The causes of poor scholastic performance can be broadly classified into two groups that involve genetics and environment. The present study exclusively aims to focus on genetic causes of PSP however, discussion on medical and varied environmental factors remains imperative. There may be genetic basis of various medical problems associated with PSP, and many problems are purely environmental in their origin. Poor scholastic performance (PSP) shows multiple etiologies. Many reasons are responsible for poor scholastic performance of children involving specific learning disability, attention deficit hyperactivity disorder, low IQ level, emotional problems and psychiatric disorders. Other reasons involve a poor socio-cultural home environment and additional environmental causes, (Bruno and Njoku, 2012).

The causes of PSP can be further divided into extrinsic or environmental and intrinsic or individual factors. School difficulty (SD) and learning disability (LD) are two different manifestations of some school attending children. Former is related to pedagogical difficulties. Apart from diseases and related disabilities, pedagogical difficulties can also pose poor scholastic performance. It is extrinsic in nature with no possibilities of organic impairment (Siqueira and Gurge-Giannetti, 2011). Environmental reasons may infuse lack of interest in studies and distraction among some children may results in disappointment, frustration, low self-esteem and failure (Karande and Kulkarni, 2005).

Emotional causes are also important while considering poor scholastic performance being secondary to environmental factors involving lack of inspiration, low self-esteem and lack of sympathy and unresponsiveness (Valiente et al. 2012). It is argued that emotions influence school performance of children and that integrating cognition and emotion can demonstrate school performance and scholastic achievement in early formal education (Blair, 2002; Raver, 2002). Therefore, for a flourishing learning process, numerous cognitive skills associated with proper opportunities are essential.

Present work exclusively focuses on genetic causes of Poor scholastic performance hence; this largely involves Mental Deficiency (MD) which is again a developmental disability characterized by sub-mental level or lower than average intelligence of the age of a child. This is chiefly associated with biological causes that may show developmental delay or/and involvement of genes or chromosome. The investigation of the genetic basis of mental deficiency focuses mainly on identification of smaller and smaller chromosome variations associated with disease, (Raynham et al., 1996; Lucy Raymond and Tarpey, 2006).

The modest beginning of the investigation of the genetic basis of mental deficiency started long back in 1938 with a preliminary study of patients confined to hospital institutions (Penrose, 1938). In later years focus was on identification of smaller and smaller chromosome variations associated with disease (Raynham et al., 1996; Raymond and Tarpey, 2006).

Abnormal development of a child that leads to mental retardation may be due to trauma before birth caused by an infection or exposure to alcohol, drugs, or other toxins and trauma during birth caused by deprivation of oxygen or premature delivery of a new born child. Inherited disorders involve point mutation(s) and gross chromosomal abnormalities. Certain point mutations cause metabolic disorders that lead to mental retardation phenotype such as phenylketonuria (PKU). On the

other hand, chromosomal abnormality such as Down syndrome demonstrates peculiar morphological and abnormal behavioral traits. The average IQ of matured persons with Down syndrome remains 50 that remain widely variable, (Malt et al. 2013).

Prevalence

Social integration of a moderately mental retardate will be more difficult in a competitive, industrialized community than in a rural environment with the long-term support of an old fashioned, extended family (Durkin et al. 1995). Almost all studies dealing with mental subnormality in children report a higher incidence in males than in females. Moreover, in addition to the data collected from population and institution surveys, recent studies of family pedigrees more specifically demonstrate that X-linked recessive disorders represent a substantial proportion of mentally retarded males (Raymond, 2006). The prevalence of mental retardation is influenced by a great number of environmental factors such as community, age, racial and ethnic background, geographic region, and sex (Hernandez and Blazer, 2006).

According to WHO estimates globally more than 450 million people suffer from mental disorders. Currently, mental and behavioral disorders account for 12% of the global burden of disease. This is likely to increase to 15% by 2020. The major proportions of mental disorders come from low- and middle-income countries. The problem is further complicated by a lack of adequate trained manpower and a low priority of mental health in health policy (Reddy et al. 2013). The severely retarded are mostly identified before the age of one year, especially in the presence of physical abnormalities such as hydrocephaly, spasticity, and sensorial disturbances. Mildly mentally retarded individuals with IQ's ranging between 50 and 70 are recognized at school age (Boat and Wu, 2015).

In India, mental disorders have a prevalence of 1.05%. Urban population has slightly higher rate being at 1.1% as compared to rural being at 1.008%. Age was found to be highly correlated with prevalence among children of rural areas (Lakhan et al. 2015). Once the critical period of adolescence and school attendance is over, however, many of the mildly mentally handicapped are assimilated into society and join the ranks of the dull-normal, living for the most part in marginal socio economic circumstances.

Genetic Disorders

Genetic disorders are divided into two main groups. The first group includes chromosome disorders, such as Down syndrome, which may involve an entire chromosome including thousands of genes, while the second group involves only a single gene. Single gene disorders are divided into three main categories based on the mode of inheritance of the abnormal gene. The categories are autosomal recessive, autosomal dominant, and X-linked (Thompson and Thompson, 1986; Pradhan et al., 2011).

Involvement of Chromosomes in Mental Disability

There are genetic components to mental disability. Examination of persons with chromosomal variations and mental disabilities may be a way of overcoming difficulties faced with the proper diagnostic processes. Unfortunately, chromosomal analysis is rarely undertaken in subjects with psychiatric disorders. However, the rate of chromosomal abnormality has significantly increased in persons with learning disability, and may be as high as 20% in those with mild learning disability (Gostason et al. 1991).It has been established in many other medical conditions with a genetic basis that chromosomal variations, either by direct gene disorder or by positional effects, can produce identical or similar phenotypes to those caused by point mutations and their existence has greatly facilitated the physical mapping and cloning of candidate genes (Collins, 1992, 1995).

Once a chromosomal anomaly is detected in a subject with mental disability, it may be considered non-coincidental and related to the disorder if one or more of the following criteria are met: (a) the chromosomal abnormality is rare and there are independent reports of the abnormality being associated with mental disability; (b) there is proximity of the abnormality with a region of suggestive linkage findings; or (c) there is co-segregation of the abnormality with mental disability within the patient's family, (Evans et al. 2001).

Some variations in chromosomes are very small and they only involve a single gene called single gene disorders. However, when variations in chromosomes are large enough and can be seen under light microscope, they are called chromosome anomalies or aberrations. There are many types of chromosome anomalies. Chromosome anomalies usually occur when there is an error in cell division following meiosis or mitosis. They can be organized into two basic groups viz. Numerical anomalies (aneuploidy or an abnormal number of chromosomes) and Structural anomalies. Numerical anomalies occur due to nondisjunction where abnormal numbers of chromosomes may find their way into gametes, and a disorder of chromosome numbers may result. Alteration in chromosome structure can take several forms described as under:

- Deletions: A portion of the chromosome is missing or deleted.
- Duplications: A portion of the chromosome is duplicated

- Translocations: A portion of one chromosome is transferred to another chromosome. There are two main types of translocations:
  Reciprocal translocation: Segments from two different chromosomes have been exchanged.
  Robertsonian translocation: An entire chromosome has attached to another at the centromere
- Inversions: A portion of the chromosome has broken off, turned upside down, and reattached, therefore the genetic material is inverted.

There are two main types of Inversions:
  Paracentric inversions: Both breaks occur in one arm of the chromosome and do not include the centromere
  Pericentric inversions: Breaks occur in each arm of the chromosome and involved the centromere

- Insertions: A portion of one chromosome has been deleted from its normal place and inserted into another chromosome.
- Rings: A portion of a chromosome has broken off and formed a circle or ring. This can happen with or without loss of genetic material.
- Isochromosome: Formed by the mirror image copy of a chromosome segment including the centromere.

The chromosomes observed at metaphase stage possess two chromatids called sister chromatids. Chromatids of two different chromosomes are called non-sister chromatids. Conventionally, all the chromosomal aberrations are broadly divided into two groups.

Chromosome-type: In this type, breaks and re-joins always involve both sister-chromatids at any one locus.

Chromatid-type: In this type, breaks and re-joins always involve only one of the sister-chromatids at any one locus.

Common Chromosomal Anomalies in Mental Deficiency

Down syndrome

Down syndrome is unique in its prominent role in exploring biology of mental retardation for the first time in 1866 by John Longdon Down whose contribution was significant in understanding biology of normal and abnormal development (Down, 1866). The discovery of an extra 21 chromosome (trisomy 21) in the cells of individuals with Down syndrome exhibiting 47 chromosomes in place of 46 normal numbers by Professor Lejeune in 1959 established role of chromosome variations in development.

This was the discovery of chromosome aneuploidy in man that firmly established study of chromosome called cytogenetic as bona fide medical discipline (Smith and Warren, 1985; Patterson, 2009). Karyotype of normal human exhibits that chromosome 21 is one of the smallest autosomes, comprising nearly 1.9% of human DNA, Non-disjunction of this autosome during formation of the gametes at meiosis I or meiosis II in one of the parents result in Down syndrome. Down syndrome is the most common genetic form of mental retardation followed by X-linked mental retardation.

Fragile X syndrome

Fragile sites are heritable points on a chromosome which are susceptible to breakage and are consistently found on certain human chromosomes (Sutherland, 1982a, 1982b). These sites may represent structural chromosome mutations. Fragile X syndrome (FXS) causes learning disabilities and cognitive impairment. Usually, the penetrance of this genetic condition is higher in males as compared to females because males are hemizygous having single X-chromosome (McKusick, 1983). The maximum numbers of single genes that cause mental retardation are located on X chromosome. The first identified gene was *FMR1* that causes fragile X syndrome being the commonest single gene abnormality. The fragile site on the long arm of the X (Xq 27.3) is associated with a form of familial X-linked mental retardation (Lubs, 1969). It has been estimated that from one third to one half of all families with (nonspecific) X-linked mental retardation express the fragile site in some proportion of their cells (Brookwell et al, 1982).

The fragile site can be detected in chromosome preparations from lymphocytes grown in tissue culture media lacking folic acid and thymidine. Specific culture conditions can significantly alter the frequency with which the fragile site is expressed. Female carriers of this disorder may or may not express the fragile Xj some express it in only a small number of their cells. Thus, Xq fragile site demonstration in such carriers and in some affected males may be difficult due to a low level of expression. As with most X-linked recessive disorders, carrier detection is an important: aspect of genetic counseling for families with this syndrome.

Males with fragile X syndrome show mild to moderate intellectual disability whereas considerable proportions of females with this disorder being nearly one-third remain intellectually disabled. Majority of males and nearly half the females with fragile X syndrome show characteristic morphological features involving long and narrow face, prominent jaw and forehead, flat feet, large ears and in males additionally enlarged testicles after puberty. A mutation of FMR-1 known as fragile-X mental retardation gene located on the X- chromosome causes this syndromic condition. The FMR1 gene codes a protein known as fragile X mental retardation protein (FMRP) required for normal brain development. Incidence was noted in all races and ethnic groups. Nearly

10% affected males have severe intellectual disability (Hagerman and Hagerman, 2002).

In fragile X syndrome, CGG pattern in a part of DNA in FMR1 gene is repeated many times. In majority of persons, the number of repeats remains small at 5 to 44 repeats, which is common whereas; when the number of repeats is very high being greater than 200 repeats, the gene turns off and protein production is halted leading to development of FXS which is also known as trinucleotide repeat disorder. This is heritable condition transmitted from parents. Intermediate number of repeats at nearly 45 to 54 may have somewhat higher probability of having some symptoms but they do not have fragile X syndrome (Willemsen et al. 2011).

Sequence repeats in the range of 55-200 do not develop FXS but there may be development of other condition known as fragile X-associated disorder. Couple with premutation can transmit this to their children with the same condition or full mutation leading to development of FXS (Gallagher and Hallahan, 2012).

The global prevalence of fragile X syndrome (FXS) in males is estimated nearly 1 in 4,000 while in females it is nearly 1 in 5,000. It has been demonstrated in both animal and human studies that changes in the environment radically impact behavior, (Restivo et al. 2005). A peaceful high quality home environment has been found associated with fewer autistic behaviors, higher IQ scores and better adaptability in children with Fragile X syndrome (Glaser et al. 2003).

The magnitude of genetic variations in mental deficiency and scholastic performance of children suggests strong genetic component. Genetic effects that influence general and verbal cognitive ability are largely responsible for scholastic performance. Remedial intervention remains a more immediately attainable goal while Subsequent research will entail more genetic analyses leading to identification of genes that influence academic achievement.

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Biochemical alterations due to carbaryl exposure in glucose content of liver and alimentary canal of *Clarias batrachus*

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ABSTRACT

Pesticides provide useful tools to agriculturists and hygienist for crop protection and disease control. The use of pesticides has undoubtedly increased the agricultural output, but on the other hand, they have also poisoned the aquatic environment. Present study indicates that carbaryl is highly toxic to fish. It produces severe damage to the organs: alimentary canal and liver, concerned with the digestion and absorption of glucose. Due to this, glucose metabolism was significantly affected throughout the exposure period. Carbaryl intoxication reduced glucose content both in liver and alimentary canal after an initial increase, generally more energy is needed to mitigate any stress conditions. This may be obtained from glucose which is one of the most available sources of energy. All these changes in glucose content may be due to cumulative effect of enzymes, hormones, and metabolic disturbance caused by the pesticide.

KEY WORDS: HYGIENIST, TOXIC, MITIGATE, BIOCHEMICAL, HEALTH

INTRODUCTION

Pesticides provide useful tools to agriculturists and hygienists for crop protection and disease control (occupational Environmental Health 1997). The use of pesticides has undoubtedly increased the agricultural output, but on the hand they have also poisoned the aquatic environment. Carabmates are comparatively of recent development in the field of pesticides. Carbaryl, which was introduced in 1956 under the trade name “Sevin” is the most widely, used carbamate today (Oluah and Agatha 2014). The influence of pesticides on the inhabitants of the water systems may manifest itself. Both direct toxification (acute chronic toxicity) and indirectly (diminishing of the content of oxygen dissolved in water, a change in the chemical composition of the water) (Singh

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Table 1. Effect of Carbaryl (0.04 PPM) on glucose content in liver of Clarias batrachus

S. No.	Time	Amount of glucose		% Age change (increase/decrease)	't' value	Probability
		Control (mg)	Treated (mg)			
1	7	5.068 ± 0.2051	7.282 ± 0.4090	43.685879	4.3276	≤ 0.01
2	15	5.192 ± 0.2514	4.854 ± 0.3529	18.4024	2.3212	≤ 0.02
3	30	5.212 ± 0.3457	3.254 ± 0.1959	37.5671	4.4164	≤ 0.01

Value expressed as mg/100mg wet-weight of tissue
Each value is the mean ± standard error of 5 individual observations.

and Singh 2010). When pesticides pass over from water into other links of biological chain, their content grows hundreds and thousands of times. Besides observed by filtering organisms, persistent poisonous chemicals may be deposited in the tissues and then get into the organism of a fish in the interconnected links of food chains, the action of the pesticides, being cumulative is amplified several times, (Omoniyi 2018).

The main stream of the earlier toxicological studies on the pesticides on fishes was confined to histopathological field. The effect of pollutants on tissue systems of fish have shown to produce gross structural changes such as atrophy, hyper trophy, necrosis, haemorrhage, liquification, cytoplasmic vacuolation and degeneration of blood vessels, (Mekkawy et al. 2016). Histological changes and histopathological studies pressurized scientists to go in for biochemical changes. Several scientists have paid significant contribution in the histochemical and biochemical field (Ahmad et al. 2015). The present attempt has been made to investigate the biological change in the *Clarias batrachus* induced by sublethal dose of carbaryl. The alimentary canals and liver were selected for the present study because digestion, absorption and metabolism are cumulatively responsible for energy production.

The amount of glucose in alimentary canal and liver of *Clarias batrachus* was measured by Anthrone method (Nicholas et al. 1956). About 100mg of tissue sample was homogenized in 5 mL of chilled de-ionized water. Then centrifuged at 5000rpm for 10min the final volume of supernatant was noted. Soon after the supernatant collection, 1mL of 0.01% Sodium fluoride solution was added to supernatant to stop the conversion of glucose to lactic acid. From the supernatant, 0.01mL was taken as test sample. The volume in the test tube was made up to 1mL with DDW. Then de-proteinization was done by mixing 0.5mL of 1% H_2SO_4 to 1mL of test sample. Then 4mL of anthrone reagent was added slowly with constant stirring. The tubes were dipped in chilled water during the mixing of anthrone reagent. The mixture was kept in boiling water bath for 4min and then cooled. Development of green color indicated the presence of glucose in the supernatant. The intensity of the color was read at on 540nm on spectrophotometer. Each experiment was repeated 5times and the mean value with standard error were calculated.

Carbohydrate, protein, lipid, enzymes and vitamins are important component of the body and play a vital role in the body construction, metabolism and detoxification. Therefore, in present investigation biochemical changes in glucose content have been studied in liver and alimentary canal of control and intoxicated *Clarias batrachus* on interval of 7-15-30 days. In the present investigation an initial increase in glucose was observed, which may be due to the greater absorption of glucose by the intestine, under created stress conditions and acceleration of glycogenolysis and gluconeogenesis, which is similar to the findings of Sharma et al. (2012). These process synthesize glucose which is the major fuel for energy production and energy demand of animal increase to face the toxicity stress. Similar observations were also made by (Singh and Singh 2017).

Besides glucose, they also observed increase of lactate dehydrogenase (LDH) activity which elevates the amount of lactic acid. These findings are supported by Michael (2018), as they have also reported an increase LDH activity. Ahmad et al 2015 also reported the significant increased value in blood glucose and significant decrease value in serum total protein level. Mekkawy et al. (2016) also observed a decrease in LDH activity which indicates pyruvate not dehydrogenated to yield acetyl CoA and converted into lactic acid due to the ele-

Table 2. Effect of Carbaryl (0.04PPM) on glucose content in alimentary canal of *Clarias batrachus*

S. No.	Time	Amount of glucose		% Age change (increase/decrease)	't' value	Probability
		Control (mg)	Treated (mg)			
1	7	0.392± 0.032	0.541 ± 0.0448	37.500	-2.41921	≤ 0.05
2	15	0.408 ± 0.0511	0.322 ± 0.026	-21.0784	1.2837	≤0.10
3	30	0.411 ± 0.051	0.286 ± 0.0308	-28.713861	1.7247	≤ 0.10

vation of LDH activity. The other cause of initial elevation in glucose level in alimentary canal may be due to immediate increase of some digestive enzyme activity which is responsible for the carbohydrate digestion. The decrease in glucose level after 15th and 13th day of exposure is probably due to the lesser secretions of hormones, enzymes and inhibition of enzyme activity, which are responsible for the carbohydrate digestion that's why the absorption of glucose is also decreased because deficiency of glucose content occur in the intestine. Present findings get confirmed with the findings of Mahmoud et al. (2013) as they have also observed depletion in glucose content in the intestine of fish *Clarias gariepinus*, after the exposure to mercury chloride. Effects of pesticidal intoxication in intestine of different fishes were also reported by workers like Siakpere et al. (2011); Kumar et al. (2011) and Ajani et al. (2018).

They have reported necrosis, vacuolation in mucosa, complete degeneration in the serosa, muscularias submucosa and broken vili in the intestines. These changes possibly deteriorate the secretion of enzyme in the gut and absorption is also decreased due to damaged vili of intestine. This is also supported by the finding of Adewoyeso (2010); Kumar and Banerjee (2016) as they have observed depletion in glucose content in the fish *Clarias garipinus*.

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