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SPECIAL ISSUE ON INDO UK PROJECT SPONSORED INTERNATIONAL CONFERENCE ON

MICROBIAL BIOTECHNOLOGY INTERACTIONS FOR SUSTAINABLE AGRICULTURE AND SOCIETAL DEVELOPMENT

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CONFERENCE PROCEEDINGS

UGC & DST INDO-UK PROJECT SPONSORED

INTERNATIONAL CONFERENCE 2020

ON

MICROBIAL BIOTECH INTERACTIONS FOR SUSTAINABLE AGRICULTURE AND SOCIETAL DEVELOPMENT

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EDITORIAL COMMUNICATION

The Department of Microbiology and Biotechnology, University School of Sciences, Gujarat University, Ahmedabad, India hosted a University Grants Commission and Department of Science and Technology New Delhi Indo UK Project Sponsored International Conference On "**Microbial Biotech Interactions For Sustainable Agriculture And Societal Development,**" bringing together renowned agricultural scientists to discuss the role of soil microorganisms in achieving gains in agricultural productivity and to explore manifolds of scientific dimensions, complex and interconnected challenges of Agriculture, Food security, Nutrition, Capacity building and Sustainable management of resources. The speakers addressed a wide range of issues and challenges, including different aspects of soil health, soil carbon storage, and environmental sustainability and soil ecosystem services in relation to food security for betterment of society.

The conference was inaugurated by Dr. S.K.Varshney, Advisor, Government of India, DST, New Delhi, and was also adorned by renowned speakers from Imperial College, London and other Institutes of eminence in India.

The Key-note address was delivered by Dr. Jorg Schumacher, Imperial College, London. The other invited speakers were Prof. Anil Prakash from Barkatullah University, Bhopal, Dr. Tushar Roy from Babha Atomic Research Centre, Mumbai, Dr. Shraddha Gang, Imperial College, London, Vikram Mehta, Think Scientific, London and Dr. Harsha Shelat from Anand Agriculture University, Anand.

The conference showcased the role of microbial interactions in agricultural productivity, microbial mediated nutrient transformations in soil, molecular and genetic bases of soil microbe interactions. This conference besides having renowned speakers has a panel discussion with organic farming produces, suppliers, exporters and NGO's who help in BIO organic farming. Many rural farmers were invited to share thoughts, experiences, questions and problems they face. As a global community we need to deliver more food of a higher quality with a less environmental impact than ever before bioactive molecules, antibiotics, endophytes and farmers problems with organic farming and other important aspects of microbial biotechnology.

The live telecast of all the sessions of the conference was arranged to DTH-Direct To Home Channel No-9 of MHRD, Government of India. The conference got overwhelming response and was attended by the researchers and delegates from six different countries and many different states of India

The conference was structured in three technical modules along with Invited lectures of stalwarts of the fields viz. (i) Two oral sessions covering 21 presentations, (ii) Three poster sessions showcased 117 posters divided in to 5 different sections and (iii) A panel discussion comprising 18 Academicians and Agriculture Industry Experts with 35 leading farmers practising organic farming, where practical problems of farmers practising organic farming were addressed with elaborative technical discussion in global and local language too. Farmers as a whole are benefited with ecology, environment and economics and accepted the sustainable scientific developments in the field of organic farming and decided to practise the same in their farming.

With a view to leave a long lasting impact and to make scientific knowledge available to young and budding researchers, it was envisioned to publish full length papers in form of a proceeding of this International Conference in the reputed International Journal "**Bioscience Biotechnology Research Communications**", where total 38 full length research papers were accepted for publication as Proceedings of the conference Microbial Biotech Interactions For Sustainable Agriculture And Societal Development . It has been divided in to three thematic areas viz. (A) Agricultural Microbiology – 11 papers, (B) Enzymology – 16 papers and (C) Environmental Microbiology – 11 papers.

We are looking forward to highly technical learning of microbial biotechnology interactions and their use for the betterment of society is incomplete.

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Development of Sustainable Biofortification Strategy in *Phaseolus vulgaris* Through Selenorhizobacteria Under Selenium Deficient Region

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ABSTRACT

Plant Growth Promoting (PGP) selenorhizobacteria have the multi-capacity of colonizing the roots of plant and enhance the growth by various mechanisms. They are predicted eminent soil microbes for enhancing the plant growth. Study conducted with the aim to development the biofortification strategy in agriculture for increased selenium content in *Phaseolus vulgaris* plant through selenorhizobacteria bioinoculants under selenium deficient regions. Selenorhizobacteria were isolated from rhizospheric soil and characterized for their multi-trait plant growth promoting efficacy. Selected best three isolates MPJ2, MPJ8, MPJ10 of selenorhizobacteria were applied on seeds of *Phaseolus vulgaris* under pot study and after harvesting micronutrient present in *Phaseolus vulgaris* were determined by atomic absorption spectrophotometer. Among isolated selenorhizobacteria, MPJ10 was found to be the best to produce 99 µg/ml IAA producing bacteoria. MPJ10 has been identified as Rhizobium selenitireducens on the basis of 16S rRNA sequencing. MPJ10 treated seeds of *Phaseolus vulgaris* showed increased seed germination capacity, longer root length, increased fresh weight of the plant. At the harvesting time (i.e. 75 Days after sowing) enhanced selenium content of 1.52 ppm has been recorded in the fruits of MPJ10 treated plant. This is found to be 4.3-fold and 2.1-fold higher from the control plant and 1 mM Na_2SeO_3 treated plant, respectively. Selenorhizobacteria had capacity to develop selenium biofortified plant under selenium deficient soils as a sustainable agriculture.

KEY WORDS: BIOFORTIFICATION, *PHASEOLUS VULGARIS*, PGPR, SELENIUM, SELENORHIZOBACTERIA.

ARTICLE INFORMATION

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INTRODUCTION

Biofortification is the process by which the nutritional quality of food crops can be improved through agronomic practices (Patel et al., 2018a; Shaikh and Saraf 2017, Trivedi et al., 2019). Around 30% of the world's population suffers from either a lack of one or more essential micronutrients, or the overconsumption of these minerals. Selenium (Se) is an essential element for humans also found to be beneficial for crops and its deficiency may cause serious problems (Patel et al., 2018a). The movement of selenium across soil, crop and environment interfaces is thus of crucial importance for improved selenium status in humans. Selenorhizobacteria are plant growth enhancing rhizobacteria. They are beneficial soil bacteria that inhabit plant roots and promote plant growth. Techniques used for selenium biofortification of plants include supplementing of selenium in soil and foliar treatment, etc. (Pezzarossa et al., 2012). Selenium is present in trace amounts with various organic and inorganic forms in soil and environment which can be taken up and translocate in different parts of plants (Kikkert and Berkelaar 2013).

Some organic forms like Selenium methyl-selenocysteine (SeMeCys) and selenocysteine (SeCys) seem to be very effective sources of dietary selenium (Rayman 2012). Selenium concentration in food sources depends upon soil Se content, Se accumulating capacity and uptake by crops, which modify according to geographical regions (Mehdi et al., 2013). The efficacy for biofortification of crops with Se strongly depends on the biological, chemical and physical properties of the soil (Zhao et al., 2005). Se status of soil of Gujarat state is very poor and it is considered as Se deficient soil. Total Se in soil is reported to be 0.142–0.678 mg/kg with an average of 0.375 mg/kg (Patel and Mehta 1970; Patel et al., 2018a). Beans are soil improvers, which capable of fixing nitrogen in soil to improve soil fertility. In India, French bean is one of the most important leguminous plant, it is interesting to study French bean (*Phaseolus vulgaris*) because selenium content of bean is very low.

Potential biotechnological applications of selenorhizobacteria as plant growth promoting rhizobacteria can play role as carrier for plant selenium biofortification of wheat crops which is an effective strategy (Acuna et al., 2013; Patel et al., 2018a). Selenium in plants can alleviate through use of microorganisms such as selenorhizobacteria as seed inoculant which are capable of metabolizing the inorganic selenium and stimulate natural processes to enhance nutrient uptake and productivity in selenium deficient soil areas. Study was conducted with the aim, development of biofortification strategy in agriculture for increase in selenium content in *Phaseolus vulgaris* in selenium deficient area.

MATERIALS AND METHOD

Sample collection and Isolation of Selenorhizobacteria: Soil sample was collected from rhizospheric soil of leguminous plant. Bacteria were isolated by adding 10 g of rhizospheric soil to 100 ml of distilled water and shaking at 150 rpm for 10 min. Soil sample was diluted and 0.1 ml aliquots were plated on different media like Nutrient Agar supplemented with 2 mM sodium selenite (Acuna et al., 2013) as selenium enriched medium and incubated at 37^{0}C for 48 h (Ghosh et al., 2008). After growth, different red colour colonies were isolated (Zheng et al., 2014; Torres et al., 2012). Due to the sensitivity of selenite to high temperature during autoclaving, it was added to the medium as filter-sterile solution after autoclaving the medium.

Growth profile and selenite reduction by different Selenorhizobacteria: To determine optimum concentration of selenium favourable for the bacterial growth, a 250 ml flask having 100 ml nutrient broth supplemented with different concentration of 0.1, 1, 2, 5 and 10 mM Na_2SeO_3 were used (Tetteh et al., 2014; Zheng et al., 2014). Cultures were inoculated and the flasks incubated on shaker (150 rpm) at 37^{0}C for 48 h. After incubation development of red colour in medium indicates sodium selenite reduction and the formation of elemental selenium, the absorbance was measured at 600 nm by spectrophotometer.

Selenium content present in Selenorhizobacteria: Quantitative analysis of selenium content present in selenorhizobacteria was performed. 1 ml of bacterial suspension in sterile saline solution was centrifuged it 1500 rpm for 10 min. Collected cell pellet was digested with 10 ml of tri-acid mixture of HNO_3:$HClO_4$:$H2SO_4$ as per method Duran et al., (2013) Se content was measured by atomic absorption spectrophotometer with hydride generator at wavelength of 196 nm (HG-AAS, Elico, GI-639) using $NaBH_4$ as reducing agent.

Identification of isolate: Identification of isolates through 16S rRNA was done by automated DNA sequencer at Chromous Biotech Pvt. Ltd., Bangalore, India. Genomic DNA from bacterial strain was isolated using Chromous bacterial genomic DNA isolation kit and tested on 1% agarose gel electrophoresis. The Sequence data was aligned by BLAST analysis and were further deposited at NCBI with their accession numbers through sequin software. Molecular phylogenetic tree analysis was constructed through MEGA 4.1 software (Tamura et al., 2007).

Characterization of plant growth enhancing efficacy: Selected selenorhizobacterial isolates were individually activated in nutrient broth supplemented with 1 mM Na_2SeO_3 as below followed by characterization for their multi-trait Plant Growth Promoting (PGP) efficacy. Quantitative analysis of siderophore production was also performed by CAS-shuttle siderophore assay (Patel et al., 2018b). Phosphate solubilization capacity was checked on liquid Pikovskyaya's medium (Zhu et al., 2011). In liquid medium total amount of phosphate solubilized was calculated from the standard graph. IAA production was evaluated by tryptone yeast broth basal medium with added 1% tryptophan using Salkowski's reagent at 536 nm. IAA produced was measured as per Sarwar

and Kremer (1995). Ammonia production was studied in peptone water broth and measured the absorbance at 540 nm by spectrophotometer (Agbodjato 2015).

Application of selenorhizobacteria on Phaseolus vulgaris: Pot experiments were carried out with selected isolates to enhance soil containing micronutrients particularly selenium and iron in crop. Isolates MPJ2, MPJ8 and MPJ10 were individually grown in 100 ml of nutrient broth supplemented with 1mM of sodium selenite (Na_2SeO_3) and incubated at 37°C for 48 h with continuous shaking at 150 rpm to obtain required biomass. After incubation bacterial cells were harvested by centrifugation at 1,500 rpm for 10 min, rinsed twice with sterile normal saline (0.85% NaCl) and resuspended in 30 ml of sterile saline solution having 1-2 × 10^9 CFU/ml (Acuna et al., 2013). This suspension was used as selenorhizobacteria inoculum for pot experiments of *Phaseolus vulgaris*.

Phaseolus vulgaris seeds were surface sterilized using 0.1% $HgCl_2$ followed by washing several times with sterile distilled water to remove traces of $HgCl_2$. Sterilized seeds were immersed for 60 min in 48 h grown culture of individual selenorhizobacteria as inoculum for coating the seeds with cells (Yasin et al., 2015a). Control was prepared without treatment with culture. Coated seeds were sown in pots having sterilize soil prepared by finalization process. Moisture in the pot was maintained by sprinkling water to pots taking care not to disturb inoculants. At the flowering stage, 10 ml of respective selenorhizobacteria culture as inoculum was foliar sprayed to each pot. The plants were grown under natural day light conditions. The experiment was performed in triplicates. Observations like seed germination, seed vigour index, shoot and root length, fresh and dry weight (Tank and Saraf 2009, Jha and Saraf 2012) were recorded at 45 days after sowing (DAS) and 75 DAS.

Quantitative analysis of selenium content of selenium-enriched plant: At 45 DAS and 75 DAS plant of *Phaseolus vulgaris* were rinsed with distilled water and dried. 1 g of fruits (seeds) of plant was acid digested as described above. Acid digest was analysed for selenium content present in fruits by HG-AAS (Elico, GI-639) at wavelength 196 nm using $NaBH_4$ as reducing agent (Duran et al., 2013).

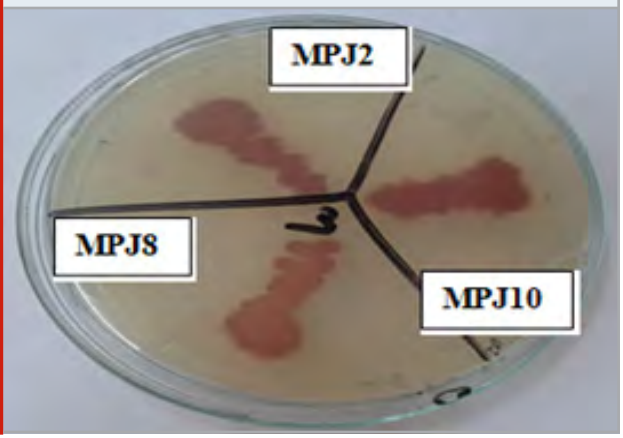
Statistical Analysis: Data were analysed by a one-way analysis of variance (ANOVA). All experiments were carried out in triplicate and the values were presented as means ± standard errors. Differences were considered to be significant when the P value ≤ 0.05.

RESULTS AND DISCUSSION

Isolation of Selenorhizobacteria: A total of sixteen morphologically different colonies were isolated from rhizospheric soil. Among them five bacterial isolates were found to grow on sodium selenite supplemented Nutrient agar medium as they had a capacity to reduce selenium from inorganic sodium selenite to elemental Se (Se0) and visualized as red colour colonies on agar plates. Best three isolates were selected on the basis of their capacity to accumulate selenium and labelled as MPJ2, MPJ8 and MPJ10 (Figure 1).

Growth profile and selenite reduction by different selenorhizobacteria: Growth curve and selenite reduction by different selenorhizobacteria with different concentration of sodium selenite at 48 h of incubation is shown in Figure 2. Selenium is an essential element for the bacterial growth. Growth stimulating effect at different concentration ranging from 0.1 to 10 mM Na_2SeO_3 was evaluated. The growth curve revealed 1 mM Na_2SeO_3 concentration could stimulate the bacterial growth and reduced the sodium selenite (Na_2SeO_3) into the red colour elemental selenium (S0) (See Figure 2). The increased absorbance is because of not only the increased biomass but also the accumulation of elemental selenium. Moreover, as concentration of Na_2SeO_3 increased the stimulatory effect of it on the bacterial growth was found to decrease (See Figure 2). The growth curve showed decrease in the growth of bacterial culture, above 1 mM Na_2SeO_3 concentration (Figure 2). It suggests that the selenite reduction was decreased under the increased Se concentration, which could be because of extremely low number of bacterial cells. Based on the above results, 1 mM Na_2SeO_3 concentration for growth was selected as a required concentration for the further studies.

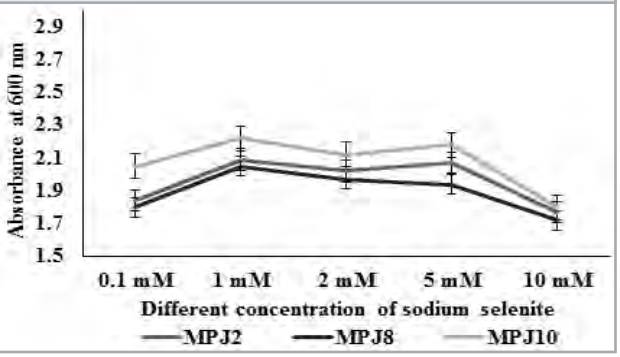
Figure 1: Isolates isolated on Nutrient agar plate with 1mM sodium selenite



Our results revealed that in presence of 1 mM Na_2SeO_3 concentration, the bacterial growth increased. Studies have reported that, the lower concentration of sodium selenite can promote bacterial growth whereas increased selenium concentration inhibits the bacterial growth (Tetteh et al., 2014). The reduction of Se (selenite) through bacterial activity precipitates Se^0 as above a tolerance mechanism, which is exhibited by development of red colour in culture medium (Vallini et al., 2005). Our studies found *R. selenitireducens* (MPJ10) had maximum potential to enhance selenium in plant. Reduction of selenite into elemental selenium by *Bacillus sp.* was reported 32% when medium inoculated with 10 mM

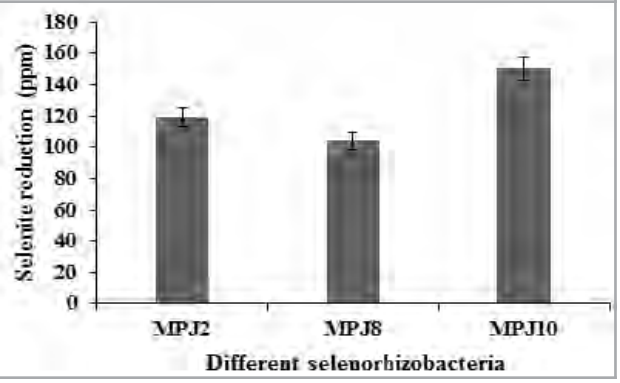
sodium selenite (Yasin et al., 2015b). Elemental selenium (Se^0) is a nontoxic form of Se and could be an adequate candidate for biofortification of cereal crops using bacteria as inoculum (Acuna et al., 2013).

Figure 2: Growth curve of different selenorhizobacteria with different sodium selenite concentration at 48 h of incubation



Selenium content present in Selenorhizobacteria: Quantitative results showed that MPJ10 culture was able to accumulate selenium up to 88.4% which may have significant capacity to reduce sodium selenite to elemental selenium. Whereas, MPJ2 isolate was able to accumulate selenium up to 70.2% followed by MPJ8 isolates was showed 61.1% (Figure 3). Our study showed when medium inoculated with 1 mM sodium selenite, about 88.4% reduction by *Rhizobium selenitireducens* (MPJ10) was recorded.

Figure 3: Selenium concentration in Selenorhizobacteria



Characterization of plant growth enhancing efficacy: All the isolates were characterized for their plant growth promoting efficacy. Phosphorus is the second most important nutrient required for plant growth. In the present study, the isolates were found to solubilize insoluble mineral phosphate such as tri-calcium phosphate in liquid Pikovskaya's broth. Tri-calcium phosphate solubilisation in liquid medium was calculated by $SnCl_2$ method. Higher liquid phosphate solubilization was observed by isolate MPJ10 as 61 µg/ml, whereas MPJ2 and MPJ8 solubilized 23 µg/ml and 22 µg/ml, respectively after 7 days. Results show that isolate MPJ10 was good phosphate solubilizer (Table 1).

Bacterial strains produce varied range of siderophore like catecholate, hydroxamate, carboxylate etc., that have a higher affinity for iron. It was observed that all the three isolates showed positive activity for siderophore production in the range of 32% to 80%. Among that MJP10 isolate was found to be the best siderophore producer. Quantitatively the isolates MPJ10, MPJ8 and MPJ2 showed 80% followed by 61% and 32% of siderophore production after 48 h of incubation, respectively (Table 1). Study indicates siderophore produced by bacteria can show biocontrol mechanism.

IAA plays a key role in root initiation, elongation and a number of other processes concerned with the differentiation and proliferation of plant tissue. It has been reported that more effective auxin producers are normally associated with rhizosphere soil. IAA production was found in the range of 52 µg/ml to 99 µg/ml. All the three selected isolates showed remarkable production of IAA. MPJ10 was reported highest IAA production of 99 µg/ml after 96 h of incubation in dark. The estimation was carried out by Salkowski's reagent which develop red colour after incubation in dark for 30 min. Further production of IAA by isolates MPJ2 and MPJ8 were 74 µg/ml and 52 µg/ml, respectively (Table 1).

Ammonia production by isolates were studied for ten to thirteen days of incubation as per method and estimation was done by Nessler's reagent. The development of brown to yellow colour indicated ammonia production. All the isolates showed appreciable ammonia production by MPJ10 isolate as 39 µg/ml whereas, MPJ8 and MPJ2 reported as 25 µg/ml and 21 µg/ml, respectively at eleven days (Table 1). Results indicate isolates showed most important trait of PGPR which beneficial for plant vegetative growth.

Selenium tolerant bacteria can promote plant growth which may be associated with a capacity to produce plant growth promoting substances (Yasin et al., 2015a). Studies reported that Plant growth promoting rhizobacteria has capability to produce plant growth regulator indole acetic acid, siderophore activity, ACC deaminase activity, etc. which could improve plant growth in forms of root length and in general enlarged the plant ability to cope with environmental stresses (Barret et al., 2011). The bacteria isolated from Se-enriched wheat plant did not found to solubilize phosphate and siderophore production (Duran et al., 2014). Whereas, our study finding reveals *R. selenitireducens* (MPJ10) applied with sodium selenite has great potential to produce plant growth enhancing substances such as IAA production (99 µg/ml), phosphate solubilization (61 µg/ml) and siderophore production (80%). IAA is involved in the promotion of growth of lateral roots (Patel et al., 2019). Studies have reported Klebsiella E2 isolated from Se-enriched plants showed high IAA production 94 µg/ml, as compared with isolates from plant without selenium that showed lower IAA production of 35 µg/ml concentration (Duran et al., 2014).

Identification of selenorhizobacterial isolate through 16S rRNA sequencing: The traditional identification

of bacterial isolates on the basis of phenotypic characteristics is usually not as precise as identification based on genotypic methods. Bacterial genomic DNA was extracted from whole cells by using a standard method at Chromous Biotech Pvt. Ltd. A PCR product of about 500 or 1,500 bp of the 16S rRNA gene sequence was produced. The purity of PCR product was checked on 1% agarose gel electrophoresis. The 16S rRNA analysis confirmed that isolate MPJ10 is a strain of *Rhizobium selenitireducens* having 99% similarity with the reported gene sequence. *Rhizobium selenitireducens* having similarity with *Rhizobium sp*. strain AKU19 with GenBank accession No. MF173085.

The sequence of potential strain MPJ10 isolate was deposited at NCBI GenBank with their accession number MG676682 (Table 2). Phylogenetic tree of MPJ10 isolate was constructed through MEGA 4.1 software. Phylogenetic analysis is based on 16S rRNA gene sequences and aligned with data available from National Centre for Biotechnology Information (NCBI) data library. Finally, after multiple alignment of data by Clustal X the evolutionary history is studied through Neighbor-joining method. The evolutionary distances were computed using the maximum composite likelihood method.

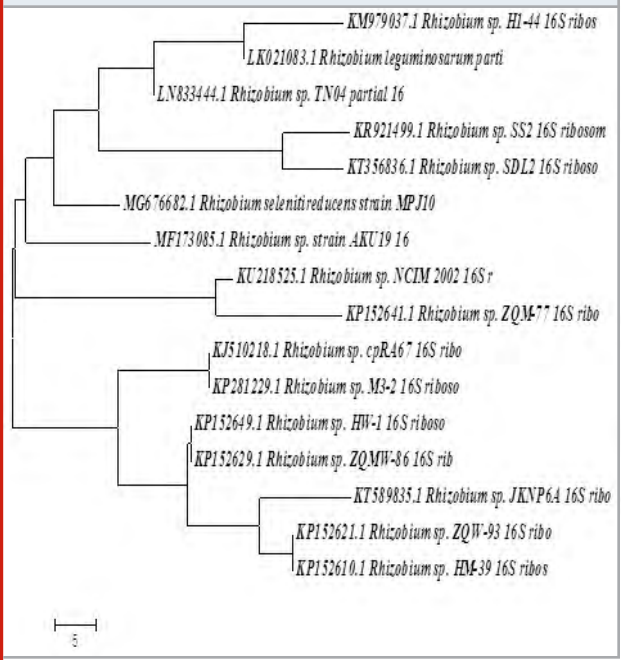
Table 1. Plant growth promoting efficacy of selected isolates

Isolates	Phosphate solubilization (µg/ml at 7 days)	Siderophore production (% at 48 h)	IAA production (µg/ml at 96 h)	Ammonia production (µg/ml at 11 days)
MPJ2	23	32	74	21
MPJ8	22	61	52	25
MPJ10	61	80	99	39

Table 2. Phylogenetic similarity of isolates by 16S rRNA sequencing

Isolate	Genera	Strain	Similarity (%)	Accession No.
MPJ10	*Rhizobium sp.*	*Rhizobium selenitireducens*	99	MG676682

Figure 4: Phylogenetic tree of MPJ10 isolate based on 16S rRNA gene sequence



Application of Selenorhizobacteria on Phaseolus vulgaris: Effect of selenorhizobacteria on Phaseolus vulgaris was studied with the aim of plant growth promotion and selenium biofortification. Seed germination percentage showed remarkable difference among various inoculant strains. Results showed that maximum seed germination 80% followed by 73.3% and 70% was obtained by MPJ10, MPJ2 and MPJ8 isolates in the inoculated plants respectively, whereas in control plant and 1 mM Na_2SeO_3 treated plant 60% and 66.6% seed germination was observed (Table 3). Results indicate seed germination showed good quality of seed and better seedling were produced by selenorhizobacteria treated plant as compared to control plant.

Vegetative parameters showed maximum shoot length and root length (31.4 cm and 8.2 cm) with MPJ10 culture inoculated plant followed by, MPJ2 culture treated plant (29.05 cm and 6.9 cm), MPJ8 culture treated (29 cm and 6.6 cm). It was observed to be higher than shoot and root length (25 cm and 5.08 cm) of the control plant and 1 mM Na_2SeO_3 treated plant (28.05 cm and 6.3 cm) (Table 3).

Seedling vigour index was calculated as germination percent × seedling total length (shoot length + root length). Higher seed vigour index of 3168 was observed for MPJ10 culture treated plant followed by, 2635.1 by MPJ2 culture treated plant and 2492 by MPJ8 culture treated plant which were better than the control (1804.8) and 1 mM Na_2SeO_3 (2287.7) treated plant (Table 3). Seed vigour index revealed the fitness of the seedling produced as considering the germination percentage and total length of plant. It indicates increased in the seed vigour index can help in the improvement of seedling fitness.

Results showed significant enhancement in fresh weight and dry weight of selenorhizobacteria inoculated plants and were recorded in the range of 10.65 g to 15.15 g and 7.76 g to 8.41 g, respectively. Maximum fresh weight and dry weight of plant 15.15 g and 8.41 g were observed when plants were inoculated with MPJ10 isolate (Table 3). Results observed fresh weight and dry weight of MPJ10 treated plant was increased between 53.8% and 46.5% followed by, MPJ2 treated plant between 22.4% and 37.2% and MPJ8 treated plant between 8.1% and 35.1%, 1 mM Na_2SeO_3 treated plant between 18% and 31% than the control plant. Moreover, fresh and dry weight of MPJ10 treated plant showed increase up to 30.2% and 11.8% than the 1 mM Na2SeO3 treated plant. Results indicate positive effect of selenorhizobacteria on higher biomass production than the control plant and 1 mM Na_2SeO_3 treated plant.

Our study showed maximum shoot length (25.6% and 11.9%) and root length (61.4% and 30.1%) increase by *Rhizobium selenitireducens* (MPJ10) isolate treated plant as compared to negative control plant and 1 mM Na2SeO3 treated positive control plant. Studies reported that in presence of Se Bacillus YAM2 inoculated from wheat plant showed improvement in shoot length 16% as compared to un-inoculated plants (Yasin et al., 2015a). Researchers reported that when sodium selenite or sodium selenate is applied as selenium source to the plant like broccoli, rice respectively, it upregulates the chlorophyll protein which could improve photosynthetic capacity of plant and regulate pathways related to various biotic and abiotic stresses (Sepulveda et al., 2013; Wang et al., 2012). Studies have also reported that in presence of selenium Bacillus YAM2 inoculated wheat plant showed improvement in dry weight 34.5% compare to un-inoculated plants. Whereas, our study showed 46.5% increase in dry weight by MPJ10 treated plant than the control plant which is again higher than Bacillus YAM2. This indicate that MPJ10 *Rhizobium selenitireducens* is a better candidate for Se biofortification.

Table 3. Effect of selenorhizobacteria on vegetative parameters of inoculated *Phaseolus vulgaris*

	Seed Germination (%)	Seed Vigour Index	Shoot Length (cm)	Root Length (cm)	Total Fresh Weight (g)	Total Dry Weight (g)
Control	60	1804.8	25±0.17	5.08±0.10	9.85±0.15	5.74±0.20
1mM Na_2SeO_3	66.6	2287.7	28.05±0.02	6.3±0.13	11.63±0.19	7.52±0.25
MPJ2	73.3	2635.1	29.05±0.02	6.9±0.12	12.06±0.18	7.88±0.11
MPJ8	70	2492	29±0.09	6.6±0.16	10.65±0.19	7.76±0.29
MPJ10	80	3168	31.4±0.10	8.2±0.10	15.15±0.09	8.41±0.08

* Mean ± standard error (n=3), significant difference P≤0.05

Enhancement of selenium in *Phaseolus vulgaris* can occur significantly through the application of seed bacterized with selenorhizobacteria and foliar spray of those bacteria at flowering stage as inoculants. The improvement may be because of their various multi-traits plant growth promoting efficacy. Researcher reviewed that foliar spray application of Se is better and efficient means of Se biofortification in plant than the application of Se fertilizers in soil, because foliar spray does not depend on root to shoot transfer of Se (Winkel et al., 2015; Gupta and Gupta 2017).

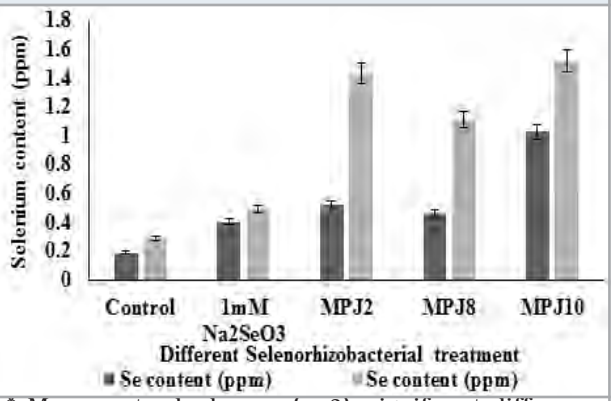
Use of selenium fertilizers in soil have low rates of Se enhancement in edible part of plants, and long-term use may be toxic to the habitat. Studies have reported that selenobacteria (selenorhizobacteria) have been studied for their potential for bioremediation of Se from polluted environments, but now a days the use of selenobacteria as a tool for improving Se content in cereal crops (Duran et al., 2013), is more practiced. Overall maximum growth in all vegetative parameters of plant was shown by MPJ10 isolate. Results indicate selenorhizobacteria could promote the vegetative parameters of plant growth as compared to the sodium selenite treated plant (Positive control) and control plant (Negative control).

Quantitative analysis of Selenium content of biofortified plants: Pot experiments were carried out with the selenorhizobacteria for improved availability of selenium in plants. Results showed significantly enhanced selenium content in fruit of Phaseolus vulgaris inoculated with the selenorhizobacterial strains. At 45 DAS the range of Se content was found to be 0.46 ppm to 1.03 ppm in selenorhizobacteria treated plant fruit whereas, at 75 DAS, the Se content was observed in fruit to be between 1.11 ppm and 1.52 ppm (Figure 5). At 45 DAS, maximum Se content present in fruits of MPJ10 culture treated plant (1.03 ppm). Whereas, after 75 DAS increased Se content was present in fruits of MPJ10 culture inoculated plant (1.52 ppm) followed by MPJ2 culture inoculated plant (1.43 ppm) and MPJ8 culture inoculated plant (1.11 ppm) (Figure 5).

At the time of harvesting of crop after 75 DAS, the results of MPJ10 treated plant showed 4.3-fold and 2.1-fold increased selenium content compared to the control plant and 1 mM Na_2SeO_3 treated plant, respectively (Figure 5) also it indicates fruits of MPJ10 selenorhizobacterial treated plant had best potential to increase selenium content in *Phaseolus vulgaris* plant.

In our studies on *Rhizobium selenitireducens* (MPJ10) strain could be used as selenorhizobacteria for biofortification with selenium and other micronutrients in *P. vulgaris*. Moreover, *Stenotrophomonas sp*. and *Bacillus sp*. were also studied and it was found to improve the selenium content in plant. Previous studies have reported that *Bacillus sp., Paenibacillus sp., Pseudomonas sp*. and *Enterobacter sp*. can promote Se transformation via oxidation, reduction and methylation (Fordyce 2007). Several other studies have shown various aerobic selenium tolerant strains viz., *P. aeruginosa*, *Bacillus sp., Enterobacter sp., Stenotrophomonas sp., Acinetobacter sp*. and *Klebsiella sp*. have ability to accumulate selenium and the potential of these bacteria being used as inoculants for selenium biofortification in wheat (Acuna et al., 2013). Studies by Acuna et al., 2013 have shown that inoculation with *Stenotrophomonas* sp. with sodium selenite can increase the selenium content in plant tissue like leaves and root of wheat. Inoculation with *Bacillus sp*. with sodium selenate has potential for plant growth, selenium biofortification in wheat and other crops (Yasin et al., 2015a). It has been shown that Se biofortified wheat through *Bacillus sp*. contain Se up to 32-55 µg/g and 82-89 µg/g in stem and kernels, respectively. *Bacillus sp*. with sodium selenate have significantly enhanced Se and Fe content in wheat kernels 1.67-fold and 0.7-fold, whereas in stems 2.52-fold and 1.47-fold as compare to uninoculated plants (Yasin et al., 2015a).

Figure 5: Selenium content enhanced in fruits of Phaseolus vulgaris by different Selenorhizobacteria at 45 DAS and 75 DAS Se content (ppm) 45 DAS
Se content (ppm) 75 DAS



* Mean ± standard error (n=3), significant difference P≤0.05

Our study indicates that the potentiality of plant growth promoting selenorhizobacteria as potential microbial inoculant for *Phaseolus vulgaris* crop as it has capability to enhance plant growth via diverse mechanisms and therefore its application in sustainable agriculture is of great significance.

CONCLUSIONS

Results of pot study of selenorhizobacteria treated plant and its comparison with control plant and 1 mM Na_2SeO_3 treated plant revealed MPJ10 culture could had significantly improved seed germination capacity and other vegetative parameters like root length, total fresh weight also recorded to be increased in the experimental plant *Phaseolus vulgaris*. At the harvesting time 75 DAS, *Rhizobium selenitireducens* (MPJ10) treated plant showed significantly enhanced selenium content in fruits up to 4.3-fold and 2.1-fold as compared to control plant and 1 mM Na_2SeO_3 treated plant, respectively. *Rhizobium selenitireducens* had capacity to develop selenium biofortified plant in selenium deficient soil which can be applicable as a sustainable agricultural practice for crop improvement. This may play an important role in the selenium bioavailability representing a stimulating biotechnological alternative to micronutrients biofortification which progressively offer an attractive way to replace the use of chemical fertilizers and other supplements. Selenium biofortified crops can be a good source of selenium to satisfy the daily requirement and prevent disease in selenium deficient soils.

Conflict of interest: Authors declare that they have no conflict of interest.

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Assessing the IAA Production by Various *Trichoderma spp.* and Evaluating its Root Colonization During Early Root Development of Soybean Plant

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ABSTRACT

Trichoderma strains a major cultivable plant growth promoting fungi in soils, significant promote plant Growth and Yield and an anamorph of Hypocreaces, is a known bio-control agent and Biofertilizer. Morphological Characteristics of four different (GFT1, GFT2, GFT3 and GFT4) strains were appraisal by grown on six disparate media like Bengal gram flour medium (BAM), Wheat agar medium (WAM), Rose Bengal agar medium (RBA), Potato dextrose agar medium (PDA), Soya bean agar medium (SAM) and Czapek's dox agar medium (CZDA). One of the most Plant growth enhancing traits is Indole-3-acetic acid (IAA) Mickle Ordinary phytohormones that regulate many aspects of growth, yield and development of plants. In-vitro assessment of Indole 3- acetic acid production from four *Trichoderma* strains, among this strains GFT1 showed maximum production of IAA (328 µg/ml) in Bengal gram broth and GFT4 showed maximum production of IAA (380 µg/ml) in Soybean broth supplemented with Tryptophan 100 µg/ml was obtained after 7 days. In the present study, we evaluate the positive and negative effect of *Trichoderma* strains Such as GFT1 and GFT4 on early stage of root development of Soybean plant and further research work we will check the impact of *Trichoderma* during nodules formation on root, their particular effect on rhizobium bacterial community of leguminous crop.

KEY WORDS: IAA, LEGUMINOUS PLANT, ROOT COLONIZATION, *Trichoderma spp.*

ARTICLE INFORMATION

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INTRODUCTION

The Food and Agriculture Organization of the United Nations has recently estimated world's population to reach up to 10 billion by 2050 (Vainio, 2020), imposing an increasing demand for food that comes together with an increasing concern on environment and food security. Agricultural prospectus to feed all of these individuals are an Imperious challenge in twenty-first century (Ando et al., 2020). Accumulative world human population and lessening in land available for cultivation are two threats for agronomic sustainability (Shrivastava and Kumar, 2015).Under this pressing scenario for agriculture, the widely acknowledged beneficial role of legumes in cropping systems, by increasing N2 fixation by nodules, reducing energy costs, improving soil physical conditions, fertility and biodiversity, is more needed than ever (Kulkarni et al., 2018). Grain legumes are a good source of proteins and lipids. Soybean (*Glycine max L. Merril*) is the world's most important seed legume, which contributes to 25 % of the global edible oil, about two-thirds of the world's protein concentrate for livestock feeding and it has recently become the crop of interest in India (Zhang et al., 2018).

Soybean (*Glycin max L.*) is a subtropical member of the leguminous family and one of the most important oil seed crop in the world (18 to20 % oil) which protein (40 to 42 %) provide all eight essential amino acid in the amount need for human health (Yang et al., 2019).The contribution of India in the world soybean area is 10 %, but the contribution to total world soybean grain is only 4 % indicating the poor levels of productivity of the crop in India (1.1 t/ha) as compared to other courtiers (world average 2.2 t/ha) (Tiwari et al., 2019). The well-studied cases of microbes able to instantaneously exert positive effects on plant development and bout directly on fungal phytopathogens are the filamentous fungi of the genus *Trichoderma* are effectively marketed worldwide as biocontrol and biofertilizers on various crops (Kohl et al., 2019). In this research we investigate the impact of *Trichoderma* strains on early stage of root development of Soybean plant.

MATERIALS AND METHOD

Microbial Strains and Culture Conditions: In this work, four different fungal Strains (GFT1, GFT2, GFT3 and GFT4) were used. Fungal culture were isolated from rhizospheric soil of Soybean plant were collected from Ahmedabad, Gujarat, India. Fungal isolated culture cultivated on Rose Bengal agar medium (HI- MEDIA, Pvt. Ltd., Mumbai, India) at 25 °C. Isolated Fungal Spore suspensions were adjusted with sterile distilled water to the desired concentrations. Soybean seeds were procured from Sarthi Agro. Pvt. Ltd. Ahmedabad, Gujarat, India.

Morphological characteristics of Trichoderma strains: The characteristics of *Trichoderma* Strains, like colony appearance and sporulation pattern were examined from cultures grown on six media: Potato dextrose agar medium (PDA), Rose Bengal agar medium (RBA), Czapek's dox agar medium (CZDA), Wheat flour agar medium (WAM), Soyabean agar medium (SAM) and Bengal gram flour medium (BAM) at 28 ^{o}C for 4 days. For observing colony characteristics and growth rate, inoculum was taken from the actively growing margin of 4 days' culture, grown on Rose Bengal agar medium. A 7 mm mycelia disc was placed at center of all Petri dishes. The dishes were kept dark condition for incubation at 28°C. Radial growths were measured at 24 h intervals until colony covers the whole Petri dish. All micro morphological data were examined on cultures grown on Bengal gram flour and wheat flour medium for four days at 28°C. The microscopic examination and measurements of conidiophores, conidia and Phialide were made from slide preparations stained with lactophenol-cotton blue /1 % Methylene blue were observed at 40X magnification of objective lens (Kim et al., 2019).

In vitro Assessment of PGP trait (IAA) of Fungal isolates: Spectrophotometric estimation of IAA was performed as per the method developed by Brick et al. (1991) with modifications adapted by Goswami et al. (2015) , 48h, 96h, and 144 h old Culture supernatant of the fungal isolates grown in the Bengal gram flour broth medium supplemented with L- tryptophan (200 µg/ml^{-1}) with Salkowski reagent (50 ml, 35% of perchloric acid, 1ml 0.5M $FeCl_3$ solution) in the ratio of 1:1. Development of pink color indicates production of IAA and its optical density was recorded at 530nm. Concentration of IAA produced was estimated against standard curve of IAA (Hi-media) in the range of 10–100 µg/ml^{-1}.

Assessment of root colonization ability: Root colonizing ability of PGPF strains under study were accessed by closed test tube assay as described by Lojan et al. (2017) with modifications. To start with, distinct layers of clay and soil were prepared in test tubes. Test tubes containing such soil and clay were sterilized using autoclave. Seeds were sown with biofertilizer (105 spores /ml) and watered with 5 ml of sterile water. Spores were counted by Neubauer chamber. Mouth of the test tubes were sealed with parafilm. Seeds were allowed to grow for 15 days, seedlings were then uprooted with forceps, and roots were properly excised and excess soil on the root was removed by tapping, inoculated Wheat flour agar medium plates. Fungi were allowed to grow around the roots and were examined.

Identification of screened fungal isolate: Of all the fungi under study the *Trichoderma sp.* that showed best IAA production with the most efficient root colonization was identified based on its of 18s RNA gene sequencing. The stain was sequenced by GSBTM (Gujarat State Biotechnology Mission, Gandhinagar, Ahmedabad, Gujarat, India) the sequence so obtained was deposited in GenBank and its Accession ID was obtained. The sequence was analyzed using BLASTn algorithm to identify the maximum similar fungi from previously deposited data in GenBank.

RESULTS

Fungal Isolates: Total 10 fungal isolates were isolated from rhizospheric soil samples of *Glycine max L* collected from Ahmedabad, Gujarat, India. For further study four fungal (GFT1, GFT2, GFT3 and GFT4) isolates from collection site were selected on the basis of the best growth (Figure1).

Figure 1: Photographs showing colony appearance of four *Trichoderma strains* grown for 4 days and 7 days on BAM, WAM, RBA, PDA and CZDA. A, B, C, D- Photographs showing microscopic observation of conidiophore of *Trichoderma strains* (A- GFT1, B-GFT2, C-GFT3, D-GFT4).

Morphological Identification of Fungal Isolates: Morphological identification of (GFT1, GFT2, GFT3 and GFT4) isolates were carried out using of staining method (Figure 1). The colony appearance and microscopic morphological details revealed by 1 % Methylene blue staining are summarized in Table 1.

Assessment of PGP traits: Quantitative analysis of IAA depicted that Four Fungal strains (GFT1, GFT2, GFT3 and GFT4) produced this phytohormones. IAA from culture supernatant of four fungal strains were determined at 48 h, 96 h and 144 h of incubation. Maximum IAA produced by GFT1 was 328 µg ml-1 after 144 h grown in Bengal gram broth and GFT4 produced 380 µg ml-1 of IAA after 144 h grown in Soybean broth supplemented with 100 µg ml-1 of L-tryptophan (Figure 2). As the concentration of supplemented L-tryptophan increased, IAA production was also increased.

Assessment of root colonization ability: Assembly for root colonizing assay was developed as described above. Assay performed is known as closed test tube assay. Briefly, assay was performed in a test tube which contained sterile layer of sand at the bottom covered by the layer of sterile clay on the top. Soybean seeds treated with PGPF were sowed and allowed to grow for 15 days (Figure 3). Seedlings were then uprooted with sterile forceps, and roots were properly excised and inoculated on Wheat flour plates. Fungal colonies were allowed to be grown around roots and examined (Figure 3). From picture it can be estimated that both the fungal strains (GFT1 and GFT4) showed root colonization on Soybean root.

Figure 2: Comparison Production of IAA from four Fungal isolates .A- IAA standard, B – IAA production by four different fungal isolates in BGB medium, C-IAA production by four different fungal isolates in SB medium.

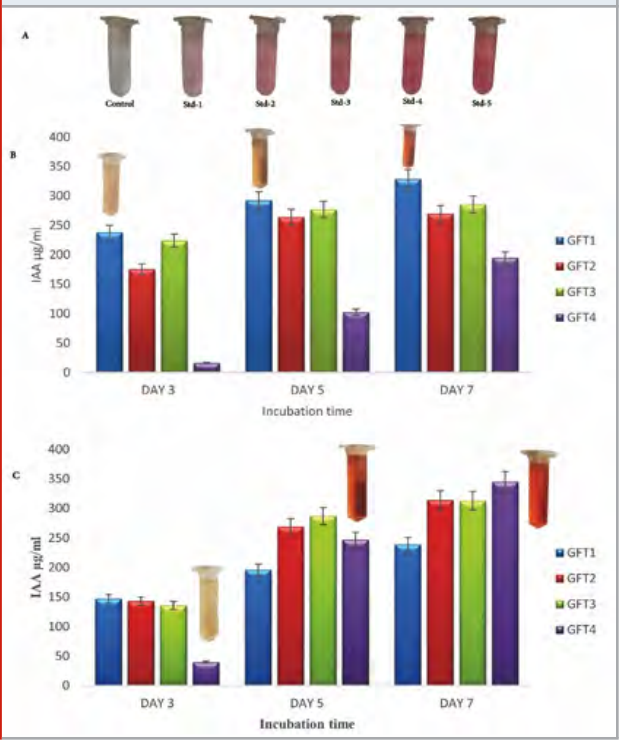
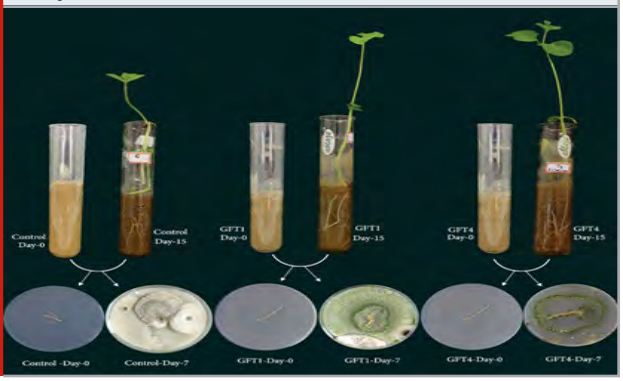


Figure 3: Photographs shows close test tube assembly to determine root colonization ability of PGPF strains under study.



Identification of screened fungal isolate: Based on all the parameters, GFT1 was found to be the most robust, based on its potentials to grow in various media, and could produce maximum IAA from all the strains under study. It was also able to colonize the soybean root as observed by root colonizing assay. Hence GFT1 was screened and was elected for its molecular identification using 18s rRNA gene sequence analysis. The sequence so obtained was deposited in GenBank under the accession ID.FJ904855. The gene sequence when analyzed using BLASTn algorithm, it should maximum similarity with *Trichoderma viride* (with accession ID).

DISCUSSION

The present study reveals the primary work done to isolated plant growth Enhancing and biocontrol fungi from rhizospheric soil of *Glycin* max L. Rhizoplane and rhizospheric environment of root is suitable survival for PGPF due to rich of Rhizodepositon avaibility in soil. Community of microbiome residing in a particular niche is specific plant species and dependent on environmental factors and biotic factors (Sergaki et al., 2018). Isolating PGPF from rhizospheric soil and screening them on the basis of PGP traits was the focus of present research work. *Trichoderma Viridi* have been reported that to exhibit plant growth-Enhancement activity on various cultivated leguminous plants (Dutta and Thakur, 2017). The *Trichoderma* genus comprises a large number of soil borne filamentous fungi that are widely used as inoculants for their health benefits to plants, such as conferring better growth, disease resistance or tolerance to abiotic stress for their host. The production of phytohormones by the PGPF is another important mechanism often associated with growth stimulation (Goswami et al., 2016).

Table 1. Microscopic and culture morphological characteristics of fungal isolates

Sr. no	Growth Media name	Culture name	Colony growth (mm) after 2 days (28 °C)	Culture color	Conidia Shape	Pigmentation and concentric ring	Sporulation initiate After (hrs.)	Margin	Texture	Phialide Shape	Conidia color
1	CZDA	GFT1	39.5	White Green	ROUND	1	98	Filamentous	Loose	Flask Shaped	Green
	BAM		39.9	Green		2	98		Comp.		Green
	PDA		64	Green		1 /Yellow Pigm	98		Comp.		Green
	RBA		39.9	White Green		2	48		Loose Puffy		Green
	WAM		39.9	White green		2	98		Loose		Green
	SAM		81	White green		2	48		Loose		Green
2	CZDA	GFT2	52	Green	OVAL	1	98		Loose	Subg lobose	Green
	BAM		78.8	Yellow Green		1	98		Loose		Green
	PDA		79.8	White green		2 /Yellow Pigm	98		Loose Puffy		Green
	RBA		50.2	Yellow		1	48		Loose		Green
	WAM		64.2	Green		2	98		Loose Puffy		Green
	SAM		52	White green		1	98		Loose		Green
3	CZDA	GFT3	51.2	White green	ROUND	1	98		Loose	Flask Shaped	Green
	BAM		57	White green		3	98		Comp. Loose		Green Green
	PDA		59.1	White green		1 /Yellow Pigm	98		Puffy		
	RBA		37.2	White green		2	98		Loose		Green
	WAM		56.0	White green		3	98		Loose Puffy		Green
	SAM		69	White		3	48		Loose		Green

4	CZDA		49.2	Green		1	48		Loose		Green
	BAM		69.5	Green		2	48		Loose		Green
	PDA	GFT4	59.2	Green		2 /Yellow Pigm	48		Comp.		Green
	RBA		39.2	Yellow	OVAL	2	48		Loose Puffy	Subg lobose	Green
	WAM		69.2	Green		1	98		Comp.		Green
	SAM		63	Green		2	48	Loose			Green

The balance between vegetative and reproductive growth is controlled by hormone signaling within the plant and can therefore be highly influenced by it (Ripa et al., 2019). Previous works have reported that the synthesis of IAA is often associated with plant growth stimulation by fungi, including Trichoderma spp. In this sense, our results are being in agreement with studies where *Trichoderma* species were reported as producers of IAA being part of their metabolism (Nieto-Jacobo et al., 2017). In this study, results showed that *Trichoderma* are able to synthesize IAA from different nutrient sources supplemented with tryptophan. Microbes produced IAA in higher amount they may be inhabited easily to root of plant and improvement of growth. *Trichoderma* both strains GFT1 and GFT4 were produced higher IAA so inhabited to root easily.

CONCLUSIONS

The present study demonstrated that four fungal isolates (GFT1, GFT2, GFT3 and GFT4) had the potential as plant growth enhancer. *Trichoderma* strains ability to produce PGP trait such as IAA and also improved plant growth by production of phytohormones. Improvement plant health and yield farmer use huge amount of *Trichoderma* as biocontrol and biofertilizers, they don't know the impact of *Trichoderma* strains on the vegetative growth of root of Soybean plant. According to our experiment we can conclude that Trichoderma strains had no any negative effect on early stage of root development of Soybean plant till date and further research work may be helpful for positive and negative effect of *Trichoderma* strains on nodules formation of the Soybean.

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Analyzing the Role of Phosphate Solubilizing Bacteria Isolated from Rhizospheric Soil of Cyamopsis in Improving Agricultural Productivity

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ABSTRACT

Approximately more than 95% of the phosphorus present in soil is in the form of insoluble phosphates that cannot be utilized directly by plants. Many bacteria present in the Rhizospheric soil can solubilize the insoluble phosphorus in that can be easily assimilated by plants. Phosphate Solubilizing Bacteria (PSB) plays a vital role in improving plant nutrition through making phosphorous in available form to plants and thus increasing phosphate uptake by plants making them suitable to be used as biofertilizers for agricultural crops. In the present research isolation of PSB was done from the Rhizospheric soil collected from different regions of Gujarat. Pikovskaya's agar medium, having insoluble tricalcium phosphate (TCP) was used for isolation of Phosphate Solubilizing bacteria and a total of 30 bacterial colonies were isolated. Out of 30 microbial isolates, 10 isolates showing highest Phosphate Solubilization Index (PSI) were selected for further qualitative as well as quantitative studies. Among these 10 potent isolates, 4 strains (MN 40, KM1, KM6 and AK17) showing maximum PSI as well as high soluble phosphate production were selected. These isolates were further identified as Bacillus & Pseudomonas species on the basis of their morphological & biochemical behavior. The efficiency of purified PSB was evaluated from pot experiments on Cluster beans under greenhouse condition for their positive role in plant growth promotion. A significant increase in plant height, number of leaves, root length, dry matter contents etc. was recorded in PSB inoculated plant over that in the non-inoculated control

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KEY WORDS: BIOFERTILIZERS, CYAMOPSIS, PHOSPHORUS, PSB.

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INTRODUCTION

Phosphorus is the second most growth limiting macronutrient for plants next only to Nitrogen. Phosphorus is found in many molecules such as nucleic acids, phospholipids, ATP etc. and plants cannot grow in absence of adequate supply of phosphorus. Flower and seed formation, Root development, stalk and stem strength, Nitrogen fixation in legumes, resistance to plant diseases and improvement in crop quality and production are some of the attributes associated with phosphorus nutrition [Khan et al.].

However a large part of phosphorus present in soil is in the form of insoluble phosphates that cannot be utilized by plants directly [Chen et al.]. Plants obtain ionic form of phosphorus from the soil solutions, of which the dihyrogen form of orthophosphate ion (H2PO4-) is readily absorbed by roots [Schachtman et al.]. However, because phosphate ions are very reactive they get immobilized by precipitation with cations like Mg2+, Fe3+, Ca2+, and Al3+ [Chen et al.]. Further in contrast to nitrogen, Phosphorus cycle is "sedimentary," because there is no interchange with the atmosphere and no large atmospheric sources can be made biologically available [Walpola et al.]. For good productivity plants require approximately 30 µmol L-1 of phosphorus, but phosphorus that is naturally available in many soils is only about 1 µmol L-1. Therefore, the unavailability of phosphorus in many soils has been recognized as a major growth limiting factor in agricultural systems [Daniels et al.].

One way of increasing the phosphorus intake in plants is through addition of soluble forms of phosphorus in the soil by application of phosphate fertilizer. However phosphate anions present in chemical fertilizers are too reactive and are rapidly immobilized (fixed) to insoluble forms upon its application in the soil due to its reaction with aluminum and iron minerals. The efficiency of applied phosphorus is less than 30% due to its fixation in soil [Sharma et al.]. There is also loss through leaching and run-off, leaving only 10–20% available for utilization by plants [Sashidhar et al.]. It is thus imperative to explore for other ways of making phosphorus available in soluble form to plants.

There are many studies that suggest that soil microorganism play a key role in transforming soil phosphorus to forms that can be absorbed by plants. Phosphate solubilization takes place through various microbial processes / mechanisms, principal mechanism being by the production of organic acids synthesized by soil microorganisms [Walpola et al., Rodríguez et al., Satyaprakash et al.]. In organic acid production mechanisms, gluconic acid(GA) is major organic acid that acts as agent of inorganic phosphate solubilization. Alternative mechanism for solubilization of mineral phosphates include production of chelating substances by microorganisms and production of inorganic acids such as carbonic, nitric and sulphidric acids [Kalayu et al.]. As such PSBs have a potential to be used as bio fertilizers to enhance plants growth and increase crop yield.

The aim of the this study was isolation and purification of PSB from the rhizospheric soil of Cyamopsis and to identify the PSB, using morphological and biochemical tests. Pot experiments were performed to estimate the efficiency of PSB to positively affect the growth parameters of Cyamopsis in green-house conditions.

MATERIAL AND METHODS

Soil Sampling and Soil Characteristics: The Rhizospheric soil samples of Cyamopsis (Cluster Beans) were collected from different regions of Gujarat. Sampling site was selected in the interior area of the field and the top soil contains dry matter was removed from the sampling site. With the help of sterile equipments, the entire root system along with the rhizosphere soil was collected by digging up to 12- 15 cm in depth. The soil samples were collected in sterile air- tight plastic bags and transferred to the research laboratory. These samples were stored at 4 ^{0}C ± 1 ^{0}C until further use. Physiochemical properties of soil such as pH, EC, NPK, salinity etc. was determined.

Isolation of Strain: For isolation of Phosphate Solubilizing Bacteria (PSB), 5 gm of soil sample collected from different sites was diluted with 95 ml of sterile distilled water, dispersed equally by shaking at 150 rpm/min for 30 min at 28 ^{0}C and further serial dilution were done upto 107 fold. Aliquots (100 µl) of the diluted sample were spread in Pikovskayas agar plates and incubated at 28 ^{0}C for 24-48 hrs. Colonies showing phosphate solubilizing zone around them were considered as PSB.

Analysis of Phosphate Solubilizing Activity: The isolates producing large halo zone were selected for further study. Selected isolates were test for both qualitative & quantitative analyses of phosphate solubilizing activity using plate screening method and broth culture method.

a.) Qualitative assay: Phosphorus solubilizing capacity of isolates were determined on Pikovskayas agar plate medium, having tricalcium phosphate [Ca3(PO4)2] as insoluble phosphate source and bromophenol blue dye. The plates were incubated at 28 ± 2 ^{0}C for 6 days. Isolates capable of solubilizing insoluble phosphate form halo zone around colonies. Diameter of clearance zone was measure successively after 24hrs for 6 days. Phosphate solubilizing index (PSI) was calculated by using the Formulae mentioned below

$$PSI = \frac{\text{Colony Diameter + Halozone Diameter}}{\text{Colony Diameter}}$$

b.)Quantitative assay: Phosphate solubilizing capacity of PSB was determined in 100ml Pikovskayas broth medium containing insoluble TCP [Ca3(PO4)2]. After sterilization, this liquid media was inoculated with

1ml of each bacterial isolates. Inoculated medium were incubated for 21 days at 28 ± 2 ^{0}C on rotary shaker. 5 ml from the inoculated medium was withdrawn aspectically at 7 days interval from each flasks and centrifuged at 10,000 rpm for 20 mins. The supernatant was analyzed for P2O5 content by Chlorostannous reduced molybdophosphoric acid blue method using Systronic 166 spectrophotometer. The pH of the supernatant was also checked along with phosphate content at regular interval of time. Standard graph was prepared using KH2PO4 as standard reagent.

Identification of bacteria: Selected bacterial strains were identified by biochemical and morphological characteristic which includes gram staining, capsule formation, urease activity, catalase test, oxidase test, production of H2S, sucrose, starch hydrolysis, lactose fermentation, Nitrate reduction and Gelatin hydrolysis.

Pot experiment: Plant growth promotion ability of isolated PSBs were assessed in Cyamopsis (Cluster Bean) under green house condition. Cyamopsis seeds were surface sterilized by soaking in 0.02% sodium hypochlorite for 02 minutes and then washed with sterilized distilled water. Seeds were then coated with 1% carboxymethyl cellulose as adhesive agent and inoculated with bacterial strain for 30 minutes. Soil was properly sterile for pot study. There were a total of 5 treatments:

1. Control
2. Control + MN40
3. Control + KM1
4. Control + KM6
5. Control + AK17

Inoculated and uninoculated plants were harvested after 45 days of sowing to study their physiological parameter such as root length, shoot length, no. of leaves, fresh weight, dry weight etc.

RESULTS AND DISCUSSION

Soil Sampling and Isolation: Soil samples were collected from 3 different regions of Gujarat viz. Mehsana, Kutch and Ahmedabad. Physiological charectiristics of the soils were studied (Table 1). Pikovskayas agar medium was used to assess the collected soil sample for phosphate solubilizing microbes. A total of 30 bacterial strains were obtained showing phosphate solubilizing zone.

Table 2: Results of pot experiments and extract

Sr. No.	Characteristics	Unit	Soil Sample		
			Mehsana	Kutch	Ahmedabad
1	pH	Direct	8.6	7.9	7.80
2	Electric Conductivity	Milli.moh/cm	0.60	0.79	0.62
3	Org – C	In %	1.37	1.95	0.95
4	Nitrogen	In %	0.12	0.14	0.09
5	Av. - P	ppm	3.62	3.86	4.09
6	Av. - K	ppm	26	37	23
7	Cu	ppm	1.82	1.12	1.56
8	Zn	ppm	2.32	1.56	1.12
9	Mn	ppm	5.94	6.09	7.23
10	Fe	ppm	58.75	17.0	35.5
11	Boron	ppm	1.34	1.09	0.94
12	Sulphur	ppm	21.7	34.9	27.0
13	Salinity	ppt	39	50	43
14	Bulk density	mg/m3	1.33	1.35	1.44
15	Soil moisture	In%	11.60	12.70	12.09

Analysis of Phosphate Solubilizing Activity: Out of 30 bacterial colonies, the bacterial colonies producing larger halo zone were selected for further study. Both qualitative and quantitative analysis were done for selected bacterial colonies.

a.)Qualitative Analysis: Selected 10 bacterial strains showed Phosphate Solubilization Index (PSI) ranging from 1.14 to 2.38. Among these 10 potent isolates, 04 strains showing maximum PSI were MN40, KM1, KM6 and AK17(Figure 1).

b.) Quantitative analysis: Pikovskayas broth medium was used for quantitative analysis of PSB. Selected bacterial strains showed the phosphate solubilization ranging from 15.06 µgm/ml to 42 µgm/ml (Figure 2)

Identification of Bacteria: On the basis of Qualitative and Quantitative analysis, 04 best bacterial strains showing highest phosphate solubilization(i.e MN40, KM1, KM6 and AK17) were selected for further study. They were identified as bacillus and pseudomonas species on the basis of their morphological and biochemical test.

Pot Experiment: During pot study a significant increase in physiological parameters in treated plants as compared to control were observed.(Table 2). Among the 04 isolates KM1 showed the highest growth promoting activity.

Figure 1: Isolates showing clear zone on Pikovskaya agar plates

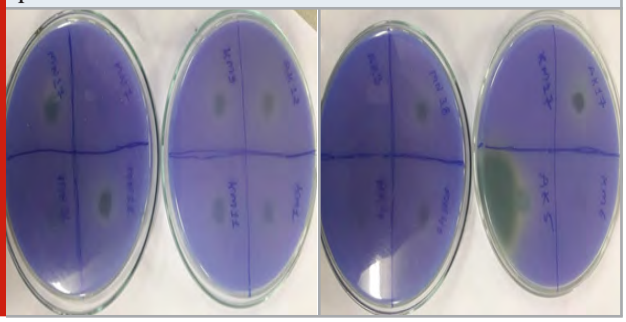

Figure 2: Quantitative estimation of phosphate solubilization by PSB

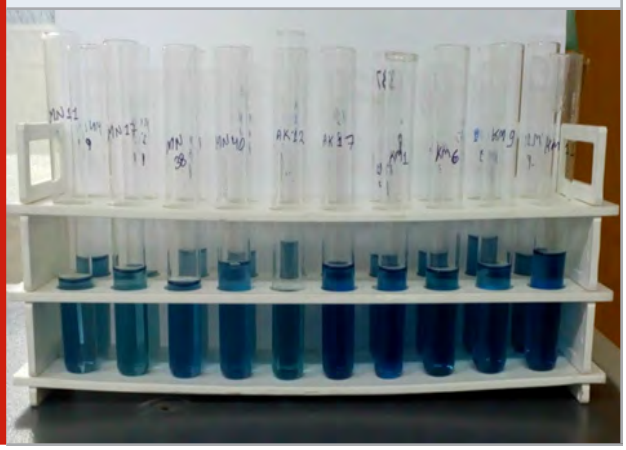

Table 2. Effect of bacterial treatment on different growth parameters in Cyamopsis in green house pot study

Sr. No.	Physiological Characteristics	Soil Nature Control	Control + MN40	Control + KM1	Control + KM6	Control + AK17
1.	No. of plant germinated (%)	50%	50%	100%	75%	100%
2.	Shoot Length (cm)	30	32	38	35	33
3.	Root Length (cm)	4.5	8.5	13.5	10.0	9.0
4.	No. of Leaves	12	12	18	16	14
5.	Root Fresh Weight (gm)	0.49	0.63	1.36	0.55	0.77
6.	Root Dry Weight (gm)	0.17	0.24	0.50	0.18	0.28

CONCLUSION

In the present study, 04 bacterial isolates showing best phosphate solubilising abilities were used as inoculants for plant growth promotion in Cyamopsis. Results indicate that the selected isolates induced a significant level of enhancement in the physiological parameters of Cyamopsis. Owing to their efficient phosphate solubilisation activity, these bacterial strains have potential to support plant growth and be used as biofertilizers and bioinoculants.

Conflict of interest: The authors have no conflict of interest concerning this article.

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Biotechnological Communication

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Performance of Liquid Bio-Formulations on Rhizospheric Soil and Growth of *Pisum sativum L.*

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ABSTRACT

Liquid biofertilizer is increasingly available in the market as one of the options to carrier-based fertilizer, as organic farming can promote the growth of plants. The effect of commercially available herbal liquid bioformulation was studied. In the present work, the efficiency of commercially available herbal liquid bioformulation was assessed in the greenhouse (pot study) on growth enhancement of *Pisum sativum* L. (Green pea). The application treatments included urea formulation (UF), herbal liquid bioformulation (HLF) and herbal formulation coated urea formulation (HCLF) and control. The physicochemical parameters such as pH, electric conductivity, organic carbon, available potassium and available phosphorus were tested before and after treatment. After treatment of different formulation, HLF showed higher organic carbon (1.94%), available potassium (85.56 kg/hectare) and available phosphorus (518.88 kg/hectare). Seed germination rate, root length, shoot length, number of leaves, number of nodes, internodes distance, plant biomass and chlorophyll content were measured at the time of harvest. Liquid herbal bioformulation gave good results when average shoot length, root length, leaf count, nodes and internodes distance were analyzed in controlled environments whereas seed germination was better in control treatment. Though overall growth parameters studied showed enhancement upon application of herbal liquid bioformulation. Total count of bacteria was also highest in the rhizospheric soil of the liquid herbal bioformulation treated plants.

KEY WORDS: BIOFORMULATION, CARRIER BASED FERTILIZER, PLANT GROWTH PARAMETERS, *PISUM SATIVUM* L, RHIZOSPHERIC DIVERSITY.

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INTRODUCTION

Soil microorganisms are considered to play an essential role in maintaining soil health, quality, productivity and sustainability (Zhao et al., 2014). Rhizospheric diversity of microorganism is the important component which affects the growth of the plant (Upadhyay et al., 2019). They are the driving force in organic matter decomposition, nutrient cycling like carbon, nitrogen, phosphorus, sulphur and other nutrients, and other chemical conversions (Murphy et al., 2007).

India is predominantly an agricultural country and green revolution has brought a major change in Indian agriculture. The successes were mainly due to the use of high yielding varieties, fertilizer responsive crop cultivars and increased fertilizer use. However, overemphasis on chemical farming has led to the deterioration of soil health resulting in a decline in productivity, besides causing soil and water pollution problems and health risks (Pandey et al., 2008). Though chemical fertilizers and pesticides help in getting substantial yields, its indiscriminate use and continuous cropping have resulted in deterioration of soil health, which in turn resulted in poor crop productivity (Khandare et al., 2020). In the field of agriculture, many experts found that the model of chemical agriculture will only prove counterproductive in the long run and cause irreversible damage to soil health and the environment.

Organic fertilizers are obtained from animal and plant matter such as compost and green manure. They improve soil structure, increase organic matter content of the soil, promote nutrient mobilization, increase moisture retention capacity of the soil, promote the formation of soil aggregates, suppresses certain plant diseases and soil-borne pathogens and encourage the growth of useful microorganisms (Chen et al., 2006). The advantages of using organic fertilizers over chemical fertilizers are that they do not leave a toxic effect in the soil environment and their use is cost-effective. Appropriate use of organic manures and biofertilizers maintain the sustainability of crop productivity and improve soil health besides complementing chemical fertilizers of the crops. There are several reports which show that the combined and/or sole application of organic manures and biofertilizers increase yield and influence the quality attributes in vegetables (Bahadur et al., 2006) such as green pea.

Bioformulation application to the field is a cost-effective and eco-friendly method as it supplies nutrients like nitrogen and phosphorus (Agrawal et al., 2004; Kumar et al., 2014). The liquid biofertilizers can be better described as "A preparation comprising requirements to preserve organisms and deliver them to the target regions improve their biological activity or a consortium of microorganisms provided with suitable medium to keep up their viability for certain period which aids in improving the biological activity at the target site" (Pindi and Satyanarayana, 2012). Liquid biofertilizer is more cost-effective and easy to use as the production of carrier-based fertilizers is expensive and energy-consuming. Study of the liquid bioformulation is limited only to grain legume crops (Brahmprakash et al., 2007; Sahai and Chandra, 2010). In the present work commercially available liquid bioformulation was used to study its effect on chickpea plant and was compared with urea fertilizer.

MATERIALS AND METHODS

Sample collection: Liquid herbal bioformulation and urea fertilizer samples were collected from Divya Bioscience Ltd., Jamnagar, Gujarat, India using sterile plastic bottles.

Soil analysis: The soil used for the pot study was collected from the greenhouse of Botany Department, Gujarat University, Ahmedabad. The soil was air-dried, grounded manually and was sieved through 2 mm mesh (B.S.S.) to remove stones and large particles. The soil in the pot study was analyzed before sowing and after uprooting of plants at Bhisma Agri Research Biotech Laboratory, Ahmedabad, India. The soil was analyzed for pH, electric conductivity and major chemical composition in terms of available phosphorus and available potassium following the standard protocols (Alef, 1995).

Experimental crop plant: The test plant selected for the present study was i.e. *Pisum sativum* L., one of the important pulse crop in India. It is an annual pulse crop and native to central Asia. The seeds were purchased from the local market, Ahmedabad, India. Visibly seen healthy seeds were selected for the study.

Table 1. Physico-chemical properties of bioformulation

	UF	HLB	HCUF
Physical state	Solid	Liquid	Liquid
Solubility	Water	Water	Water
pH	8.9	7.2	8.1

Evaluation of physical property of formulations: For evaluation of formulations, the prepared 10% formulation of the urea formulation (UF) and herbal liquid bioformulation (HLB) were directly taken. For herbal coated urea formulation (HCUF) 10 g urea and 1 mL of the herbal formulation were mixed and kept for 24 h. From that mixture, 10% formulation prepared in sterile distilled water was taken to check the pH of all treatments by pH meter (model 361 Systronics, India) and used for further study.

Pot culture: Pot culture study was taken up in the greenhouse of the Department of Botany, Gujarat University, Ahmedabad. The pot study was carried out in triplicate. The pots used in the study were having 7 kg soil capacity and in each pot, 5 kg of soil was filled. *Pisum sativum* L. seeds were sown in 12 pots (10 seeds/pot). The seeds of *Pisum sativum L.* were transplanted in twelve pots of equal size. The pots were provided with deionized

water facilities and at every 48 h of the interval, watering was carried out to maintain moisture at approximately 60% water holding capacity of the soil. Control was also maintained without any formulation treatment. On the 45th day, the seedlings were harvested.

Treatment: Total of twelve pots were used for the study. The pots were maintained in the open shade at the room temperature of 26±6 ˚C. The experiment set up was as follows:

T1: Urea formulation treatment; T2: Liquid herbal formulation treatment; T3: Herbal formulation coated with urea; T4: control (without any treatment)

Enumeration of the bacterial population: On 1st, 15th and 45th days of growth, the soil was taken for the enumeration of the bacterial population. Enumeration and isolation of N-fixing bacteria, PO_4-solubilizing bacteria and heterotrophic bacteria from soil samples were conducted using serial dilution technique on agar plates with differentiating media (HiMedia, India). For N- fixing bacteria: Azotobacter agar, for PO_4-solubilizing bacteria: Pikovskaya's agar and for heterotrophic bacteria: High plate count agar media were used. All the dehydrated media were used that were purchased from HiMedia, India and as per instructions they were prepared.

Morphological and biochemical parameters: At the 45th day of pot run, all the plants from pots were removed and studied for the morphological parameters namely per cent seed germination, height of the plant (in cm), shoot length (in cm), root length (in cm), number of leaves (per plant), number of nodes, distance between internodes, plant biomass and chlorophyll content.

Chlorophyll content was estimated using the spectrophotometric method as described by Dere et al., (1998). First 0.1 g of leaves were taken from the plants of each treatment pots and crushed using mortar and pestle with 10 mL of acetone. The mixture was filtered through Whatman filter paper no. 41 and filtrate volume was made up to 25 mL by adding acetone. The mixture was kept in dark condition for 30 min and optical density was measured at 645 nm and 662 nm. Chlorophyll A, chlorophyll B and total carotene content were calculated using the formula described by Dere et al., (1998).

$$\text{Chlorophyll a} = 11.75 * A_{662} - 2.350 * A_{645}$$
$$\text{Chlorophyll b} = 18.61 * A_{645} - 3.960 * A_{662}$$

Statistical analysis: All the experiments were repeated as triplicates. The results obtained in the present investigation were subjected to statistical analysis like mean and standard deviation.

RESULTS AND DISCUSSION

The effect of liquid herbal biofertilizer treatment on vegetative growth of *Pisum sativum L.* was significantly higher than control plants. A significant variation in plant height and number of leaves due to application of biofertilizers was found. Statistically significant increase in plant height, number of leaves, shoot length, root length, number of nodes were observed. Physicochemical properties were also improved after the treatment of liquid bioformulation.

Physicochemical properties of soil

Soil pH: As shown in Table 2, a slight decrease in pH was recorded in the soil of treatment T2 (8.51) as compared to treatment without any inoculations i.e. control (8.69). The results revealed that the initial soil pH before sowing of the crop was 8.97, which decreased significantly with the treatment of different formulation. At harvest, soil pH within various treatments decreased with the application of inorganic and organic fertilizers. The lowering of pH with the addition of liquid herbal bio-formulation may be attributed to the presence of organic acids in the formulation which turned the soil pH towards acidity. Thus, they play an essential role in buffering the soil. Addition of herbal bioformulation may increase the content of organic acids through their secretions thus lowering the soil pH. The results were following findings of Gopinath et al., (2009) and Jaipaul et al., (2011) who studied the effect of biofertilizers, organic manures and inorganic fertilizers on soil properties. They found that soil pH decreased significantly with the addition of farmyard manure along with biofertilizers.

Electric conductivity (EC): The EC of soil is directly related to the ions present in it. It may be due to the production of acids or acid-forming compounds through the decomposition of organic materials, that reacted with the sparingly soluble salts already present in the soil or either convert them into soluble salts thereby increasing their solubility. Electrical conductivity was increased with the addition of urea, herbal and herbal coated urea formulations. Maximum electrical conductivity (0.38 m mho/cm) was observed in treatment with HCUF (Table 2), which was followed by treatment of UF and HLB soil sample showing 0.37 and 0.32 m mho/cm EC respectively. The electrical conductivity of 0.3 m mho/cm was observed that was minimum in the soil of control pots. The results were as per the findings of Bulluck et al., (2002) who reported that the addition of any fertilizer increases soil ion exchange capacity and thus improve soil quality.

Organic carbon: High microbial biomass production and high rhizodeposits of carbonaceous materials through root exudates may be one of the reasons for higher organic carbon in organically treated soils (Franzluebbers et al., 1995). The organic carbon content of the soil was increased in control and all treatment as presented in Table 2. Organic carbon content in control was 2.54% followed by treatment of HLF (1.94%) and HCUF (1.9%). The lowest organic carbon was found in the soil of treatment with UF (1.81%). Results were in agreement with findings of Kumar and Sharma, (2015) who reported an increase in the organic carbon content of the soil with the addition of organic manures and biofertilizers.

Available phosphorus: The maximum available phosphorus of 85.56 kg/ha was observed in the soil treatment of HLF, which was significantly higher than treatment with UF where available phosphorus content was 53.94 kg/ha (Table 2). Available phosphorus in HLF was followed by control pot having available phosphorus of 65.1 kg/ha and HCUF (57.66 kg/ha). The minimum available phosphorus content of soil (53.94 kg/ha) was observed in UF. The results revealed that there was an increase in available phosphorus content due to the treatments with the addition of herbal bio-formulation. This can be due to the presence of phosphorus solubilizing bacteria (PSB) in the formulation, which was effective in increasing the availability of phosphorus in soil by solubilization of native soil phosphorus (Jeon et al., 2003).

Available potassium: Soil potassium of 518.88 kg/ha was observed to be the maximum with HLF followed by treatment of control with an available potassium content of 517.12 kg/ha and treatment of HCUF with 506.97 kg/ha. The minimum available potassium of 503.32 kg/ha was observed in the treatment of UF. This may be due to an increase in the release of potassium from organic compounds and minerals present in soil via decomposition as well as solubilization by soil microorganisms thus increasing the release of organically bound potassium. Results also agree with the work of Tolanur and Badanur (2003) who observed positive changes in the available nutrient status of the soil with the addition of organic fertilizers in legumes.

Table 2. Effect on Physico-chemical properties of the soil before and after treatment

Test	Before treatment	After treatment Control	UF	HLF	HCUF
pH (1:2)	8.97	8.69	8.87	8.51	8.68
Electric conductivity (m mho/cm)	0.27	0.3	0.37	0.32	0.38
Organic carbon (%)	1.52	2.54	1.81	1.94	1.9
Available phosphorus (kg/hectare)	55.32	65.1	53.94	85.56	57.66
Available potassium (kg/hectare)	508.19	517.12	503.32	518.88	506.97

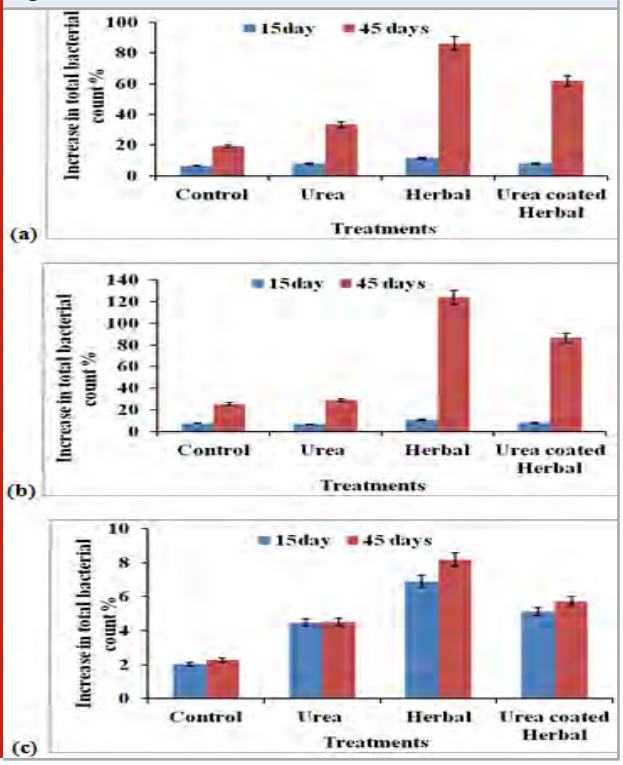

Figure 1. Evaluation of microbial population in all treatment: (a) Percent of bacterial count increase in HPC, (b) Percent of bacterial count increase in Azotobacter agar and (c) Percent bacterial count increase in Pikovskaya's agar.

Enumeration of total count from rhizospheric soil: When it was compared with the initial day, on HPC agar, the bacterial count was increased to about 7 to 10% after 15 days while 18 to 85% increase was recorded after 45 days (Figure 1). While on Azotobacter agar, the increase was of about 10 to 15% after 15 days while 30 to 120% increase after 45 days was noticed. These results are in agreement with the findings of Boomiraj and Christopher (2007) who reported such increased bacterial and fungal population in soil due to application of poultry manure. On both media, HLF showed about 2 to 8 fold increase in bacterial count as compared to control, which was the highest under the experimental condition. In Pikovskaya's agar, when compared with the 0-day bacterial count, it did not increase significantly after 15 and 45 days.

Plant growth parameters

Seed germination (%): The per cent seed germination (%) was highest in treatment of control, which was 76.66% followed by urea and HCUF having 73.33% and the minimum was observed in herbal bio-formulation, which was 63.33%. From the results (Table 3), it can be seen that HLF had an adverse effect on seed germination rate.

Root length: After 45 days of sowing, maximum average root length (17.99 cm) was observed in plant samples with the treatment of HLF followed by control and HCUF having shoot length of 16.4 cm and 16.29 cm respectively. The minimum root length of 14.47 cm was observed in the treatment of urea formulation.

Shoot length: Shoot length showed a significant increase with the addition of HLF (Table 3). After 45 days of sowing, the average maximum shoot length of 41.07 cm was observed in plant samples that received herbal treatment followed by HCUF having shoot length of 37.5 cm. The minimum shoot length of 36.4 cm was observed in the treatment of urea and control.

Leaf count: At harvest, the average maximum number of leaves of 22 were found in treatment HLF followed by treatment of HCUF and urea formulation having 20 and 17 leaves per plant respectively. The average minimum number of leaves at harvest having 16 leaves per plant was found in control. The results revealed that bio-formulation does not significantly increase the number of leaves per plant.

Plant biomass: Treatments had influenced plant biomass differently as shown in Table 3. Average maximum weight of plant was obtained in the HLF treatment that was 3.77 g/plant, followed by HCUF treatment and urea treatment showing 3.11 and 3.04 g/plant respectively. The average weight of plant was recorded in control plants was 2.78 g/plant.

Chlorophyll content: Chlorophyll A and B were maximum recorded in HLF which were 17.34 (mg/g of a leaf) and 9.66 (mg/g of a leaf) respectively followed by HCUF treatment having 17.13 and 9.55 mg/g of a leaf respectively. Minimum chlorophyll A and B content observed in urea formulation were 14.95 and 7.26 mg/g of a leaf respectively. Ansari et al. (2015) and Saghafi et al. (2013) have also reported the increase of chlorophyll content of bio-formulation treated plant.

Table 3. Influence of liquid bio-formulations on various green pea plant growth parameters in the pot study

Plant growth parameters	Control	UF	HLF	HCUF
Germination (%)	76.66±3.2	73.33±4.2	63.33±3.9	73.33±2.2
Root length (cm)	16.4±1.09	14.47±1.08	17.99±1.0	16.29±1.9
Shoot length (cm)	36.4±2.2	36.4±1.2	41.7±0.89	37.5±3.2
Number of nodes	14.92±1.34	17.0±2.5	22.33±2.2	19.75±2.6
Internodes distance (cm)	2.42±0.23	2.14±0.38	1.87±0.11	1.9±0.09
Leaf count	16±1.26	17±1.32	22±1.55	20±1.39
Plant biomass (g/plant)	2.78±0.03	3.04±0.05	3.77±0.09	3.11±0.07
Chlorophyll A (mg/g of leaf)	16.92±2.1	14.95±1.7	17.34±2.09	17.13±1.4
Chlorophyll B (mg/g of leaf)	9.44±0.09	7.26±0.2	9.66±0.56	9.55±0.90

CONCLUSIONS

Treatment of liquid bio-formulation to the seeds showed a positive effect on all the plant growth parameters except the per cent seed germination. It increases the rhizospheric bacterial count of phosphate solubilizer and heterotrophic bacteria. Moreover, it helps in improving the overall soil characteristics which in turn is beneficial for the plant growth.

Conflict of interest: There is no conflict of interest.

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Salinity Alleviation in *Trigonella foenum-graecum* by Employing Cytokinin from *Rhizobium sp.*

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ABSTRACT

Salinity stress is one of the brutal abiotic stress which is increasing at very rapid pace throughout the globe which has profound effect on the plant health and yield causing heavy losses. A wide range of adaptations and mitigation strategies are required to cope with this stress which are simple, low cost and short term biological methods for salinity stress management. Halo-tolerant plant growth promoting rhizobacteria are capable to mitigate salt stress and promote plant growth along-with enhanced synthesis of secondary metabolites including phytohormones (cytokinin), enzymes and volatile compounds. The present work describes isolation, characterization and identification of cytokinin producing halotolerant PGPR from *Trigonella foenum-graecum* (fenugreek) rhizosphere cultivated in saline areas of Little Rann of Kachchh, Western Region of Gujarat, India. Initially, the cytokinin secreting ability was assessed for 20 bacterial isolates and one potential isolate that had ability to synthesize cytokinin under saline conditions (2% w/v NaCl) was selected for further studies. The culture was identified as *Rhizobium sp.* by partial sequencing of 16S rRNA gene. The cytokinin producing ability under salt stress was assessed for *Rhizobium sp.* was observed to be 18 µg/ml. The total estimation of cytokinin biosynthesis by *Rhizobium sp.* was done by TLC and FTIR of extracted cytokinin and reference synthetic cytokinin. Further, confirmation of the activity of extracted bacterial cytokinin was done by in-vitro plant study at pot, field and plant tissue culture trials. This is the first time that *Rhizobium sp.* is the being selected for cytokinin production under salt stress and development of Halo-tolerant PGPR for field based studies and applications. The isolate showing the production of plant growth enhancing phytohormone (cytokinin) can be commercialized as potent bioformulations.

KEY WORDS: CYTOKININ, HALOTOLERANT PGPR, PHYTOHORMONE, *Rhizobium sp*, SALT STRESS.

ARTICLE INFORMATION

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INTRODUCTION

Crop productivity is severely affected by major abiotic factors such as drought and salinity which imposes osmotic stress that limits the growth and development of any given crop. Salinity is an adverse condition in arid and semi-arid areas around the world which is one of the most serious environmental issue that hampers the food security of the growing human population of the world, causing an annual loss of 1–2% of arable land (Shrivastava and Kumar, 2015). Salinity alters cellular processes causing several physiological, morphological, biochemical, and molecular changes in plants (Gupta and Huang, 2014). Salinity drastically influence plant growth and development by accumulation of toxic ions such as Na+ and Cl– in the cells, nutrient uptake imbalances, and oxidative stress damage (Munns and Tester, 2008).

The rhizosphere is a repository of plant growth–promoting rhizobacteria (PGPRs), which can enhance plant acclimitization and growth under high salt stress. PGPRs play an essential role in promoting plant growth even in stress conditions by both direct and indirect effects on plant growth. The direct mechanisms involve biosynthesis of phytohormones(Cytokinin,IAA etc) enhanced nitrogen fixation, phosphate solubilization,HCN, HCN production etc (Ansari and Ahmad, 2018).Bacterial species belonging to *Azospirillum, Alcaligenes, Arthrobacter, Acinetobacter, Bacillus, Burkholderia, Bradyrhizobium, Enterobacter, Erwinia, Flavobacterium, Pseudomonas, Rhizobium* and *Serratia* have already been reported for their Plant growth promoting activities (Yasin et al., 2018;Khan et al., 2018).

Cytokinins are a class of pytohormones that play a key role during the cell cycle and influence numerous developmental programs (Skoog et al., 1965). The extremely low levels of the endogenous cytokinin is found in plant tissues(Letham, 1994).The ability of rhizobacteria to produce phytohormones cytokinin, is major factor affecting plant growth. Cytokinin distributed widely in higher plants, algae, and bacteria which are also produced by plant related bacteria (Tirichine et al.,2007). This hormone is a key factor in the formation of plant tumors (Liu et al. 2013). Cytokinin regulate cell division, cell enlargement, and tissue expansion, implicates beneficial effect on plant growth and yield (Hanano et al.,2006; Weyens et al., 2009) and play a major role from seed germination to leaf and plant senescence and harmonize important physiological processes throughout the lifecycle of the plant (Mok, 1994; Timmusk S et.al., 1999).

The cytokinin also improves senescence inhibition, cell growth, secondary-compound metabolism, respiration inhibition during senescence and stimulation of respiration during development under stress condition. The present work deals with isolation, identification and characterization of cytokinin producing bacteria from the rhizospheric soil of *Trigonella foenum-graecum*. Here we highlight the isolation of potent Halo-tolerant cytokinin producing bacteria and its competence as an alternative ecofriendly bio-enhancer for enhanced crop production under elevated salinity.

MATERIALS AND METHODS

Sample collection: Soil sample was collected from rhizosphere of *Trigonella foenum-graecum* commonly known as fenugreek, Little Rann of Kachchh, western region of Gujarat, India. The geographical location of the sampling site is latitude 23.31'67"^{0}N and longitude 69.62'89"^{0}E and the sampling was collected during winter- summer transitional period (Shah et al., 2020). The estimation of various physico-chemical parameters viz. pH, electric conductance, salinity, bulk density, soil texture, moisture, redox potential etc. was done by standard methods.

Chemicals and media used in present study viz. NaCl, $MgSO_4$, Na_2HPO_4,KH_2PO_4 and $CaCl_2$ were procured from CDH Fine chemicals (P) Ltd., New Delhi, India. While ethyl acetate, methanol, kinetin, methanol, sodium hypochlorite, glycerol and carboxy-methyl-cellulose were purchased from SRL Pvt. Ltd, Mumbai, India and rest of the microbiological media viz. nutrient agar, Trypton Yeast Broth (TYB), pre-coated TLC plates, casamino acid, thiamine and biotin were procured from HiMedia Laboratories, Mumbai, India.

Isolation and Screening of potential halotolerant strain RM3 cytokinin producer: Salt-tolerant strain RM3 was isolated from soil sample collected from rhizosphere of *Trigonella foenum-graecum*. Of all the strains isolated, strain RM3 was showed significant tolerance to Sodium Chloride (NaCl) and thus it was selected for further studies. Salt tolerance assay was performed by supplementing NaCl in the Tryptone culture medium with 670mM concentration. The growth of strain RM3 was determined by turbidometric assay using spectrophotometer. Of all the strains under study, RM3 exhibited eminent tolerance to 670mM NaCl concentration.Colonial and Morphological characterization of the isolate RM3 was done after incubating the Nutrient agar plate streaked with the pure culture of RM3 from preserved slants.The strain was cultivated using Nutrient Broth (NB) (HiMedia MM244, India) supplemented with 670mM NaCl for 20 - 24 h at 30 ± 2 °C. The culture was activated by inoculating a loopful of culture in fresh M9 medium supplemented with 0.2% Casamino Acids, 0.01% thiamine, and 2 pg of biotin per liter (Akiyoshi et al.,1987) following incubation at 28 ± 2 °C up to 5d with agitation at 150 ± 5 rpm (Remi,India). Turbidity of activated culture with an optical density of 0.9 ± 0.1 at 600 nm was used as inoculum with an aliquot equivalent to 0.1 %(v/v). Growth was determined spectrophotometrically at 600 nm using Systronics 166 spectophotometer

Determination of cytokinin synthesis in M9 medium: M9 medium supplemented with 0.2% Casamino Acids, 0.01% thiamine, and 2 pg of biotin per liter (Akiyoshi et al., 1987) was used for cytokinin synthesis.100 ml of sterile M9 medium in 250 mL flask Erlenmeyer flask was inoculated with 1 mL of activated RM3 culture and

incubated at 28 ± 2°C, 160 rpm(Remi,India) for five days. The experiment was conducted in triplicates.Cytokinin synthesis was quantified spectrophotometrically at 665 nm on 72, 96 and 120 h, respectively with uninoculated M9 medium served as control.

Extraction of crude cytokinin: Bacterial cells were separated from the supernatant by centrifugation (Remi, India) at 10,000 rpm for 20 min at 4°C. The cell free supernatant was filtered through Millipore Filter of 0.45µm size (Hi-Media, India) and extracted thrice with ethyl acetate.The insoluble fraction of extract was dissolved in 1 ml of HPLC grade methanol and kept at –20 °C till further analysis(Patel and Saraf, 2017)

Thin layer chromatography of extracted cytokinin: A small volume (10 µL) of ethyl acetate extract and standard cytokinin(Kinetin) were spotted on TLC chromatogram(Silica gel G f254, thickness 0.25 mm) standard cytokinin (Kinetin) using mobile phase n-butanol: acetic acid: water (12:3:5 v/v/v) and was observed under the UV light (254 nm) using UV transilluminator (Biorad,India) (Patel and Saraf, 2017).

Molecular characterization of extracted cytokinin by FTIR: Molecular characterization of cytokinin produced by RM3 was confirmed by FTIR spectra performed as per methods described by Kamnev et al., 2001 using BRUCKER Alpha ECO-ATR (Attenuated Total Reflectance) with 16 scans and 4000-400 cm−1 using extracted cytokinin dissolved in HPLC grade methanol.

16S rRNA Analysis: Predefined standard protocol provided by HiMedia Hipura Bacterial Genomic DNA Purifcation Kit (HiMedia-MB505, India) was used for extraction of genomic DNA (g-DNA) of the selected isolate RM3(Shah et al.,2019). The activation of culture was proceeded by growing in Luria Bertani media for 18–20 h. The PCR reaction was carried out in Veriti Thermal Cycler (Applied Biosystems) and the 16S rRNA gene was amplified using universal primers. The sequence was then locally aligned using NCBI BLAST (Altschul et al., 1990). The identification was done based on the maximum percentage similarity with the already existing similar sequence in the database . The 16S partial gene sequences obtained from this study were submitted to Genbank. Phylogenetic and molecular evolutionary analyses were conducted by Maximum Likelihood method, using MEGA version X with the bootstrap value of 1000 replications (Kumar et al., 2018).

Pot and field trials of RM3 isolate on Trigonella foenum-graecum: The seeds of fenugreek (Gujarat Methi-2) *Trigonella foenum-graecum* were used for the study since they have short germination time(110-120d).The seeds were surface sterilized in 70% ethanol for 2 min and in 2% sodium hypochlorite for 5 min and then washed ten times in sterile distilled water. The selected potent isolate RM3 was grown in M9 medium supplemented with 670mM NaCl, 2% casamino acids, 0.01% thiamine, and 2 pg of biotin per liter (Akiyoshi et al. 1987), pH 7.0 and incubated at 28±2 °C for 5d. The surface sterilized seeds were soaked overnight in culture inoculated M9 medium containing sterilized carboxy-methyl-cellulose (CMC 1%) as an adhesive at room temperature and later on were air-dried for further studies. Seeds treated with sterile distilled water amended with CMC alone served as control. A 25 cm pots were taken for pot trials and 250 g sterile soil were sown with 5 seeds each. The vegetative profile of the plant was monitored after 30d for pot trials (Shah et.al.,2019). The experiment was performed in triplicates. Similar method of seed sterilization and seed bacterization was used for field trials where field was located at Nakhtarana near Bhuj ,western region of Gujarat ,India with the geographical location 23° 21' "0" N and 69° 15^0 48 "E. The vegetative profile of the plant was monitored after 60d for field trials.

Plant Tissue Culture (PTC) bioassay of extracted cytokinin from *Rhizobium sp.*: Plant tissue culture bioassay was done in order to analyze the activity of bacterial extracted cytokinin on *Trigonella foenum-graecum* seeds.The seeds were surface sterilized by the method used for pot and field trials seed sterilization. Jensen seedling agar (HiMedia,India)was used as a PTC medium instead of Murashige and Skoog(MS) medium. Seedling agar medium supplemented with 670mM NaCl and bacterial cytokinin extract (0.1%v/v) was inoculated with the surface sterilized fenugreek seeds.The Jensen seedling agar supplemented with standard kinetin and 670mM NaCl was also inoculated with surface sterilized fenugreek seeds in order to get the comparative results and slants were incubated in PTC laboratory with 19±2 C temperature.

RESULT AND DISCUSSION

Physicochemical characteristics of soil sample: Physicochemical characteristics of Rhizospheric soil sample were estimated in terms of its appearance, soil temperature, color, pH, electric conductance, organic carbon, Nitrogen, Phosphorus, Potsassium, Copper, Zinc, Manganese, Boron, Sulphur, TDS replaced by nitrogen, phosphorus, potassium, copper, zinc, manganese, boron, sulphur, TDS, salinity, chloride, iron content and bulk density by standard laboratory methods. The results are as described in Table 1.

Soil sample implicates the higher value of chloride, redox potential, salinity and iron content with highest TDS values. Higher pH showed that the soil was alkaline in nature. There was presence of other soluble salts also that helps in making the soil saline in nature. This indicated that soil is not suitable for the agricultural use (Shah et al., 2019). The organic and inorganic content of soil play an key role for driving the microbial community structure(Zhou et al., 2014)

Isolation and screening of potential halotolerant strain RM3 cytokinin producer: The results of colony characteristics of potential halotolerant bacteria are compiled in Table 2. The salt tolerance was checked in Tryptone medium supplemented with 670 mM NaCl and the response activity was monitored from24 h to 96

h. Figure:1 shows the growth of the selected isolate at different NaCl concentration. Phytohormones improves the protective response of plants under biotic and abiotic stresses (Shah et.al, 2020). Bacterial isolate was selected depending upon distinct colony morphology and was taken up for further cytokinin biosynthesis sc. The halo-tolerance assay of selected isolate was tested. Remonsellez and his collegeaus defined halotolerant bacteria are those capable of growing in the absence as well as in the presence of relatively high salt concentrations (F Remonsellez et.al, 2018).A total of 20 different isolates grown at 670 mM salt were selected primarily and potent three, cytokinin producing bacterial isolates were selected. A most potent one RM3 isolate was found for secretion of cytokinin under 670 mM NaCl concentration. Previous studies reported that salinity can increase cytokinin synthesis at higher concentrations (300mM) of NaCl in plants enhance photosynthesis and related processes (Pavlu J at el.,2018) Present studies highlight the cytokinin synthesis by bacterial isolate RM3 at NaCl concentrations as high as 670 mM.

Table 1. Physicochemical analysis of soil (Shah et al., 2019)

Sr. No.	Parameter	Unit	Soil Sample
1.	Color	-	Brown
2.	Temperature	^{0}C	35 ±^{0}C
3.	pH	Direct	8.6
4.	Electric Conductivity	Milli. moh/cm	0.60
5.	Organic C	In %	1.37
6.	Nitrogen	In %	0.12
7.	P	ppm	3.62
8.	K	ppm	26
9.	Cu	ppm	1.82
10.	Zn	ppm	2.32
11.	Mn	ppm	5.94
12.	Fe	ppm	58.75
13.	Boron	ppm	1.34
14.	Sulphur	ppm	21.7
15.	Cl-	mg/K	300
16.	TDS		250
17.	mV		252
18.	Salinity	ppt	50
19.	Bulk density	mg/m3	1.33
20.	Soil moisture	In%	12.60

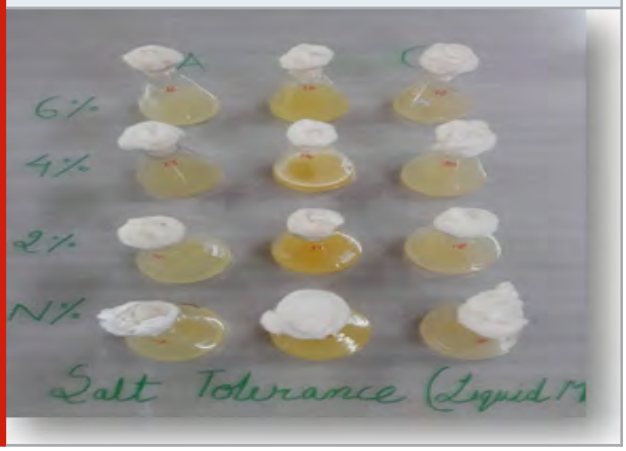
extracted cytokinin sample in the lane was determined to be same as that of reference standard for cytokinin. The results are in order with those earlier reported by our lab (Patel and Saraf, 2017; Tranová L et al., 2019)

FTIR analysis of extracted cytokinin: The FTIR graph shown in Figure:3 indicates the presence of three intense peaks in the stretching f region. A complete overlapping of standard and sample peak points confirms the presence of cytokinin along with the other compounds with different functional groups that were produced by the RM3 under salt stress and can help plant to mitigate

Table 2. Colonial and Biochemical characteristics of isolate RM3 growing on 670 mM NaCl concentration

Characteristics	Results	Characteristics	Results
Size	Medium	Citrate Utilization	-
Shape	Round	Starch Utilization Test	+
Margin	Even	Glucose peptone agar Test	+
Surface	Smooth	Gelatin Test	-
Elevation	Raised	Catalase Test	+
Pigmentation	Pale Yellow	Urea	+
Consistency	Butyrous	Ornithine Decarboxylase	+
Opacity	Translucent	H2S production	-
Gram's Reaction	Negative	Voges Proskauer	+

Figure 1: The growth of the selected isolate at different NaCl concentration



Determination of cytokinin synthesis in M9 medium: Quantification of cytokinin secretion by spectrophotometric method as described by (Patel and Saraf,2017). The cytokinin production was observed in RM3 i.e. 18± 0.1 µg/ml under standard assay conditions. Till to date there have been no reports for cytokinin production from RM3 under salt stress we first time report the synthesis of higher levels of cytokinin under salt stress. Rhizobium sp. had been extensively explored as its halotolerance ability (Franzini V I et al., 2019) and its Cytokinin production(Kumar et al.,2019). Many strains of Rhizobium are capable of producing cytokinins (Wang et al., 2019) in pure culture at high cell mass. Till to date there have been no reports for cytokinin production from Rhizobium sp. we first time report the synthesis of higher levels of cytokinin under salt stress.

Figure 2: TLC of Cytokinin observed under UV light, where Lane1: Standard (RF=1.7), Lane2: Isolate C extract (Rf=1.6)

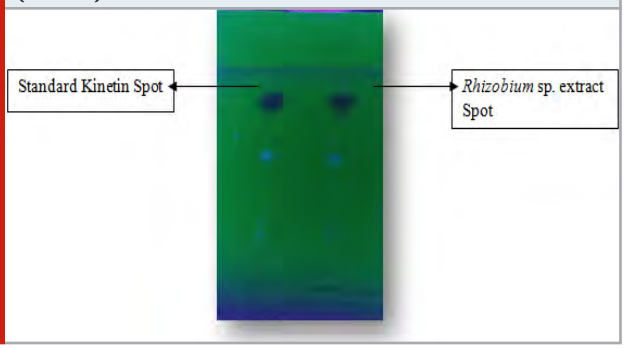
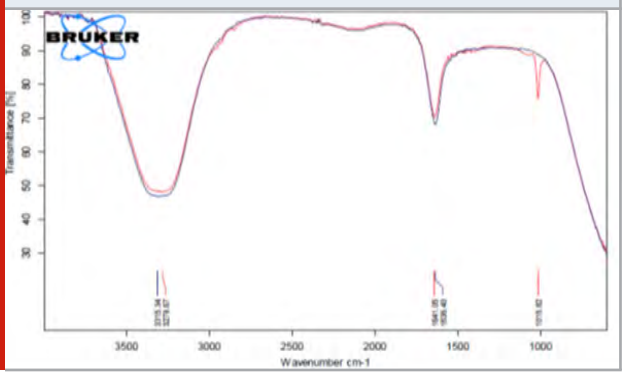


Figure 3: FTIR of bacterial cytokinin



Extraction & detection of cytokinin by thin layer chromatography: TLC plates was spotted with ethyl acetate fractions of cytokinin and developed using mobile phase n-butanol: acetic acid: water (12:3:5 v/v/v). Blue spots were identified under UV light which detected the cytokinin compound(Figure:2). The Rf values of a salt stress. The FTIR analysis of cytokinin in aqueous solution was reported by DS Hart and his coworkers in order to check the stability of cytokinin at various pH (Khatri et al.,2019)

16S rRNA Analysis: The genus and species level identification was done based on the maximum percentage similarity with the already existing identical sequence in the database .The sequence is being submitted to GenBank with accession no. MK603478 *Rhizobium sp.*(Shah et al., 2020).The tree with the highest log likelihood is shown in Figure 4. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value (Shah et al.,2019). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018)

Figure 4: Phylogentic analysis of *Rhizobium sp.*

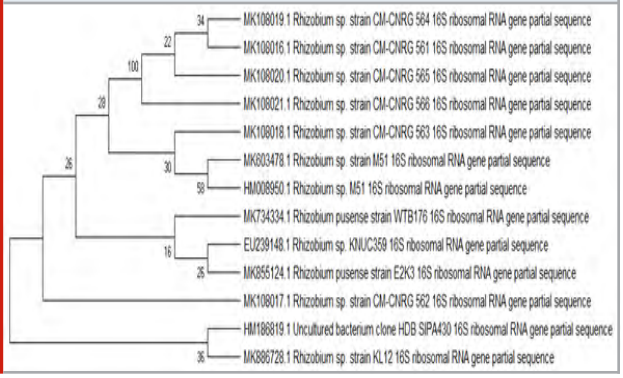


Table 3. Vegetative parameters of Plantlet recorded from pot and field study

Treatments	Wet weight(g)		Dry Weight(g)		No. of Leaves	Length(cm)	
	Shoot	Root	Shoot	Root		Shoot	Root
			Pot study				
Control	0.05	0.03	0.01	0.01	10	10.5	5.5
Rhizobium sp.	1.89	0.23	0.80	0.10	20	15.5	8
			Field study				
Control	0.28	0.55	0.10	0.28	7	10	5.3
Rhizobium sp.	0.80	2.11	0.58	0.43	20	14	44.6

Pot and field trials of *Rhizobium sp.* on Trigonella foenum-graecum: An increase in both wet weight and dry weight of shoot as well roots was noted along with the increased length of shoot and roots, after treatment with bacterial culture producing cytokinin. The results are shown in Figure: 5 (a) and (b) with treated and untreated plant samples and recorded results over a 30d growth cycle in pot study and 60d growth cycle in field study. A comparative data of vegetative parameters of control plant and *Rhizobium sp.* treated plantlet is summarized in Table: 3 which clearly implicated the positive effect of *Rhizobium sp.* on Plant development. Plant growth enhancing activity by halotolerant consortia is also reported in *Triticum aestivum* L. (Rajput et al., 2018). PGPR activity of halotolerant bacteria is also reported in Durum Wheat (*Triticum turgidum subsp.* durum) (Albdaiw et al., 2019)

Figure 5: Plantlets of *Trigonella foenum-graecum* uprooted from (a) pot(30d) and (b) field(60d)[Co=Control,C=*Rhizobium sp.]*

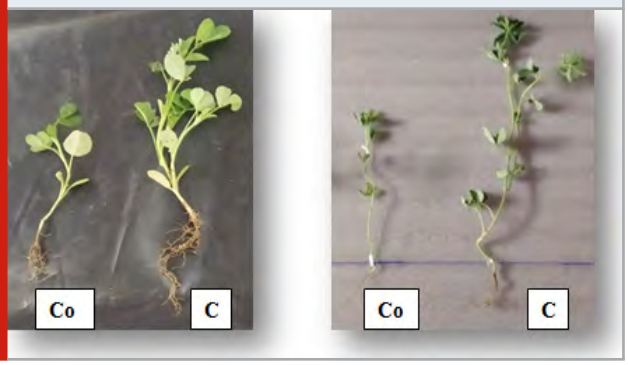
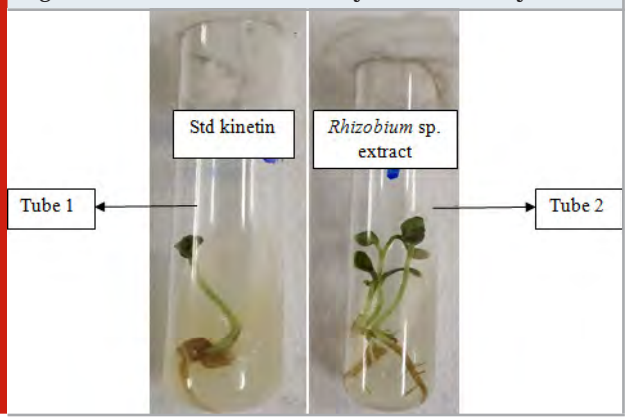


Figure 6: Tissue Culture Bioassay of extracted cytokinin



Plant Tissue Culture (PTC) bioassay of extracted cytokinin from Rhizobium sp.: PTC Bioassay is the best method for assessing the activity of any phytohormone on plant under standard conditions.Result of PTC bioassay on Fenugreek seed by bacterial cytokinin extract was shown in Figure:6.Tube 1 having standard cytokinin shows partial seed germination without leaves and less developed root,whereas Tube 2 having extracted *Rhizobium sp*. cytokinin shows a prominent fast response of seed germination along with healthy leaves,elongated shoot and well developed roots. This bioassay was attempted for the first time with addition of 670mM NaCl concentration.

The results in present studies are reassuring since higher salinity tolerance at 670mM as well as increased fenugreek plant growth by bacterial isolate RM3 is better than all of those that are previously reported. Moreover present studies are the first of kind reports from Little Runn of Kachchh, Gujarat, Western India, a semi-arid alkaline,saline dessert area, where very minor or no cultivation of crops is possible. The reports depicted a novel way of breaking headway for entrenching newer and effective farming concepts in such salt affected areas. *Trigonella foenum-graecum* being a medicinal plant in major part of world the reports of enhanced growth and developmental activities will also revolutionize sustainable agriculture in upcoming time.

CONCLUSION

The present work is path made for isolating the beneficial bacteria and to investigate its plant growth promoting potential under salt stress.Plant growth and development is mostly dependent on PGPR,this work focuses on biosynthesis of cytokinin by the isolated bacterium ,an important phytohormone that play a vital role in survival of plant under abiotic stress like salinity and drought by helping in division and differentiation of plant cells. In present study isolation of such multifarious bacterial isolate would be a positive approach towards higher yield and productivity of crop plant under salt stress.The future applications of isolate having enhanced cytokinin synthesis will certainly give an idea for developing bio-based products that have application to be used as bio-enhancer .The direct cloning of cytokinin synthesis gene in plant varieties can be useful for attaining sustainable agriculture approach with higher productivity of crops and reduces bad effects of salinity stress.

Conflict of interest: The authors declare no conflicts of interest in preparing this article.

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Bacterial Endophytes and Extraction of Secondary Metabolites of Euphorbia caducifolia from Tropical Dry Regions in Gujarat

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ABSTRACT

Endophytes are the microorganisms that can reside within robust plant tissues by just having a symbiotic association. Main aims were to isolate endophytic bacteria and extraction of phytochemicals as secondary metabolites to explore abundances of endophytes. *Euphorbia caducifolia* was from semi arid dry regions of banaskantha Gujarat, India The endophytic bacteria has an important role on the plant, such as a growth promoter with the production of plant growth regulators, besides that it can supply the plant nutrient and they are necessary for the growth and development of the plant. A plant and bacterium association between Euphorbia caducifolia and endophytic bacteria can produce secondary bioactive compounds which are helpful in many ways. Endophytic bacteria were isolated from root, stem and latex of plant *E. caducifolia*. They can globally associate and studied in all plants till date. Total ten isolated were obtain from stem, root and latex of *E. caducifolia*.

KEY WORDS: EUPHORBIA CADUCIFOLIA, ENDOPHYTIC BACTERIA, SECONADRY COMPOUNDS, PHYTOCHEMICALS.

INTRODUCTION

All plants are medically important. The plant endosphere contains a diverse group of microbial communities. Endophytes are the microorganisms may be bacteria; fungi or actinomycetes .Endophytic organisms live within the plant tissues and can promote host species tolerance to different environmental stresses. Almost all plants species have been found to harbour endophytic bacteria or fungi (Sturz and Nowark, 2000).The term Endophytes was first coined by De Berry in 1866. They are also reported to supply essential vitamins to plants (Rodelas et al., 1993), confer protection against plant pathogenic microorganisms via production of antibiotics (Struz et al., 1999) or synthesis of secondary metabolites (Long et al., 2005). . Endophyte containing plants grow faster than the non containing ones (Cheplick et al., 1989). Endophytes would have enhanced the hosts' uptake of nutritional elements such as nitrogen and phosphorus (Gasoni and Gurfinkel, 1997; Malinowski and Belesky, 1999).

Attempts are being made to isolate and identify bioactive metabolites from endophytic organisms because of its economical impotents. *E.caducifolia* popularly known as a 'dandi thor' or 'Haines' is a cactus, mainly found in all over india. Thus, it is observed that it has a vast range of therapeutic claims and is used in skin disorders; gastro-intestinal diseases, gynecological and obstetrical

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problems; respiratory diseases; musculo-skeletal disorders, ENT disorders, immune disorders and as an antidote in snake-bite, scorpion bite. *E. caducifolia* has also been reported for its wound healing, antimicrobial, antibacterial, antifungal (Goyal, Sasmal and Nagori, 2012) and anti nematologiacal activities (Maqbool, Hashmi and Ghaffar, (1987). Phylloclade dried biomass of *E. caducifolia* has potential as a renewable source for biofuel (Patan Shaik et al., 2013). Laticifers in its phylloclade have also been reported for potential hydrocarbon yielding (Rajeswari, et al., 2014). Its juice inhibits leukocyte migration which acts as a catalyst in the development of inflammatory diseases; it may also provide instant relief from Dysmenorrhea (Mirabi et al., 2014).

MATERIAL AND METHODS

2.1) Sampling: Mature and asymptomatic plant of *Euphorbia Caducifolia* was collected from latitude of 23.9944° N and longitude of 72.4099° E Pasvadal village located in Vadgam Tehsil in Banaskantha district of Gujarat state in December 2018. The sample was collected in sterilized polythene zip lock bag, transported to laboratory in the Department of Microbiology Gujarat University, Gujarat, India within eight hours and processed immediately.

Figure 1: Plant of *Euphorbia Caducifolia*

2.2) Isolation: Zin et al., 2007 described method for surface sterilization. Plant was firstly washed with running tap water for removal of soil and other impurities and then its outer surface was peeled off with knife to remove cactus spines which are actually modified leaves. Then samples were washed with running tap water and for dislodging soil and organic materials sonication was carried out for 10 minutes and immerse in 75% ethanol for 5 minutes and then in 2% hypochlorite solution for again 5 minutes and again swill them with 10% Sodium bicarbonate for 10 minutes and then flood with sterile distilled water and wait up to sample dry. Then different parts of plant were cut i.e. stem and root. Samples of stem parts were selected by aseptic cutting in vertical sections and horizontal sections in between the Bunsen burner in laminar air flow and inoculated on respective media. For the checking of effectiveness of surface sterilization final rinsed water was spread on Nutrient agar medium at 37°C. After drying of plant materials horizontal and vertical sections of stems and root were inoculated on nutrient agar, Actinomycetes agar, potato dextrose agar and Tryptic soy agar. Latex was scrapped off using sterile scalpel and then spread on all plates, all plates incubated at 28 ± 2^{0} for 6-7 days.

2.3) Physiological characterization: At first all isolates were tested for their phenotypic features. Gram's reaction was performed and the cell shape was observed under light microscope by taking adequate amount of bacterial suspension in sterile physiological solution. Antimicrobial activity against the test pathogens was checked.

2.4) Bacterial extraction: Selected bacterial isolates were grown in nutrient broth and for the extraction of bioactive substances ethyl acetate was used as a solvent. 10 ml activated broth was taken in separating funnel, equal amount of ethyl acetate was added and continuous strokes for 5 minutes were given. After the settling of layers lower layer was discarded and process was repeated for three times and extracts were collected in screw cap bottles and kept those in refrigerator (Das et al., 2017) For the Phytochemical analysis various tests were performed (Banu et al., 2014, Galib et al., 2017, Tambe et al., 2014).

Biochemical characterization: Three bacterial isolates giving best results in terms of antimicrobial activity were subject to biochemical identification. Different media were prepared, then inoculated and incubated for 24-48h at 37°C, results and observations were recorded (Table 1). Interpretations were done by according to the Bergey's manual Vol. III 2nd edition.

Sugar Utilization test: Peptone 1.0g/L, NaCl 0.5g/L, Sugars 0.5 g/L, Andrade's indicator 0.005% inoculate cultures, after incubation observed colour changes in pale yellow to pink indicates positive test.

Oxidative fermentative test: Inoculate test organism by stabbing agar in Hugh & leifson's media, after incubation colour changes in green to yellow indicate positive test.

Methyl red Test: Inoculate Glucose Phosphate broth after 24 h add few drops of methyl red development of red colour indicate positive test.

Voges Prosauer Test: Inoculate Glucose Phosphate broth after 24 h add few drops of 40% KOH mix vigorously and add alpha naphthol development of brick red colour indicate positive test.

Citrate utilization Test: Inoculate Simmom citrate agar, incubate it for 24h it if colour changes to blue indicates positive test.

Indole Production Test: Tryptone 10.0g/L, NaCl 5.0g/L, pH 7.5; inoculate medium with test culture after 24 h of incubation, add 2-3 ml xylene by shaking vigorously and add 2 ml Ehrlich's reagent if development of pink ring at the junction of broth and xylene then test is positive.

Hydrogen sulphide production Test: Peptone 20.0g/L, NaCl 5.0g/L, pH 7.6; In2% peptone broth inoculate test culture, place soaked white filter paper strip in saturated lead acetate solution observe the result for blackening of paper.

Phenyl alenine deamination Test: Streak phenyl alanine slant and incubate it for 24 hour, after that add 4.0 ml 10% ferric chloride development of green colour indicates positive test.

Urea hydrolysis Test: Urea broth: Yeast extract 0.1g/L, K2HPO4 9.1g/L, K2HPO4 9.5 g/L, Urea 20.0 g/L, Phenol red 0.01 g/L, pH 6.8; development of purple red colour indicate positive test.

Nitrate reduction Test: Peptone 5.0 g/L, Meat extract 3.0 g/L, Potassium nitrate 1.0 g/L, Final pH 7.0 ± 0.2 (25°C), after incubation add 2 ml of sulfanilic acid and 2 ml of a α-naphthylamine. Development of red colour shows positive test.

Starch hydrolysis Test: Streak Starch agar plate as a straight line and incubate it, after flood plate with iodine solution, observes if blue colour developed surrounding the growth.

Casein hydrolysis Test: Streak line on a casein agar and after incubation observe clear zone surrounding growth.

Lipid hydrolysis Test: Tributyrene agar; Peptone 5.0 g/L, Yeast extract 3.0 g/L, Tributyrene 10.0 g/L, Calcium carbonate 0.01 g/L, Agar 20.0 g/L, pH 7.4, streak test culture and observe clear zone of solubilisation next day.

Catalase Test: Streak test culture on nutrient agar slant and incubate it for 24 hours, after that add 1.0 ml H2O2 over the growth appearances of gas bubbles indicate positive test.

Dehydrogenase Test: Inoculate nutrient broth and add diluted methylene solution, incubate for 24 hours. Disappearances of Methylene blue shows positive test.

Haemolysin production Test: Blood agar; 10.0 ml blood in, 100 ml melted nutrient agar, solidify it and streak as straight line with test organism. Clear zone around the colony and certain growth indicates positive test.

Triple sugar iron test: Beef extract 3.0 g/L, Peptone 20.0 g/L, Yeast extract 3.0 g/L, Lactose 10.0 g/L, Sucrose 10.0 g/L, Dextrose monohydrate 1.0 g/L, Ferrous sulphate 0.2 g/L, Sodium chloride 5.0 g/L, Sodium thiosulphate 0.3 g/L Phenol red 0.024 g/L Agar 12.0 g/L; stab and strek slant and incubate it. Changes in colour of medium also acid and gas production indicates positive test. Qualitative and quantitative test for phytochemicals as Secondary metabolites. Preliminary Qualitative tests (Banu et al., 2015)

Phenols: For Ferric chloride test extract was collected, 5 ml distilled water and few drops of 5% ferric chloride solution is added for the development of blue/green colour. For lead acetate test 3 ml 10% lead acetate was added in 1 ml extract. White precipitates show the positive test.

Steroids: Libermann Buchard test was performed, Add 2 ml acetate acid + 2 ml concentrated sulphuric acid in 1 ml extract, violet to blue/green colour appear. Few drops of glacial acetic acid and concentrated hydrochloric acid were added in 1ml extract, violet to blue/green colour developed in Libermann sterol test.

Table 1. Results of biochemical tests

Biochemical Tests	Results		
	Ap	Ts	Tp
Sugar Utilization Test			
Dextrose	-	-	AG
Sucrose	AG	-	-
Lactose	AG	-	AG
Maltose	AG	-	AG
Fructose	AG	-	AG
Mannose	AG	-	AG
Oxidative Fermentative Test	-	-	-
Methyl red Test	+	+	+
Voges Prosauer Test	-	-	-
Citrate utilization Test	+	-	+
Indol Production Test	-	-	-
Hydrogen sulphide productionTest	-	-	-
Phenyl alenine deamination Test	-	-	-
Urea hydrolysis Test	+	+	+
Nitrate reduction Test	+	+	+
Starch hydrolysis Test	+	+	+
Casein hydrolysis Test	+	+	+
Gelatin hydrolysis Test	+	+	+
Lipid hydrolysis Test	-	-	-
Catalase Test	+	+	+
Dehydrogenase Test	+	+	+
Heamolysin production Test	-	-	-
Triple sugar iron test	A	AG	A

Key: (+) Positive test, (-) negative test, (AG) acid and gas production, (A) acid production

Flavanoids: In 1 ml extract add 1 drop of sodium hydroxide and diluted hydrochloric acid yellow colour of solution was disappear. Red precipitates were observed when Zn dust and hydrochloric acid were added in 1 ml extract.

Tannins: In 1 ml extract add 1 ml 2% lead acetate solution white precipitates indicate positive test. On the addition of potassium dichromate solution in 1 ml extract precipitates appeared.

Saponins: Frothing test; In 1 ml of extract add 5 ml distilled water and shake vigorously forth formation indicates positive test.

Alkaloids: Mayer's test; 1 ml extract add 2 ml Mayer's reagent, dull white precipitates show positive test. (Evans et al 1997). In Dragendroff test reagent was added and orange precipitates indicate positive test. Wagner's reagent was added and if reddish brown precipitation observed it show positive test (Wagner et al., 1993).

Quantitative test: The quantification of Phytochemicals present in bacterial isolates standards was performed, total Phenolic, alkaloid, Saponins and tannins compounds determined by using spectrophotometry. Folin-Ciocalteu assay method was used for the analysis of the total phenol content. Gallic acid was used as a standard solution. Test extract was taken in different aliquots (Absorbance for test 0.1 to 1 ml) and 1 ml 20% Na2CO3. 0.5 ml folin-Ciocalteu reagent was added. Solutions of standard as well as test were boiled in water bath for 10 minutes and final volume was made 20 ml by using distilled water. Absorbance was measured at 550 nm against reagent blank (Ghasemzadeh et al., 2010).

Figure 2: Endophytes growth on TSA media

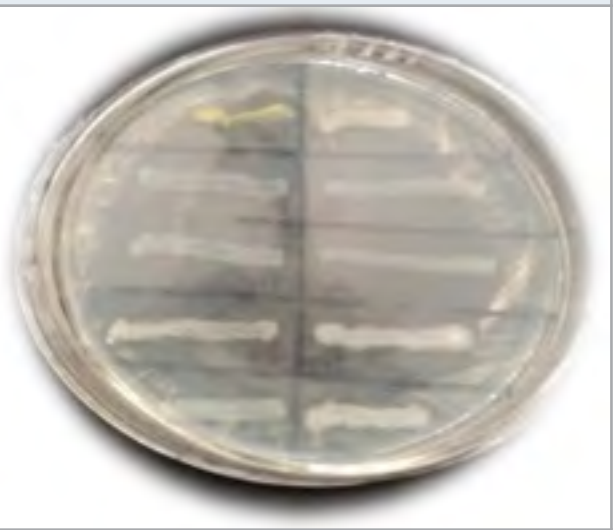
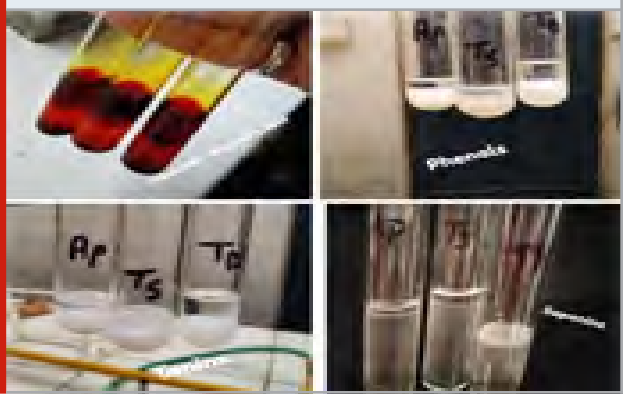


Table 2. Results of Qualitative analysis of Phytochemicals

Phytochemical Tests	Result		
	Ap	Ts	Tp
• Alkaloids:			
1. Mayer's Test	-	-	-
2. Dragendroff's Test	-	-	-
3.Wagner's Test	++	-	+ +
• Saponins:			
1. Frothing Test	++	+	++
• Tannins:			
1. Lead Acetate Test	++	+++	++
2. Potassium Dichromate Test	-	-	-
• Phenols:			
1. Ferric Chloride Test	-	-	-
2. Lead Acetate Test	++	++	++
• Steroids:			
1. Libermann Burchard Test	-	-	-
2. Libermann Sterol Test	-	-	-
• Flavonoids:			
1. Alkaline Reagent	-	-	-
2. Zn Hydrochloride Reduction Test	-	-	-

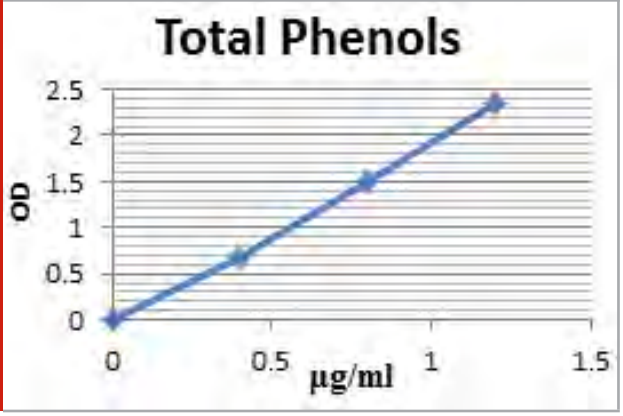
Key: (+++) Maximum, (++) moderate, (+) minimum, (-) negative

Figure 3: Tests of Phytochemicals

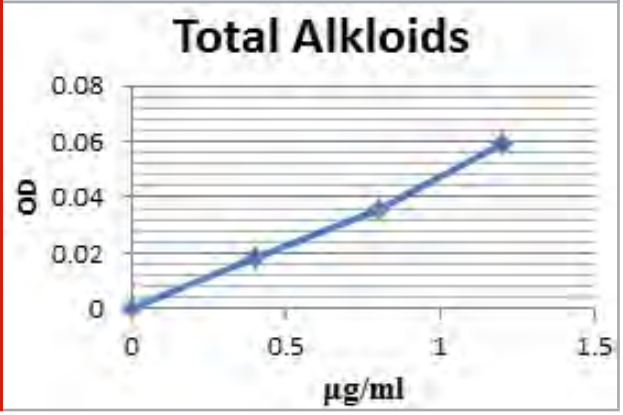


The tannins were determined by Folin - Ciocalteu method. 0.1 ml of the sample extract was added to a volumetric flask containing 7.5 ml of distilled water and 0.5 ml of Folin-Ciocalteuphenol reagent, 1 ml of 35 % Na2 CO_3 solution make up final volume 10 ml with distilled water. The mixture was shaken well and kept at room temperature for 30 min. A set of reference standard solutions of gallic acid (20, 40, to 100 µg/ml) were prepared in the same manner as described earlier Absorbance for test and standard solutions were measured against the blank at 725 nm with an UV/Visible spectrophotometer. The tannin content was expressed in terms of mg of GAE /g of extract. (Singh et al., 2012) .For the Saponins standard solution of diosgenin in different aliquote (50 to 250 µL) was taken in test tubes and volume was made up to 0.25 ml with 80% aqueous methanol. 0.25 mL of the vanillin reagent and then 2.5 ml of 72% (v/v) sulfuric acid was added slowly on the inner

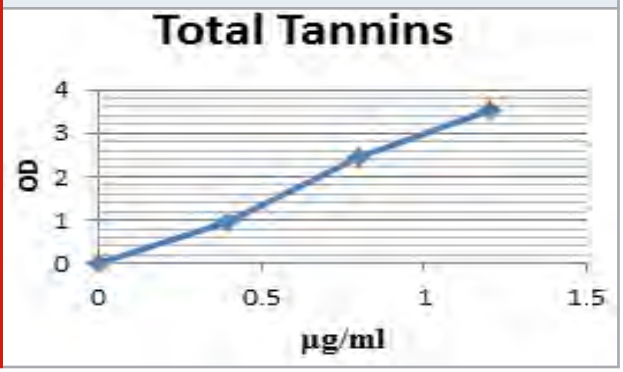
Graph 1: Gallic acid standard



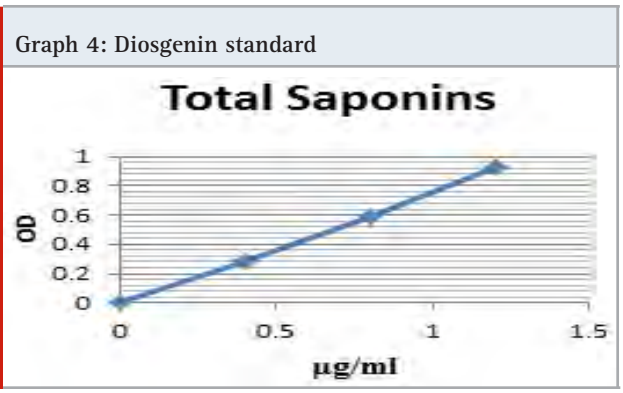
Graph 2: Caffein standard



Graph 3: Tannic acid standard



Graph 4: Diosgenin standard



side of the wall. After mixing well tubes were placed in water bath at 60°C. After 10 minutes tubes were cooled in ice-cold water for 3 to 4 min. Absorbance was taken at 544 nm against the reagent blank. (Hiai et al., 1976)

For alkaloids, aliquots (0.4, 0.6, to 1.2 ml) of caffeine standard solution was taken in funnels. 5 ml of pH 4.7 phosphate buffers and 5 ml Bromocresol green solution were added. And 1, 2, 3 and 4 ml of chloroform respectively added. The solutions were collected in a 10ml volumetric flask and then diluted to adjust volume with chloroform. The test extract was also prepared in same manner but without caffeine and absorbance measured at 470 nm against blank (Shamsa et al., 2008).

Table 3. Amount of Total phytochemicals present in sample

Compound	Ap (µg/ml)	Ts (µg/ml)	Tp (µg/ml)
Phenols	1.85	1.84	1.83
Alkaloids	2.7	3.3	3.5
Tannins	2.05	2.05	2.04
Saponins	0.48	0.39	0.52

RESULTS AND DISCUSSION

Bacterial isolates: Total 10 isolates were obtained from stem, root and latex of *E. caducifolia*. (Figure 1) Out of them three potent bacterial isolates selected on the basis of phytochemical extraction from endophytic cell was done by solvent extraction method. All the isolates were showing positive results for Phytochemicals. Apart from that Biochemical tests were also performed for the characterization. (Table 1). Endophytic bacteria are attracting increasing attention not only for their promotion of plant growth and control of plant diseases (Hallmann et al. 2011), but also for their stress tolerance and improvement of plant growth in an extreme environments (Forchetti et al. 2010; Jha et al. 2011; Karthikeyan et al. 2012; Ali et al. 2014; Naveed et al. 2014 a, b; Qin et al. 2014; Cherif et al. 2015). Endophytes play a major role in physiological activities of host plants influencing enhancement of stress tolerance, nematode and disease resistance (Carroll, 1988; Hallmann and Siora 1996; Azevedo et al 2000; Strurz and Nowark 2000).

CONCLUSION

From the present study it can be concluded that endophytes are metabolically active within their hosts and play a vital role in maintaining the host endophytic mutualistic balance. From cactus Euphorbia caducifolia was collected from latitude of 23.9944° N and longitude of 72.4099° E Pasvadal village located in Vadgam Tehsil in Banaskantha district of Gujarat state. Total ten bacterial isolates were recovered. Among them three bacterial isolates namely AP, TS, TP were showing the best antimicrobial activity. They were having capability of producing phytochemicals such

as phenol, alkaloids, tannins and saponins. Apart from that it is also having properties of good antimicrobial agent and source of secondary metabolites Phenols, Alkaloids, Tannins, Saponins and this bacterial isolate may act as anticancer, anti diabetic, antiulcerogenic and antiobessicity agents. From plant *Euphorbia caducifolia* isolated endophytic bacteria are showing the 93.43% similarity to *Micrococcus luteus*, 97.97% *Chryseomonas luteola*, and 99.41% *Enterobacter agglomerans* according to Bergey's manual of systematic bacteriology.

Conflict of interest: The authors have no conflict of interest in prepare of this article.

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Isolation and Screening of Plant Growth Promoting Bacteria from Fermented Panchagavya

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ABSTRACT

Agriculture is an important element of Indian economy. The excessive use of chemical fertilizers and pesticides has led to adverse effect on health of soil microbial community and hence the ecosystem resulting in the emergence of health hazards. It is a need of time to adopt eco-friendly bioorganic practices for sustainable agriculture. Panchagavya is a low-cost preparation useful in organic farming. Panchagavya is a mixture prepared by fermenting five cow derived components viz., dung, urine, milk, curd and ghee. The mixture contains macronutrients, micronutrients as well as important amino acids, plant growth promoting substances and beneficial microorganisms. The objective of this study is to isolate bacteria from the fermented panchagavya showing plant growth promoting abilities. Total 42 bacterial isolates were obtained on Nutrient agar and they were qualitatively tested for plant growth promoting activities like indole acetic acid (IAA), gibberellic acid (GA), ammonia, exopolysaccharides, HCN production and phosphate and zinc solubilization. Out of these, ten isolates showing multiple plant growth promoting activities were selected and further evaluated. All the ten bacterial isolates showed IAA, gibberellic acid and ammonia production. The highest IAA production of 73.71 µg/mL was yielded by RK-8 after 24 hours. Isolate RK-1 produced the highest gibberellic acid (0.8 mg/mL) after 48 hours and EPS (4.4 g%) after 24 hours. RK-6 showed the best ammonia production (4.10 µmol/mL) after 96 hours. Among the isolates studied, RK-4 showed maximum phosphate solubilizing activity. The bacterial isolates obtained from panchagavya can be used individually or as a consortia for field application.

KEY WORDS: AGRICULTURE, BIOFERTILIZER, CHEMICAL FERTILIZER, ORGANIC FARMING, PANCHAGAVYA.

ARTICLE INFORMATION

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INTRODUCTION

Rapidly incrementing population and environmental pollution have become threat to modern agriculture resulting in food shortages worldwide. The world faces the necessity to develop sustainable and eco-friendly methods to ameliorate agricultural productivity. The bioorganic formulation 'panchagavya' can be proposed as 'biofertilizer' to replace the chemical fertilizers and minimize the use of pesticides. 'Panchagavya' (Sanskrit word) is a traditional invention prepared by fermenting dung, urine, milk, curd and ghee obtained from cow. These five components are mixed in a suitable ratio and allowed to ferment up to twenty days. Fermented panchagavya contains macro and micro-nutrients, amino acids, and growth promoting hormones like indole acetic acid, gibberellins and microorganisms like *Acinetobacter, Bacillus, Azotobacter, Azospirillum, Pseudomonas, Saccharomyces, Streptomyces, Aspergillus* and *Lactobacillus* which are beneficial to the plants (Sayi et al., 2018, Amalraj et al., 2013, Nagaraj and Sreenivasa 2009). Plant growth promoting bacteria are the bacteria living in or on plant roots and enhance the plant productivity through various plant growth promoting activities such as providing efficient nutrients to plants, hormonal production and inhibition of plant pathogen (Ji et al., 2019).

The properties of panchagavya are contingent on the quality of five components used and the microbes involved in its preparation. Cow dung contains undigested residue of plants having high amount of cellulosic material, about three percent nitrogen, two percent phosphorous and one percent potassium (3-2-1 NPK) as organic matter and is rich in microbial diversity (Gupta et al., 2016). Cow urine contains nitrogen, urea, sulphur, phosphate, sodium, manganese, iron, silicon, chlorine, magnesium, maleic acid, citric acid, tartaric acid and calcium salts, vitamin A, B, C, D, E, minerals, lactose, enzymes, creatinine and hormones (Gulhane et al., 2017). Cow milk is an admirable source of amino acids, vitamins, carbohydrate, protein and microorganisms (Dhama et al., 2005). Curd is a rich source of microorganisms that are responsible for fermentation of panchagavya (Yadav and Lourduaj, 2005). Cow ghee contains vitamins, macronutrients, micronutrients and fats (Jain et al., 2014). When all components of panchagavya are combined they enhance microbial growth.

MATERIALS AND METHODS

Preparation of panchagavya: For the preparation of panchagavya, five cow products were collected from Bansi Gir Gaushala, Ahmedabad, India. On the first day, fresh cow dung (1.0 kg) was thoroughly mixed with cow ghee (0.1 L) in a plastic container. The mixture was kept for three days and mixed daily. On the fourth day, cow urine (0.6 L), cow milk (0.4 L) and cow curd (0.4 L) were added and omitted mixed thoroughly. The mixture was allowed to ferment up to twenty days and mixed daily for five minutes (Swaminathan, 2007, Natarajan, 2002).

Isolation of bacteria from fermented panchagavya: Nutrient agar was used for isolation of bacteria from fermented panchagavya sample. In order to isolate bacteria, fermented panchagavya sample was diluted appropriately and 0.1 ml of each dilution was spread onto the Nutrient agar plates. The plates were incubated at 30 ^{0}C for 48 hours. The colonies were enumerated and characteristics were recorded. Morphologically distinct bacterial colonies were selected for further study.

Maintenance and preservation of isolates: The pure culture of bacterial isolates were grown on Nutrient agar medium. They were maintained and preserved by periodic transfer onto Nutrient agar slants in refrigerator at 4 ^{0}C.

Inoculum preparation: For inoculum preparation, one loopful of culture was inoculated in sterile Nutrient broth from slant and incubated at 30 ^{0}C for 24 hours. Then, cells were separated by centrifugation at 5000 rpm for 15 minutes and supernatant was removed. Cell pellet was resuspended in sterile normal saline to get optical density of 1.0. The prepared culture suspension was used as 1% (v/v) inoculum for studying of each plant growth promoting parameter.

Screening for plant growth promoting parameters: Total 42 bacterial isolates were qualitatively tested for plant growth promoting activities like indole acetic acid (IAA), gibberellic acid (GA), ammonia, exopolysaccharides, HCN production as well as phosphate and zinc solubilization.

Indole acetic acid production: 50 mL of Luria-Bertani broth with 0.1% (w/v) L-tryptophan, pH-7.5 was inoculated with 1% (v/v) of inoculum and incubated at 25±2 ^{0}C, 150 rpm on orbital shaker. Spectrophotometric estimation of IAA was done as per the method described by Brick et al., (1991) with some modifications. 1.0 mL of culture supernatant was mixed with 2.0 mL of Salkowski reagent and incubated in dark for 30 minutes. Development of pink colour indicates production of IAA and its optical density was recorded at 530 nm. IAA quantification was carried out up to 120 hours. Concentration of IAA produced was estimated against standard curve of IAA (50 µg/mL).

Gibberellic acid production: 50 mL of Nutrient broth, pH-7.4 was inoculated with 1% (v/v) inoculum and incubated at 30±2 ^{0}C, 150 rpm on orbital shaker. Spectrophotometric estimation of gibberellic acid was performed using the method described by Graham and Thomas (1961). For quantification, equal volume of cell free extract and ethyl acetate was taken in a test tube and shook vigorously. Ethyl acetate layer was separated carefully in a glass beaker, the extraction was repeated thrice. The separated ethyl acetate extract was allowed to evaporate at room temperature. The remains were dissolved in methanol. 2.0 mL of this suspension was mixed with 1 mL of DNPH (2, 4 – Di nitro phenyl hydrazine) and incubated at 100 ^{0}C for 5 minutes. After incubation it was cooled in running tap water, to this

5.0 mL of 10% (w/v) alcoholic potassium hydroxide was added and allowed to stand till red wine colour developed. Finally, 15 mL of sterile distilled water was added, and colour intensity was measured at 430 nm. Standard calibration curve of gibberellic acid (0.8 mg/mL) was prepared in methanol.

Ammonia production: 50 mL peptone water, pH-7.2 was inoculated with 1% (v/v) inoculum and incubated at 25±2 ^{0}C, 150 rpm on orbital shaker. Spectrophotometric estimation of ammonia was performed as per the method developed by Cappuccino and Sherman (1992) with few modifications. 1 mL of culture supernatant was mixed with 0.1 mL Nessler's reagent and final volume of the mixture was made up to 5 mL by addition of ammonia free distilled water. Development of yellow to brown colour is the indication of ammonia production, and its optical density was measured at 425 nm using spectrophotometer. The concentration of ammonia was estimated using the standard curve of ammonium hydroxide solution (2 µmol/mL).

Phosphate solubilization: Phosphate solubilization was estimated using tri-calcium phosphate as insoluble form of inorganic phosphate. Each bacterial isolate was inoculated as spot on Pikovskaya's agar plate and incubated at 30 ^{0}C (Pikovskaya, 1948). Phosphate solubilization was observed in the form of a clear zone formed around the colony representing the production of organic acids as a possible mechanism of the phosphate solubilization (Ansari et al., 2015). Phosphate solubilization index was calculated up to 120 hours of inoculation. Phosphate solubilization index (PSI) was calculated using the following formula:

$$PSI = \frac{\text{Colony diameter} + \text{Zone of clearance}}{\text{Colony diameter}}$$

Exo-polysaccharide production: Each bacterial isolate was spot inoculated on Brain Heart Infusion (BHI) agar plate supplemented with 0.8 mg/L congo red and incubated at 30 ^{0}C. EPS production was observed in the form of mucoid colonies and blackening of the colony. 100 mL EPS broth supplemented with 4% (w/v) sucrose, pH-7.1 was inoculated with 1% (v/v) inoculum and incubated at 30±2^{0}C, 150 rpm on orbital shaker. Solvent extraction method described by Azeredo and Oliveira (1996) was used for quantitative estimation of EPS. For extraction of EPS, 10 mL of culture was withdrawn at an interval of 24 hours for 3 days. To this, 30 mL of chilled acetone was added and allowed to precipitate overnight at 4 ^{0}C. Precipitates were collected on a pre-weighed aluminium foil and wet weight was recorded. The precipitates were allowed to dry at 50-60 ^{0}C. Dry weight of the precipitates was recorded until three consecutive constant readings.

Hydrogen cyanide production: For the detection of HCN produced, picrate assay was used (Bakker and Schippers, 1987) with some modifications. Bacterial isolates were streaked on King's B agar plate supplemented with 4.4 g/L glycine. Whatman filter paper no.1 was soaked in solution of 0.5% (w/v) picric acid containing 2.0% (w/v) sodium carbonate and was air dried. Dried filter paper was placed in the lid of the culture plate. Plates were sealed with parafilm and incubated at 30 ^{0}C for 168 hours. Production of HCN was indicated by the colour change of filter paper from yellow to orange brown.

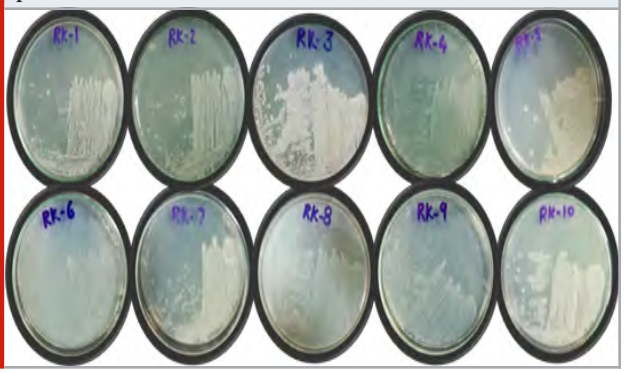
Zinc solubilization: Qualitative screening of bacterial isolate for zinc solubilization was carried out in Bunt and Rovira agar plates (pH 7.0) having 0.1% (w/v) insoluble zinc (ZnO). Isolates were spot inoculated onto the media. The plates were incubated at 37 ^{0}C for 11 days. Zinc solubilizing bacteria produce a zone of clearance around the colony indicating zinc solubilization. (Bunt and Rovira, 1955).

RESULTS AND DISCUSSION

Isolation of bacteria: From the fermented panchagavya, a total of 42 morphologically distinct bacterial isolates were obtained. These isolates were qualitatively assessed for multiple plant growth promoting activities. Among the 42 bacterial isolates studied, 22 were showing IAA production and 26 were producing GA. A total of 28 and 14 cultures were showing exopolysaccharide and HCN production respectively. All the 42 isolates were positive for ammonia production. Twenty-seven were able to solubilize phosphate whereas only 2 could solubilize zinc. Based on the multiple plant growth promoting abilities (Production of IAA and GA, Phosphate solubilization and EPS production), most potent ten bacterial isolates were selected (highlighted in Table 1) and designated as RK-1 to RK-10.

Morphological and cultural characteristics of bacteria: Colonial characteristics of the selected isolates are recorded in Table (2). To study morphological characteristics, Gram's staining was performed. Out of these ten cultures, 6 were found as Gram positive big rod, 3 as Gram negative short rod and 1 as Gram positive cocci.

Figure 1: Selected bacterial isolates on Nutrient agar plate.



Secondary screening for plant growth promoting parameters of selected isolates: Selected isolates were screened for indole acetic acid, gibberellic acid, ammonia and exopolysaccharide production and estimated quantitatively.

Table 1. Primary screening of bacterial isolates for plant growth promoting parameter

Isolate	IAA Prod.	GA_3 Prod.	PO_4 Solubilization	EPS Prod.	Ammonia Prod.	HCN Prod.	Zinc Solubilization
1	+	-	+++	-	++	-	-
2	+	-	+++	-	+++	+	-
3	-	+	-	+	+++	-	+
4	-	+	+++	+	+++	-	-
5	-	-	+++	+++	+++	+	-
6	-	-	++	+	+++	-	-
7	++	+++	+++	+++	+++	-	-
8	++	++	++	++	+++	-	-
9	-	-	++	+	++	-	-
10	-	+	-	-	+	+	-
11	-	-	+++	+	+++	-	-
12	-	++	+++	+++	+++	+	-
13	-	++	+++	+	+	-	-
14	-	-	+++	+	++	-	-
15	-	++	+++	+	+++	-	-
16	-	-	+++	+	+++	+	-
17	++	++	++	+++	++	-	-
18	-	++	+++	+	+	+	-
19	-	+	+++	-	++	-	-
20	-	-	+++	-	+++	-	-
21	-	++	-	+++	+	+	-
22	++	+	+++	-	++	-	-
23	-	-	+++	+	+++	+	-
24	+	++	-	+	+++	-	-
25	+	-	-	+	++	_	-
26	+	++	-	+	+	+	-
27	+	++	-	+	+	+	-
28	+	++	-	-	+++	-	+
29	-	+	-	-	++	+	-
30	+++	+	++	++	++	-	-
31	+	-	-	-	+	+	-
32	+++	+++	-	+++	++	-	-
33	+	-	+++	-	++	++	-
34	++	+++	+++	+++	++	-	-
35	+++	+	-	-	++	-	-
36	-	++	-	-	+	+	-
37	-	-	+++	++	+++	-	-
38	+++	+++	+++	++	++	-	-
39	+	-	+	-	+++	-	-
40	+	++	-	-	+++	-	-
41	+	-	-	+	++	-	-
42	++	+++	++	+++	+++	-	-
Positive isolates	22 Positive	26 Positive	27 Positive	28 Positive	42 Positive	14 Positive	2 Positive

+++ = Higher production, ++ =Moderate production, + =Lower production, - =No production

Figure 2: Indole acetic acid production by isolates

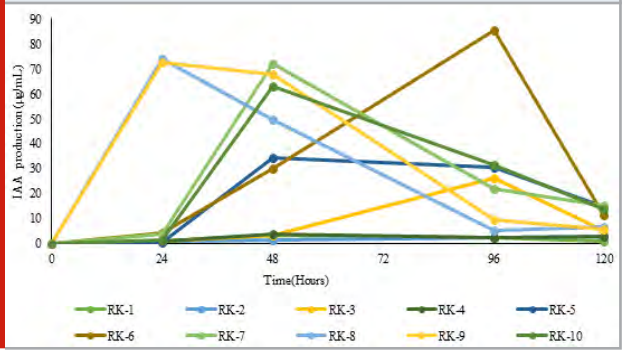


Figure 3: Gibberellic acid production by isolates

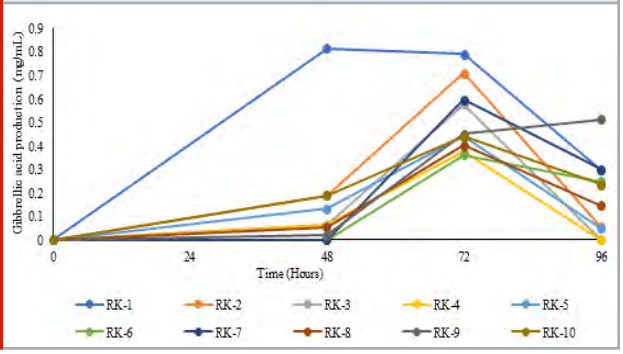


Table 2. Morphological characters of bacteria on Nutrient agar medium

Isolate		Colonial characteristics						Gram's reaction
Size	Shape	Elevation	Texture	Margin	Opacity	Pigment		
RK 1	Big	Irregular	Flat	Smooth	Entire	Opaque	White	Gram Positive
RK 2	Big	Irregular	Slightly Raised	Smooth	Entire	Opaque	White	Gram Positive
RK 3	Medium	Round	Slightly Raised	Rough	Entire	Opaque	White	Gram Positive
RK 4	Small	Round	Slightly Raised	Smooth	Entire	Opaque	White	Gram Negative
RK 5	Big	Round	Flat	Smooth	Entire	Opaque	Cream	Gram Positive
RK 6	Small	Round	Slightly Raised	Smooth	Entire	Translucent	Nil	Gram Positive
RK 7	Medium	Rhizoid	Slightly Raised	Rough	Undulate	Opaque	Off White	Gram Positive
RK 8	Small	Round	Flat	Smooth	Entire	Translucent	Nil	Gram Negative
RK 9	Small	Round	Slightly Raised	Rough	Entire	Opaque	White	Gram Negative
RK 10	Big	Irregular	Slightly Raised	Smooth	Entire	Opaque	White	Gram Positive

Figure 4: Ammonia production by isolates

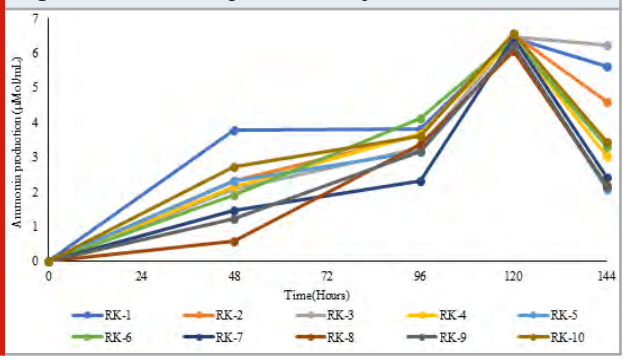
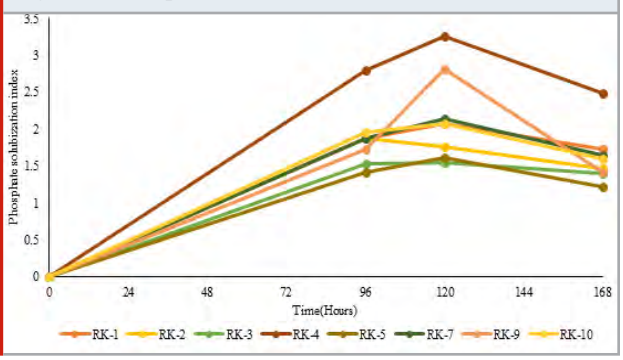


Figure 5: Phosphate solubilization index

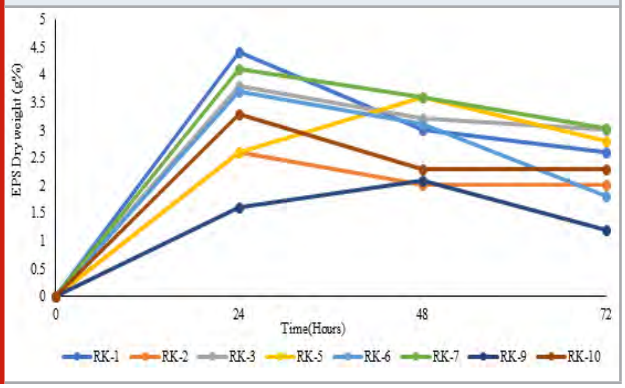


Indole acetic acid: The result of IAA production by isolates is shown in figure 2. All the studied isolates produced IAA in the range of 0.30 to 85.34 µg/mL. RK-8 and RK-9 produced 73.71 µg/mL and 72.35 µg/mL IAA, respectively after 24 hours and RK-6 produced 85.34 µg/mL IAA after 96 hours. Das et al., (2019) reported two isolates from rhizosphere which were able to produce 12.0 µg/mL and 7.0 µg/mL IAA after 72 hours. Similarly, Pal et al., (2019) reported two strains *Lysinibacillus varians* and *Pseudomonas putida* isolated from rhizosphere that

produced approximately 20.00 µg/mL IAA. Bhanderi and Khunt (2019) also reported a rhizospheric bacterium that was able to produce 55.90 µg/mL IAA. Panchal (2016) reported a bacterium isolated from panchagavya producing 75.86 µg/mL IAA after 72 hours.

Gibberellic acid: The result of gibberellic acid production by isolates is shown in figure 3. All the studied isolates were able to produce gibberellic acid in the range of 0.0004 to 0.8 mg/mL. Out of which RK-1 and RK-2 produced 0.8 mg/mL GA after 48 hours and 0.7 mg/mL GA after 72 hours respectively. Sharma et al., (2018) reported two isolates from rhizosphere which were able to produce 0.48 mg/mL and 0.419 mg/mL gibberellic acid after 72 hours of incubation. Similarly, Shravanthi et al., (2019) reported a culture from rhizosphere that produced approximately 0.159 mg/mL gibberellic acid. Ambawade and Pathade (2015) reported 0.25 mg/mL gibberellic acid by *Bacillus* siamensis BE 76 isolated from banana plant.

Figure 6: EPS production by isolates



Ammonia production: The result of ammonia production is shown in figure 4. All the studied isolates gave positive results for ammonia production. The results were monitored up to 144 hours. Ammonia was produced in the range of 0.57- 6.57 µmol/mL. Out of which RK-6 produced 4.1 µmol/mL ammonia after 96 hours. RK-10 and RK-2 produced 6.57 µmol/mL and 6.517 µmol/mL ammonia respectively after 120 hours. Goswami et al., (2014) reported 4.71 µmol/mL ammonia production by bacteria isolated from Kutch. Reports show 4.75 µg/mL ammonia production by *Azotobacter* isolates obtained from commercial biofertilizers (Ansari et al., 2015). Mazumdar et al., (2018) reported 15.21 µg/mL of ammonia production after 72 hours by a bacterial culture isolated from chickpea rhizosphere.

Phosphate solubilization: The result of phosphate solubilization index is shown in figure 5. Eight bacterial isolates were able to solubilise phosphate on Pikovskaya's agar plate. Phosphate solubilization was observed in the form of a clear zone formed around the colony representing the production of organic acids as a possible mechanism of phosphate solubilization. Phosphate solubilization index (PSI) of each bacterial isolate ranged from 1.22 to 3.25.

EPS production: The result of EPS production by isolates is shown in figure 6. All the studied isolates were able to produce EPS in the range of 1.2 to 4.4 g%, out of which RK-1 and RK-7 produced 4.4 g% and 4.1 g% EPS, respectively after 24 hours. EPS production of 2.4 g% was reported by Ansari et al., (2015) from the isolates obtained from commercial liquid biofertilizer using sucrose and mannitol as carbohydrate source. Nwosu et al., (2019) reported 0.1 g% EPS production by isolates obtained from oil contaminated soils.

CONCLUSIONS

The results of the present study revealed that fermented panchagavya is a potential bioorganic formulation which can be used to increase the crop yield and maintain the soil health. Furthermore, it contains many beneficial microorganisms having plant growth promoting abilities like production of IAA, GA, Ammonia and EPS as well as Phosphate solubilization. Thus, it can be inferred that panchagavya is definitely a promising formulation in years to come and can be an alternative to chemical fertilizers.

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Conflict of interest: The authors declare no conflict of interest.

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Influence of Bio-Fertilizers on Morphological and Biochemical Basis of Oyster Mushroom (*Pleurotus Sp.*)

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ABSTRACT

The impact of bio fertilizers was studied regarding the improvement of morphological and physio-chemical properties of *Pleurotus ostreatus* (Jacq: Fr) Kumm. Supplementing bio fertilizers during spawning gave a significantly better result at initial stage of casing. Significantly effective results in yield of *P. ostreatus* were obtained while supplementing different treatments which is Azotobacter, PSB, and *Azotobacter* + PSB (Phosphate Solubilizing Bacteria). Unaided PSB or in combination performed better in contrast to *Azotobacter*. The time taken for mycelium run is reduced (14 days), pin head initiation took (3 days) with 2 pin heads, and maximum size of fruiting bodies (4.72 cm), number of fruiting bodies (130 each bag), weight (311.05 g), yield (0.31 kg) and moisture content (87.94%), of Oyster mushroom after application of *Azotobacter* and PSB in cased. In order to assess the impact of various bio-fertilizer on growth and harvest of Oyster mushroom cultivation four different treatments control (T0), *Azotobacter* (T1), PSB (T2), and *Azotobacter* + PSB (T3) were conducted to compare the impact of different biofertilizer on growth. The fastest spawn running, primordial initiation, harvesting stage, maximum number of fruiting bodies and highest yield was detected in T2 and T3, whereas the high moisture content was found in T0 (87.94%). The maximum dry matter (36.94%) in T1 was observed. T2 showed the maximum protein (19.90%), fiber (16.80%), ash (13.77%) and fat (1.82%) whereas highest carbohydrate content was recorded in T0 (21.45%).

KEY WORDS: *AZOTOBACTER*, BIOFERTILIZER, OYSTER MUSHROOM, MUSHROOM CULTIVATION, PHOSPHATE SOLUBILIZING BACTERIA.

INTRODUCTION

Mushrooms are the spore-bearing fruiting bodies of fungus generally classified under subdivision Ascomycotina or Basidiomycotina. These fungi occur seasonally throughout monsoon globally in different habitats (Adejumo and Awosanya, 2005). The mushrooms contain an enormous heterogeneous category which include various shape, size, color, appearance and edibility (Mishra and Shukla, 2007). Mushrooms generally are rich in protein, vitamins, minerals hence are widely used in pharmaceutical and food industry.

Oyster mushrooms rich in protein (25-50%), sugars (17-47%), fat (2-5%) myco-cellulose (7-38%) and minerals (potassium, phosphorus, calcium, sodium) of about (8-12%) has been described as high-quality mushroom. Edible mushroom contains niacin, riboflavin, vitamin C, D, B1, B5, and B6 rich. *P. ostreatus* is a popular cultivated

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edible mushroom with high nutritional value (Chang, 1996; Wasser and Weis 1999). The experimental results shows that the presence of microbial population in substrate plays vital role in *P. ostreatus* cultivation. The microbial biomass present in compost affects the mycelial spread during spawn run phase, (O.P. Ahlawat and R.N. Verma, 2001). Accordingly, it can be anticipated that in order to advance morphological or physiochemical basis, oyster mushroom cultivation protocol must be supplemented by bio-fertilizers.

MATERIAL AND METHODS

Cultivation of Oyster mushroom is critical at initial level hence it requires precise technique in following:

I. Substrate
II. Spawn preparation
III. Cropping and watering.

Substrates and Spawn Preparation: The study was operated in Department of Botany, Bio-informatics and Climate Change Impacts Management, Gujarat University, Ahmedabad (Gujarat). The study aimed to experiment morphological and physiochemical parameters of *P. ostreatus* using different treatments of biofertilizers. These treatments included either Azotobacter, PSB (Phosphate Solubilizing Bacteria) exclusively or in mixture.

Substrates preparation: For sterilization process 12g bavistin, 150ml formaldehyde was added in 12kg wheat straw and soaked in 120L of water. The wheat straw (substrates) was soaked, stacked, sealed with a layer of polyethene and fermented for a day. Substrates was allowed to evaporate moisture retaining 70% by dispersing on the floor. Polypropylene bags of the size 40.64 x 45.72 cm were duly used to pack the substrate while loosely tying a knot with elastic band. Biofertilizers were added according to (table no.1).

Spawn preparation: Spawns were inoculated in the pasteurized bags at the 10 gm per bag for 24hrs. Under sterilized condition, compost bags comprising of 12 kg wheat straw and 10gm mushroom seed were sprayed with bio fertilizers. The concentration of biofertilizer were either exclusively 15 ml *Azotobacter*, 15 ml PSB or mixture of (15ml Azotobacter+ 15 ml PSB) (Kapoor, 2004). Then after the compost bags were placed in decontaminated growth chamber.

Spawn running: Growth chamber temperature was maintained between 21-25 ° C to facilitate spawn running. The humidity level (70 to 80%) was retained by spraying water on walls, floors and compost bags.

Cropping and Watering: Growth chamber was fumigated with KMnO4 and formalin for 3 days. The temperature of the growth chamber was retained at 16-25°C, after completion of the spawn run. Fruiting body started immediately as mycelial growth completely spread throughout the substratum. The growth chamber 's moisture was retained at 80-90 per cent by spraying water on the floor and on the bags ' moisture necessities were met by spraying water three times a day. Further, during the period of fruiting, moisture content was visually checked in the form of water droplets.

RESULT AND DISCUSSION

Morphological Parameter: The data were analyzed, and the following parameters were observed: Numbers of days required following spawning to complete mycelial growth, time required to form primordia after achievement of mycelial growth, time required for achieving maturity phase following to primordia formation, total numbers of fruiting bodies, total number of flushes, yield per bag and total was measured after cropping was achieved.

Spawn Running: For spawn running, noteworthy outcome was detected amongst treatments with respect to days taken for four different treatments (T0, T1, T2, T3). T1, T0 exhibited similar impact by 19 days respectively, T2 took 14 days, whereas treatment T3 took minimum number of days 17 to achieve mycelial growth. Among interaction studies, T2 has been best reported with respect to minimum time required (in days) to conduct spawn running as14 days.

Emergence of Pin head initiation: After achieving mycelial growth, treatments showed insignificant results in terms of the days required for appearance of Pin head initiation. T2 took minimum 3 days (2 pin head) followed by T3, T1 and T0 with 4(1 pin head), 5(2 pin head) and 5(1 pin head) days respectively to achieve pin head initiation. For pin head initiation T2 resulted best, by taking minimum number of days 3.

Table 1. Different Treatment Combinations Used in the Study

Treatment No.	Treatment combination
T0	Wheat Straw
T1	Wheat Straw + Azotobacter (108c.f.u./ml) @15 ml/kg (substrate)
T2	Wheat Straw + PSB (1:1) @15 ml/kg (substrate)
T3	Wheat Straw + Azotobacter (108c.f.u./ml) +PSB (1:1) @ 30ml / kg (substrate)

[T0, T1, T2, T3 are specific treatments]

Fruiting Size: As describe in table no. 1. T3 showed maximum size of fruiting bodies 5.8 cm followed by T0, T1 and T2 with the size of fruiting bodies 4.72cm, 3.7cm and 3.6cm respectively. (Table 2). For fruiting bodies T3 stood out to be the best by taking a maximum of 5.8cm. Fruiting size in the mushroom cultivation has pros that it is of good quality and highly rated in contrast the con is that fruit bodies generally disrupt while packing, hence diminishing their quality (Omyango et. al, 2011).

Table 2. Morphological Analysis of Oyster Mushroom with Different Treatments.

TREATMENT NO.	TREATMENT COMBINATION	MR	PI	NFB	FS (cm)	W (g)	YIELD (Kg)	MC%
T0	GLS + FYM + VC + Wheat Straw (2:1:1)	18	1	69	4.72	201.07	0.20	87.92
T1	GLS+FYM+VC+Wheat Straw (2:1:1) + *Azotobacter* (108c.f.u./ml)	19	2	105	3.70	180.56	0.18	76.52
T2	GLS + FYM + VC + Wheat Straw (2:1:1) + PSB	20	2	130	3.60	124.28	0.12	63.04
T3	GLS + FYM + VC + Wheat Straw (2:1:1) +*Azotobacter* (108c.f.u./ml) + PSB	18	1	95	5.80	311.05	0.31	87.39

[MR: minimum days required for mycelium run; PI: pin head initiation; NFB: number of fruiting bodies; FS: fruiting size; W: weight; MC: moisture content]

Fruiting Bodies: A shown in (Table: 2) T2 showed maximum number of fruit bodies 130 followed by T1, T3 and T0 as 105, 95 and 69 respectively. Maximum no. of fruiting bodies was recorded as 130 fruits.

Total Yield (g): With regard to total yield, the treatment T3 reported fairly significant results. T3 produced maximum yield of 311.05g followed by T0 (201.070g), T1 (180.56g) and T2 (124.28g per bag) respectively as shown in (Table 2). After application of biofertilizer significant result was obtained regarding morphological parameters. Sporophores formation in basidiomycetes is a complex phenomenon triggered by physiological and biochemical factors in the substrate and in the mushroom mycelium. The relevance of microbiological factors is not limited to fruiting; existing microbes exert unique interactive behaviours with unique mechanisms involving various types of metabolites on mushrooms. Mushroom in general produce organic acid reduce the pH of substrate (Zadrazil and Schaeidereit, 1972), which favours the spread of mycelia only.

Physio-Chemical Analysis: Physiochemical analysis of mushrooms for dry matter, humidity, crude protein, ash content, crude fiber, total carbohydrate and crude fat was conducted using the standard protocol by Helrich (Shah et.al, 2004). Treatments showed significant differences in the terms of moisture control. The moisture level was observed highest in T0 (87.94%) followed by T3 (87.4%), T1 (76.54%) and T2 (63.06%). The difference in moisture content between various mushroom species is immensely significant. For dry matter, T1 stood out to be supreme (36.94%) followed by T3 (23.47%), T0 (12.08%) and T2 (12.60%). For Protein content T2 (19.90%) was maximum followed by T1, T3and T0 as (18.15%), (17.66%) and (16.54%) respectively. Crude fiber was found maximum in T2 (16.8%) followed by T3 (16.45%), T0 (16.1%) and T1 (14.7%). The ash content outcome represented T2 (13.77 %) to be maximum followed by T1 (8.94%), T3 (4.71 %) and T0 (4.53 %). The total fat content outcome represented T2 (1.82%) to be supreme followed by T3(1.56%), T0(1.38%) and T1 (1.36%) as shown in (Table 3). For carbohydrate, T0 (21.45%) followed by T3 (19.42%), T1 (16.85%) and T2 (7.71%) respectively were obtained.

Grewel and Rainey (1991) revealed that *Pseudomonas fluroscent* migrated towards hyphal exude of *A. bisporus* facilitating colonization and propagation which appears necessary before the bacteria can provide *A. bisporus* with the stimulus to initiate basidia formation. Few literature validates the importance of bio-fertilizer in fruiting bodies in P.ostreatus is followed Singh et al., (2013), Murmu et al., (2014) and Kumar et al. (2017). In *P. ostreatus*, crude protein was found to be maximum in PSB (69.90 %) and limited in control (66.54%). Nitrogenous sources enriched materials provide high yield was observed in wheat. Researchers employ

Table 3. Physio-Chemical Analysis of Oyster Mushroom with Different Treatments.

Treatments	Moisture content (%)	Dry matter (%)	Protein (%)	Fibre (%)	Ash (%)	Fat (%)	Carbohydrate (%)
T0	87.94	12.08	16.54	16.1	4.53	1.38	21.45
T1	76.54	36.94	18.15	14.7	8.94	1.36	16.85
T2	63.06	12.60	19.90	16.8	13.77	1.82	7.71
T3	87.4	23.47	17.66	16.45	4.71	1.56	19.42

resources such as dried mango leaves, wood shavings, pigeon pea stalks, coir pith, cotton waste, *Leonits spp*., sugarcane bagasse, Lantana camara, water hyacinth, coconut coir, coffee husk,

Cassia sophera and etc. for cultivation of mushroom. *P. ostreatus* is a potentially enriched with carbohydrates (21.45%) and of protein (19.90%) on the basis of different bio-fertilizer. Very low-fat contents (1.36%) in *Azotobacter* biofertilizer represents it to be highly recommended diet for people suffering from heart diseases (Ashraf et.al 2013). The main aspect of *P. ostreatus* which is extremely beneficial to heart patients is its high fiber and low-fat content. The research also suggests that oyster mushroom nutritional value varies still every species is enriched with adequate quantities of fiber, protein and other nutrients. In developing countries, the deficiency of nutritionally essential components such as protein, fibre, and carbohydrates are a serious and major problem.

Figure 1: Comparative Evaluation of Morphological Characterization Content by Combination of Azotobacter and PSB Isolates.

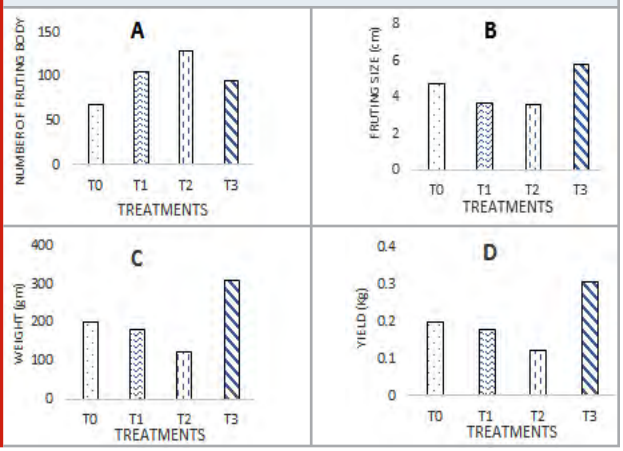
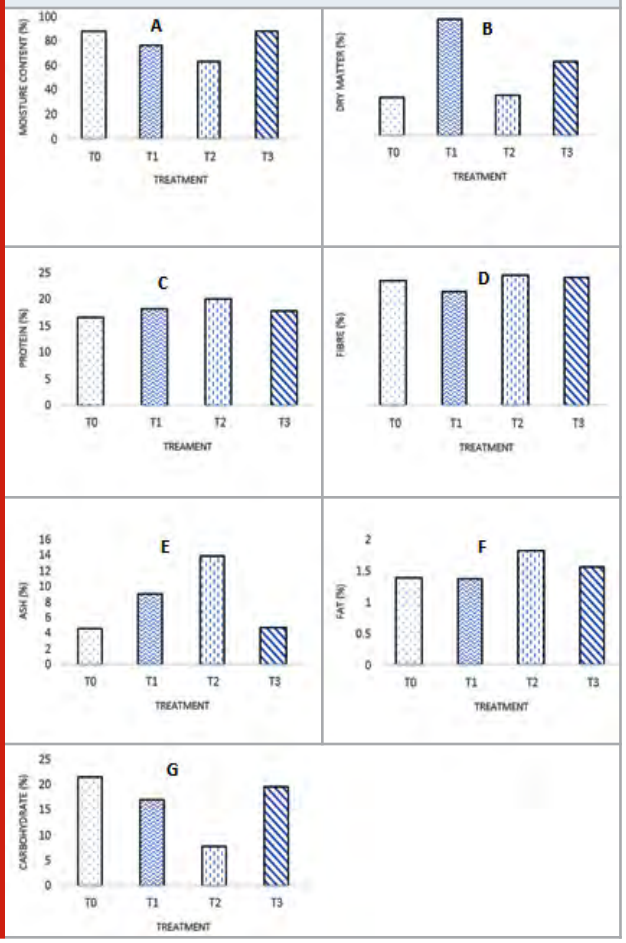


Figure 2: Comparative Evaluation of Chemical Characterization Content by Combination of Azotobacter and PSB Isolates



CONCULSION

In the present investigation, oyster mushroom cultivation was undertaken using different bio-fertilizer and a combination of it. Bio-fertilizer such as *Azotobacter*, Phosphate solubilizing bacteria (PSB) and a combination was used as treatments which yielded promising results. For the first time, *Azotobacter* as bio-fertilizer in terms of oyster cultivation was considered for the investigation. Treatment of PSB proved to outstand among all in terms of morphological and chemical analysis. Hence PSB is a promising bio-fertilizer for oyster mushroom cultivation and can be used for further large-scale industrial cultivation. Increased yield, large fruit size, increased protein, carbohydrate, fiber and less fat content manifests it to be the best Bio-fertilizer for oyster mushroom cultivation.

Conflict of interest: The authors declare that they have no conflict of interest

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Study of Secondary Metabolite Production from Bacteria Under Abiotic Stress

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ABSTRACT

Secondary Metabolite play a major role in adaptation of plants to the changing environment and in overcoming stress condition. Abiotic stresses are the major limiting factors for agricultural productivity. Microorganism have potential to synthesize several secondary metabolite to cope with the adverse effect of stress. The present research is design to study the production of secondary metabolites under salt stress condition. The Organism which was isolated from Rhizospheric soil Produced different secondary metabolites when cultured in 3%, 6% and 9% saline condition. Characterization of isolates was done by visual observation and microscopic observation. Potassium hydroxide test was done to determine the gram's reactivity of the bacterial isolates. Phytochemical extraction from endophytic cell was done by solvent extraction method by using Ethyl Acetate as solvent. Phytochemical screening of different extractions revealed the presence of phenols, flavonoids, tannins, saponins, alkaloids, steroids. In addition total phenolic content, total flavonoid content was evaluated using standard methods.

KEY WORDS: ABIOTIC STRESS, ANTIOXIDANTS, PHENOLICS, SALINITY, SECONDARY METABOLITES.

INTRODUCTION

In today's climate change scenario Environmental stresses affect the Plant growth promotion and productivity. Abiotic stresses are specifically critical in arid and semiarid regions of the world. The effect of abiotic factor on the plant depends on its quantity or intensity. The plant requires a certain quantity of abiotic environmental factor for their optimal growth. Any alteration from such optimal environmental conditions, which is deficit in the chemical or physical environment, is considered as abiotic stress and critically impacts on plant growth, development, and productivity. Abiotic stresses such as drought, salinity, heavy metal toxicity, flooding ,heat, cold etc. are affecting adversely the agricultural crops. Drought has affected 64% of the worldwide land area, salinity 6%, anoxia 13%, soil alkalinity 15%, mineral starvation 9%, and cold 57% of the world's 5.2 billion ha of dryland agriculture, 3.6 billion ha is influenced by the issues of soil erosion, degradation, and salt stress.

Salt stress is one of the major abiotic factor which reduce the Growth and development in plant. Salinity is defined as the presence of excessive amounts of soluble salts that hinder or affect the normal functions of plant growth. Salinity is measured in terms of electrical conductivity (ECe), with the exchangeable sodium percentage (ESP) or sodium adsorption ratio (SAR) and pH of a saturated soil paste extract. saline soil defined as soil which have

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saturated soil paste extracts with an ECe of more than 4 dSm^{-1}, ESP less than 15 percent, and pH below 8.5 (Waisel, 1972; Abrol, 1986; Szabolcs, 1994). Saline soils have a mixture of salts of Sulfate, Chloride, Sodium, Magnesium and Calcium ions with sodium chloride often dominant. There are two major sources of salinity: (i) Primary or natural sources Resulting from weathering of minerals and the soils developed/derived from saline parent rocks (Ashraf, 1994) (ii) Secondary salinization Caused by various human factors like irrigation, deforestation, overgrazing, or intensive cropping (Ashraf, 1994)

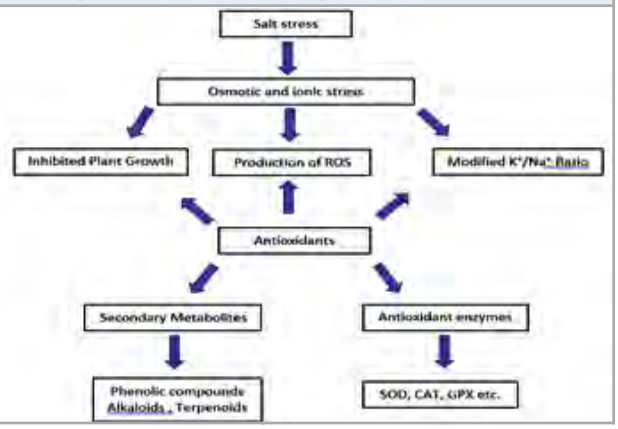
Different abiotic stress management procedures are used to reduce these stresses. However, as such strategies are long and costly, there is a need to develop simple and low-cost biological methods for managing salt stress. Microorganisms found in different environmental conditions exhibit enormous metabolic capabilities to cope with abiotic stress. Microorganism have potential to synthesize several secondary metabolite to cope with the adverse effect of stress. Microorganism manage these stresses by various mechanisms, viz., tolerance to stresses, adaptations, and response mechanisms that can be subsequently engineered into plants to deal with climate change-induced stresses.

Secondary metabolites are organic compounds produced by microorganism originating of primary metabolism, have no direct relation to its growth, development and reproduction. Secondary metabolites play an important role in defence system of an organism. Most of secondary metabolites, such as alkaloids, phenolic compounds and terpenes are classified based on their biosynthetic origin. The environmental stress factors such as salt, temperature, and soil pH are essential factors for release of secondary metabolites with potent antioxidant activity (Selvam et al. 2013). i.e there is a confirmed correlation between antioxidant activity and total phenolic content in plants of different origin (Piluzza and Bullitta 2011; Sadeghi et al. 2015). Different secondary metabolites which can have antioxidant capacity are participating in creating parameters of total antioxidant activity. These are flavonoids, tannins, anthocyanins, and phenolic acids - representatives of phenols group (Dyduch-Sieminska et al. 2015). Secondary metabolites alkaloids which containing nitrogen also have antioxidant activity (Velioglu et al. 1998) and can be connected with changes of metabolites having nitrogen in primary metabolism under salt stress. Many of secondary metabolites participate in the development of salt stress response as antioxidants (Fig. 1.1).

1.1 Role of Phenolic Compounds in Salt Stress: Phenolics are aromatic benzene ring compounds with one or more hydroxyl groups produced by microorganism mainly for protection against stress. The mechanism of phenolic compounds in plant physiology is difficult to overestimate, especially their interactions with biotic and abiotic environments. Phenolics play major roles in plant development, particularly in biosynthesis of pigments and lignin (Bhattacharya et al. 2010). However, phenolic compounds are strong antioxidants because there is a relation between antioxidant activity and presence of phenols in common vegetables and fruits (Cai et al. 2004; Fu et al. 2011).

Flavonoids are largest classes of plant phenolics that play a role in plant defence (Kondo et al. 1992). Isoflavonoids are derivatives of flavanone intermediate, e.g., naringenin which secreted by legumes and play a critical role in plant development and defence response and also in supporting the configuration of nitrogen-fixing nodules by symbiotic rhizobia (Sreevidya et al. 2006). Posmyk et al. (2009) reported that synthesis of flavonoids is an effective strategy against ROS. Under salt stress, *Azospirillum brasilense* promoted root branching in bean seedling roots and increased secretion of nod gene-inducing flavonoid species (Dardanelli et al. 2008). Anthocyanins are reported to increase in response to salt stress (Parida and Das 2005).Whereas, salt stress decreased anthocyanin level in the salt-sensitive species (Daneshmand et al. 2010).

Fig 1.1: Participation of secondary metabolites in development of salt stress response



2. MATERIALS AND METHODS

2.1 Sampling & Characterization of Isolates: Soil sample was collected from rhizosphere of Cyamopsis tetragonoloba from latitude of 23.021624° N and longitude of 72.579707° E Ahmedabad, Gujarat, India. Characterization of isolates was done by visual observation i.e. colony characteristics on agar media. Microscopic observation i.e Gram staining of bacteria for morphological characteristics. Potassium hydroxide test was done to determine the gram's reactivity of the bacterial isolates. (Suslow et al. 1982)

2.2 Production of secondary metabolite under stress condition: The Bacteria which was isolated from Rhizospheric soil Produced different secondary metabolites when cultured in 3%, 6% and 9% saline condition. By using nutrient broth having various salt concentrations. Then ethyl acetate was used as solvent for extraction using, solvent extraction method described by Das I, M.K.et al. in 2017.

2.3 Phytochemical screening

2.3.1 Qualitative analysis of phytochemical constituents: Detection of active phytochemical constituents was carried out for all the extracts using the standard procedures.(Kokte C.K. ,2005)

2.3.1.1 Phenol:

1. Ferric chloride test: Take 2 ml extract add 5ml d/w and few drops of 5% ferric chloride solution. Development of blue/green colour indicates presence of phenol. (Kokte C.K. ,2005)

2.Lead acetate test: Take 1 ml extract add 3ml 10% lead acetate solution. Development of white precipitates indicates presence of phenol.(Kokte C.K. ,2005)

Table 1. Result of Qualitative Analysis of Phytochemicals

Phytochemical Tests	Result		
	RS 1	RS 2	RS 3
• Alkaloids:			
1. Mayer's Test	-	-	-
2. Dragendroff's Test	++	++	++
3. Wagner's Test	-	-	-
• Saponins:			
1. Frothing Test	+++	+	++
• Tannins:			
1. Lead Acetate Test	++	++	++
2. Potassium Dichromate Test	-	-	-
• Phenols:			
1. Ferric Chloride Test	-	-	-
2. Lead Acetate Test	++	++	++
• Steroids:			
1. Libermann Burchard Test	-	-	-
2. Libermann Sterol Test	-	-	-
• Flavonoids:			
1. Alkaline Reagent	++	++	++
2. Zn Hydrochloride Reduction Test	-	-	-

Key: (+++) Maximum, (++) moderate, (+) minimum, (-) negative

2.3.1.2 Steroids: 1. Libermann Buchard test: Take 1 ml extract add 2 ml acetic acid and 2 ml conc. sulfuric acid. Development of violet to blue or green colour indicates presence of steroids.(Kokte C.K. ,2005)

2.Libermann sterol test: Take 1 ml extract add drop of glacial acetic acid and conc. Hydrochloric acid. Development of violet to blue/green colour indicates presence of steroids.(Kokte C.K. ,2005)

2.3.1.3 Flavonoids:

Alkaline reagent: Take 1 ml extract add 1 drop of sodium hydroxide and diluted hydrochloric acid Solution becomes yellow to colourless indicates presence of Flavonoids. (Durai M.V. et al. 2016)

Zinc hydrochloride reduction test: Take 1 ml extract add Zn dust and conc. Hydrochloric acid. Development of red precipitates indicates presence of Flavonoids. (Durai M.V. et al. 2016)

2.3.1.4 Tannins: 1. Lead acetate test: Take 1 ml extract and 1 ml 2% Lead acetate solution. Development of white precipitates indicates presence of Tannins. (Durai M.V. et al. 2016)

2.Pottasium Dichromate test: take 1 ml extract add potassium Dichromate Solution. Development of Precipitate indicates presence of Tannins.(Durai M.V. et al. 2016)

2.3.1.5 Saponins: 1. Frothing test: Take 1 ml extract add 5 ml d/w and shake vigorously. Froth formation indicate presence of saponin. (Durai M.V. et al. 2016)

2.3.1.6 Alkaloids: 1.Mayer's Test: Take 1 ml extract and 2 ml Mayer's reagent. Development of dull white precipitate indicates presence of Alkaloids.(Durai M.V. et al. 2016)

2. Dragendroff Test: Take 1 ml extract add 2 ml Dragendroff reagent . orange white precipitate indicate presence of alkaloids.(Durai M.V. et al. 2016)

3.Wagner's Test: Take 1 ml extract add 2 ml Wagner's reagent. Reddish brown precipitate indicates presence of Alkaloids..(Kokte C.K. ,2005)

2.3.2 Quantitative Analysis of phytochemicals

2.3.2.1 Phenols: The Folin-Ciocalteu Spectrophotometric method was used for determination of total phenolic content. Total phenol content was expressed as mg of GAE/gm of extract. (Ali Ghsemzadeh et al.,2010)

2.3.2.2Flavonoids: The Aluminium Chloride Colorimetric Assay was used for quantifying flavonoids in the crude extracts. The total flavonoid content was expressed as mg of QE/g of extract (Hanane et al., 2010)

3. RESULTS AND DISCUSSION

3.2 Results of Quantitative Analysis

3.2.1 Determination of Total Phenolic content (µg/ml)

3.2.2 Determination of Total Flavonoid Content (µg/ml)

Table 2. Total Phenolic content (µg/ml) of Bacterial Extract

Extract	Control	3% NaCl	6% NaCl	9% NaCl
RS 1	3	4.5	8.5	28
RS 2	4	6	8	14.4
RS 3	4	4.25	5	5.75

Table 3. Total Flavonoid Content (µg/ml) of bacterial Extract

Extract	control	3% NaCl	6% NaCl	9% NaCl
RS 1	30	61	39	32
RS 2	33	33.5	40	48
RS 3	38	54	45	19.9

CONCLUSIONS

This study shows three bacterial isolates, in which RS-1 is gram positive cocci, RS-2 is gram negative short rod and RS-3 is gram positive bacillus from cultural and morphological characteristic revealed by providing various concentration of salt under appropriate growth condition. All three bacteria shows salt tolerance and higher antioxidant activity among them RS-1 shows higher salt tolerance and higher phenolic content. These bacteria may be effective alternative strategy to overcome the limitation to crop production brought by abiotic stress and for better plant health and protection as they are environment friendly cost effective and it also provide protection against biotic stress.

Conflicts of interest: The authors declare no conflict of interest.

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Effects of Auxin Producing Endophytic Bacteria to Sustain Growth Under Drought Stress

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ABSTRACT

The aim of the present work was to characterize indole acetic acid (IAA) producing endophytic bacteria. A total 5 endophytic bacteria were selected on the basis of their drought tolerant capacity and plant growth promoting properties. Results of 16S rRNA identified endophytic isolates MGT9 as *Paraburkholderia megapolitana* and MGT16 as *Alcaligenes faecalis*. Production of IAA was confirmed through FTIR and HPLC analysis. Maximum IAA production was observed when supplemented with 1.0% L-tryptophan was applied during optimization study. Two isolates were selected based on this study and identified as *Paraburkholderia megapolitana* MGT9 and *Alcaligenes faecalis* MGT16 which were able to produce IAA 65µg/ml and 49µg/ml under drought stress respectively. Results indicate that both IAA producing endophytic bacteria were able to produce IAA under drought-stress condition.

KEY WORDS: INDOLE ACETIC ACID; ENDOPHYTIC BACTERIA; DROUGHT STRESS; L-TRYPTOPHAN; HPLC; FTIR.

INTRODUCTION

Drought stress is among the most common environmental stress that affect plant growth and influence crop quality and efficiency in the semiarid regions (Jones, 2009). The world consists of about 60-65% of the arid and semi-arid region and therefore water crisis which have a negative impact on the crop productivity particularly in most of the Asian countries with an economy mainly dependent upon irrigated agriculture (Swain et al., 2017). Like many other Asian countries, India is also facing the severe problem of drought. Plants growing under detrimental environmental conditions, like arid and semi arid lands, go through water limitation and nutrient deficiencies. Drought can affect the growth of plant as the water become the limiting factor for plants and microbes to survive (Susilowati et al., 2018).

Plant-associated endophytic microorganisms are involved in symbiotic and associative various microbial activities which help in plants to establish in their environment (Morrissey et al., 2004). These endophytes are cost-effective and harmless source of nutrition for increasing agricultural production and improving soil fertility (Patel et al., 2018). Suggested mechanisms for endophytic microbe associated plant growth comprise production of indole-3-acetic acid (IAA) improved mineralization and nitrogen availability in the soil (Coombs and Franco, 2003). Indole-3-acetic acid is quantitatively the most abundant phytohormone secreted by many plant-associated bacteria and play beneficial role for plant growth. IAA biosynthesis is related to environmental stress, including acidic pH, osmotic and matrix stress, and carbon limitation (Spaepen et al., 2007). The present study was aimed to evaluate the potential of PGPE isolated

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from Ricinus communis from semi-arid region for the production of IAA under drought stress condition.

MATERIALS AND METHODS

Plant growth promoting endophytes used in study: Plant growth promoting endophytic bacteria were isolated from the different parts of Ricinus communis by using surface sterilization method. Total five potent endophytes MGT7, MGT9, MGT13, MGT16 and MGT19 were selected on the basis of their plant growth promoting attributes like auxin production, siderophore production, ammonia production, HCN production, phosphate solubilization as per our publication (Trivedi et al., 2018).

Extraction, purification and determination of IAA from endophytic bacterial cultures by FTIR and HPLC: The extraction of indole-3-acetic acid from selected endophytic bacterial cultures was carried out by normal solvent extraction method. The supernatant was obtained after centrifugation was acidified with pH 2 with 1N HCl and extracted twice with an equal amount of ethyl acetate (Patel and Saraf, 2017). Estimation of functional group present in the extracted IAA was analysed by Fourier Transform Infrared (FTIR) spectrophotometer (ALPHA FTIR Spectrophotometer, Bruker) by diamond attenuated total reflection (ATR) based FTIR spectrophotometer (Sachdev et al., 2009).

Characterization of IAA was done by High Performance Liquid Chromatography analysis (Shimadzu prominence HPLC System, Japan). HPLC analysis of IAA was carried out on C18 reversed phase column (Phenomenex, 250 × 4.60 mm 5 micro). Detection was carried out by using HPLC grade acetonitrile- water system containing 0.1% trifluoroacetic acid and it was programmed over 20 min at a flow rate of 1 ml/min with UV detector at 220 nm at 40oC. Mobile phase consisted of methanol and water (80:20 v/v) run at flow rate was analyzed by comparison with the elution profiles of standard IAA injected separately (Patel and Saraf, 2017).

Optimization of L-tryptophan concentration for maximum IAA production: IAA production of endophytic bacterial strains were induced by supplementing tryptone yeast broth with 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 1, 1.5 and 2% (w/v) L-tryptophan and cultures were incubated in the dark on an orbital shaker at 200 rpm for 120 h (Gang et al., 2018). Samples were withdrawn after every 24 h intervals to check the growth curve and IAA production (Harikrishnan et al., 2014).

Quantitative detection of IAA activity under drought stress: IAA production was measured in culture supernatants using Salkowski reagent as modified quantification method developed by Jha and Saraf (2011). Briefly, the isolate bacterial endophytic strains were cultured for 24-96 h using tryptone yeast broth with addition of 1% tryptophan and 20% PEG was added for development of drought stress. Cultivation was performed in the dark condition on shaker at 200 rpm for 72 h. After 72 h of incubation, the cultures were centrifuged at 10,000 rpm for 10 min and 1 ml of culture supernatant was taken and mixed with 1 ml of Salkowski's reagent (1 ml of 0.5M FeCl3 in 50 ml of 35% HClO4). The mixture was incubated in dark for 30 min at room temperature to develop pink color. The quantification of IAA was recorded at every 24 h up to 120 h by observing absorbance at 536 nm using spectrophotometer (Systronics 166, India) (Jha and Saraf, 2011). A standard curve was plotted for quantification of IAA. The amount of IAA in cultures was expressed as µg/ml.

RESULTS AND DISCUSSION

Total 5 microbial endophytic bacteria MGT7, MGT9, MGT13, MGT16 and MGT19 were selected on the basis of their drought tolerant capacity and plant growth promoting abilities. These selected bacterial endophytes were further studied for their IAA production capacity under drought stressed condition and their application as bio-inoculants on soybean crop under stress condition in pot trials.

FTIR analysis of extracted IAA from endophytic bacteria: FTIR spectra of extracted IAA was carried out by endophytic bacteria MGT9 *Paraburkholderia megapolitana* and MGT16 *Alcaligenes faecalis* is shown (Table 1). Characteristic N-H indole streching moeity was observed at 3330 cm-1 and 3334 cm-1 respectively. Streching for C-N bond of indole ring was observed around 1450 cm-1 and 1403 cm-1. Strong bond of C=O observed around 1021 cm-1 and 1216 cm-1 respectively. Thus, with the help of functional group analysis, it can characterized that auxin was present in both the endophytic bacteria. All the characteristic peaks of extracted IAA from the bacterial culture supernatant of MGT and MGT16 confirms the production of IAA. Reports suggsted that indole moiety was observed around 3400 cm-1 and strong peak of IAA appeared between 1060 cm-1 to 1305 cm-1 (Goswami et al., 2014). Our results reported that indole moiety ring was observed between 1400 to 1450 cm-1 in FTIR spectra.

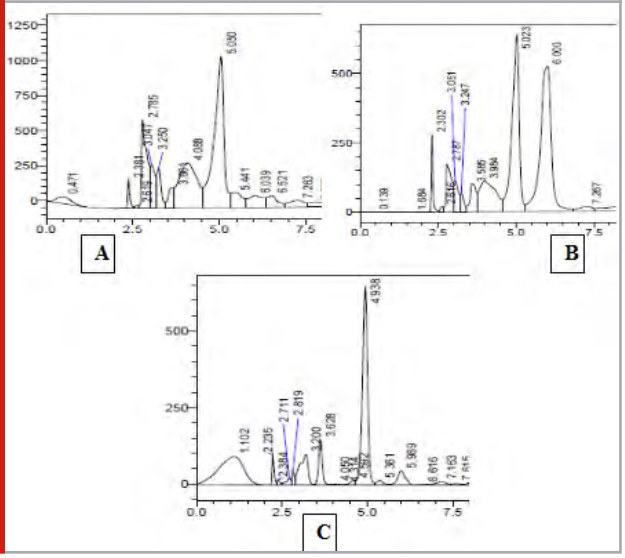
Table 1. FTIR spectra moeity of extracted IAA *Paraburkholderia megapolitana* MGT9 and *Alcaligenes faecalis* MGT16

solate	N-H bond	C-N bond	C=O bond
MGT9	3330 cm-1	1450 cm-1	1021 cm-1
MGT16	3334 cm-1	1403 cm-1	1216 cm-1

Detection of IAA by HPLC analysis: Identification of quantified IAA has been further confirmed by High Performance Liquid Chromatography. Ethyl acetate extract from the filtrate of endophytic bacterial culture strain MGT9 revealed a charcteristic at retention time of 5.050 min (Figure 1A) whereas endophytic bacterial culture strain MGT16 was shown a clear peak at retention time 5.023 min (Figure 1B). Standard of indole-3-acetic

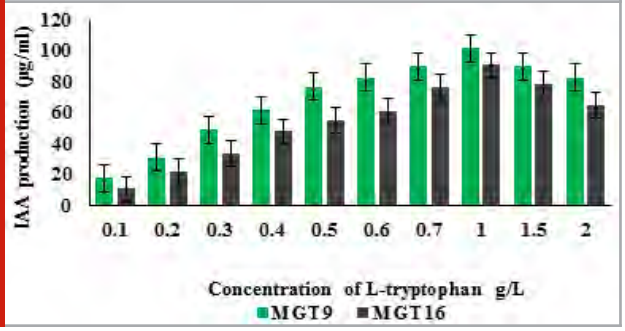
acid showed clear peak at retention time 4.93 min (Figure 1C). Results also indicated that IAA extracted from the bacterial strains was shown peak at 8.0 min where standard retention time of IAA was shown clear peak at 5.781 min (Mwange et al., 2003). Our results also showed peak retention time at 5.050 min and 5.023 min for MGT9 and MGT16 respectively containing the identity of IAA produced in endophytes.

Figure 1: HPLC profile of IAA production by: (A) *Paraburkholderia megapolitana* MGT9; (B) *Alcaligenes faecalis* MGT16 and (C) Standard indole- 3- acetic acid



Optimization of L-tryptophan concentration for production of IAA: Various concentrations of L-tryptophan between ranges of 0.1 to 2% were selected for the IAA production. From the spectrophotometric readings, it was observed that production of IAA was increased gradually with respective L-tryptophan concentration. Maximum IAA production was observed when medium was supplemented with 1.0 g/L of L-tryptophan (Figure 2).

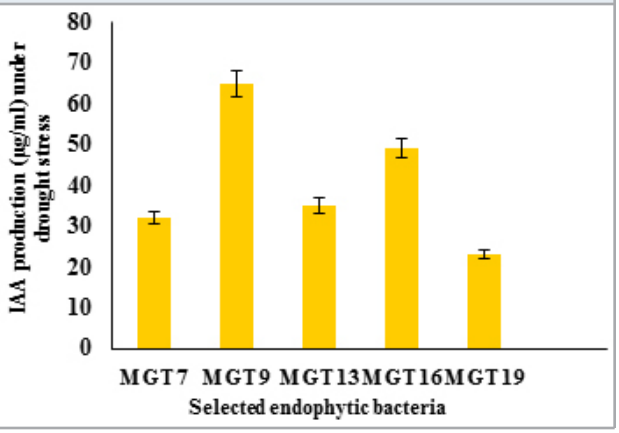
Figure 2: Effect of different concentration of L-tryptophan on IAA production



Our results reported that at 1.0% of L-tryptophan concentration, maximum IAA production was observed (Harikrishnan et al., 2014). In agreement with our results, another study stated that maximum IAA production was observed when medium was amended with 1 and 1.5% of L-tryptophan (Mohite, 2013). There is a significant different level of L-tryptophan for different species. Based on these results, it may be suggested that IAA producing endophytic bacteria result in better growth of plants.

Quantitative detection of IAA under stressed condition: Auxin production was determined in endophytic bacterial isolates under drought stress condition. *Paraburkholderia megapolitana* MGT9 and Alcaligenes faecalis MGT16 produced more IAA. In this study, *Paraburkholderia megapolitana* MGT9 produced highest IAA 65µg/ml whereas *Alcaligenes faecalis* MGT16 produced 49µg/ml under drought stress condition. *Bacillus pumilus* MGT7 produced 32µg/ml, *Achromobacter xylosoxidans* MGT13 produced 65µg/ml and *Stenotrophomonas maltophilia* MGT19 produced 49µg/ml auxin under drought stress condition (Figure 3).

Figure 3: IAA production of selected bacterial endophytes under non-stress and drought stress condition



From the above results, among the endophytes two potent endophytic bacterial isolates MGT9 *Paraburkholderia megapolitana* and MGT16 *Alcaligenes faecalis* showing significant growth and IAA production at -1.09 MPa were chosen for further studies. Development of plants was regulated by producing phytohormones in various parts of plant, while endophytic microorganisms also contributing in the production of these phytohormones (Khan et al. 2016). It has been confirmed that IAA can be produced by large variety of endophytes (Saxena, 2014; Duca et al., 2014). Various types of endophytic bacteria were characterized for the synthesis of IAA; exploit various IAA production pathways and single bacterial strain at times including more than one pathway (Duca et al., 2014). With reference to previous reported data, production of IAA by bacteria can vary among different species and strains as well as it is also subjective by condition of culture, growth stage and substrate availability (Firakova et al., 2007).

CONCLUSION

Endophytic bacteria maintain good drought tolerant activities which gave much promising results in drought stress condition. The endophytic bacteria gave its effect throughout the life under drought stress condition.

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Role of Diazotrophic Bacteria in Elevating Growth of Hordeum vulgare

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ABSTRACT

Eighty per cent (80 %) of the atmosphere is nitrogen gas (N2). All the living organisms utilize nitrogen for building blocks of the biomolecule to sustain life. Numerous acetylene reduction assay showing positive bacteria have involved their presence in nitrogen fixation of root and stem like Enterobacter cloacae, *Klebsiella oxytoca, Bacillus, Erwinia*. Chemical nitrogen fertilizer has been implemented in the field for a long term which causes soil pollution as well as decreases the fertility of soil. To replace that focus has deviated on Biological Nitrogen Fixation through application for various diazotrophic bacteria to enhance the total nitrogen content of the soil. To study the effect of non-symbiotic diazotrophs, soil samples were collected from the rhizosphere of barley in Gujarat, India. Differential media has been reported for isolation of diazotrophic bacteria and to distinguish nonsymbiotic bacteria from symbiotic, hence one of such Glucose nitrogen free media utilized in our study on the mechanism of ammonia production. Recently reported method for characterization of total nitrogen biomass from bacteria cell, in the two different part of nitrate nitrogen and amino nitrogen. A standard strains M5a1 is reported for nitrogen fixation from many years and nifH which is knockout for nif genes was consider for comparative study in all experiments.

KEY WORDS: BIOMASS, DIAZOTROPH, GFNM, NITRATE, PGPR,.

INTRODUCTION

Free living soil bacteria that are indirectly connected to plants and involved in a sufficient amount of nitrogen fixation in plants. Such free living diazotrophs can be found in association with organic matter and plant residue. The nitrogen fertilizer demand has hike from 112 million in 2015 to 117 million in 2019, still goes on increasing (Monokoane P.C et al., 2016). Many free living diazotrophic bacteria has been reported for biological nitrogen fixation which involves the conversion of atmospheric N2 into different available forms for plants. Some of the bacterial strains have ameliorated to fix nitrogen and can be replaced to chemical fertilizers, these are *Panebacillus, Klebsiella, Pseudomonas* which are enabled to penetrate roots of plants. (Reinhold-Hurek, B., and Hurek, T., 1998).

The number of methods has been documented to find out the appearance of free living bacteria which involves detection of bacteria immunologically, tagging

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with fluorescence dyes or proteins, various types of microscopy and use of particular oligonucleotide probes (Rosenblueth and Martínez-Romero., 2006).

Diazotrophic bacteria also possess other plant growth-promoting traits such as auxin and gibberellin phytohormone production, iron-chelating agent siderophore, many droughts tolerant osmolyte production resulting in enhanced root and shoot development with increase amount of nitrogen source. (Steenhoudt and Vanderleyden, 2000; Dobbelaere et al., 2003).There has been significant evidence about BNF by *A. amazonense*, Enterobacter sp in a sugarcane field with a maximum increase of 170 kilograms of nitrogen ha-1 year-1 and increase in total biomass of yield by 39% (Mirza et al., 2001). The standard method to detect the activity of nitrogenase enzyme is acetylene reduction assay which is based on the fact that nitrogenase enzyme converts acetylene into ethylene which is eventually calculated by Gas Chromatography. The research article mention the method to determine nitrogen activity in the lab without any cost affecting methods like acetylene reduction assay (Orji et al., 2018)

MATERIALS & METHODOLOGY

I. Soil sample collection: To potentially isolate free-living diazotrophic plant growth promoting bacteria samples from roots and soil were collected from the rhizosphere of barley at Dholka region of Gujarat from three distinguishing sites. Collected soil and root samples were placed in zip lock bag stabilized at 4°C until they were transferred and processed in the laboratory. Soil samples were send for physiochemical analysis at Indian Farmers Fertiliser Cooperative (IFFCO).

II. Isolation: Before the root samples were cut into pieces, all the samples were surface sterilized with 5% sodium hypochlorite for 5 mins and rinsed thrice with sterile distilled water. With the help of motor pestle root sample was grounded in 10 ml sterile distilled water following subsequently serial dilutions, samples were plated on Jensen agar media Sucrose 20.000g L–1; Dipotassium phosphate 1.000 g L–1; Magnesium sulphate 0.500 g L–1; Sodium chloride 0.500g L–1; Ferrous sulphate 0.100 g L–1; Sodium molybdate 0.005g L–1; Calcium carbonate 2.000 g L–1 for solid media add 4 g L–1 of agar. (Bag et al 2017. Soil samples showing the high amount of nitrogen in physicochemical properties were weighed 10 gm and dissolved in Erlenmeyer flask of 250ml containing 90ml of sterile distilled water and kept for 30 min at 200rpm. After vigorous shaking, all the soil samples were serially diluted and plated on the Jensen media agar and incubated at 30°C for 4-5 d.

III. Screening: For screening nitrogenase activity of all the selected isolates, Glucose Nitrogen Free Media (GFNM) was prepared, Dipotassium phosphate 1.0 g L–1, 1.0 Calcium chloride g L–1, 0.5 Sodium chloride g L–1, 0.25 Magnesium sulfate heptahydrate g L–1, 0.01 Ferrous sulfate heptahydrate g L–1, 0.01 Sodium molybdate g L–1, 0.01 Manganese sulfate pentahydrate g L–1 and glucose as a carbon source g L–1. Solid medium was prepared by adding 4% agar media and supplemented with 0.01% of Bromothymol blue (BTB) to visualize the colour change from green to blue. Based on the mechanism that if isolate is capable to produce extra ammonia can fix atmospheric nitrogen to plants, NFB plates which are initially green will turn blue after incubation 5 to 6 d at 35°C and compared with our standard strains M5a1, SGM 81 and nif H mutant.

IV. Total nitrogen from cell biomass: A) Nitrate nitrogen was estimated by broth culture experiments from the initial d of inoculation 0 and 7th d of incubation. 100 ml of Jensen media broth was inoculated with selected isolates and every d total nitrate content of culture was determined by modification with Cataldo's method (Orji et al., 2018). On the initial d, 5 µl of broth sample and 20 µl of transferred in 2 ml of Eppendorf tube and incubated at room temperature for 20 minutes. Later 500 µl of 2M NaOH was mixed with this previously prepared sample and incubated again for 20 minutes. The samples were diluted to 1ml at final concentration and O.D was recorded at 410nm.

B) To determine the amino nitrogen content the sample was in the same concentration and mixed with 150 µl of citrate buffer subsequent addition of 120 µl ninhydrin solution. Once the sample has been stabilized for 20 min at room temperature 600 µl of ethanol was mixed. The colour change was observed and after bringing final concentration to 1ml absorbance was recorded at 570 nm and compared with a standard serially diluted solution of asparagine. Total nitrogen concentration was calculated formula mention in (Orji et al., 2018). For standard for positive and negative control samples were also prepared for M5a1, SGM 81 and nifH mutant. Amount of nitrated produced by each bacterial mass was compared with standard nitrate solution (Orji et al., 2018)

RESULT AND DISCUSSION

Soil sample has been appeared to shown a diverse variety of bacteria rather than root samples uprooted from barley field. 28 % of the colony morphology appear gram positive bacilli and the lowest number was recorded for 4% of cocci Table no: 1physciochemical properties of soil. Around seventy-six isolates were selected form GFNM media plate prepared from both root and soil samples. Colonies appearing opaque and slimy were purified on the nutrient agar medium. Isolates showing distinguish cultural characteristics were selected for screening process, assuming the fact that GFNM is plate assay for visualization of nitrogen fixing bacteria, among all the selected isolates were streaked on GFNM only twenty-three was potentially able to turn colour of media from green to blue indicating high production of ammonia in the plate which increases the pH of the medium. (Khin Mya Lwin et al., 2012).

Yellow colour colonies were able to show effective growth on GFNM media but devoid of colour change indicating such colonies were unable to fix nitrogen. SGM81

and M5a1 have been efficiently shown production of ammonia and was used as a positive control also nif H mutant was used as a negative control. One of the preliminary and conventional methods reported by (Orji et al., 2018).

Has been significantly proved for the quantification of the total nitrogen content of cell biomass. The amount of total nitrate and amino content of the bacterial cell mass was recorded and calculated for abundant quality of nitrogen produced by bacterial biomass. As shown in fig 2 indicating total nitrogen biomass production, the highest amount of nitrogen yield was recorded for SSM1N culture which is 0.72ppm compared with standard isolates showing SGM81 0.65ppm and M5a1 0.62ppm. It was also observed that the increase in bacterial biomass in Jensen media there is a significant amount of nitrogen yield till 7 ds but after reaching stationary phase there were no changes for recorded results. nif H mutant was able to grow in Jensen media broth but no nitrogen yield was recorded even after seven ds of incubation also there was no colour development for nif H alike other strain was developing yellow colour fig 3.

Table 1. Physicochemical properties of soil

Soil properties	0-20 cm
Sand (%)	36.1
Slit (%)	38.6
Clay(%)	31.6
Texture class	Loam
EC (ds m-1)	0.74
pH	6.5-7.0
Total N (%)	0.045
Available P (mg kg-1)	2.65
Extractable K (mg kg-1)	201.5

Table 2. Identification of isolate

No of isolates	Name	Morphology	PGPR
1	SSM1N	Short rods	Klebsiella
2	SSM2N	Large oval	Aeromonas
3	SSM9N	Bacillus	Bacillus subtilis
4	SSM17N	Short rods	-
5	SSM19N	Bacillus	Panebacillus
6	SSM20N	Short rods	Klebsiella
7	SSM5N	Large oval	Azotobacter
8	SSM26N	Bacillus	Bacillus subtillis
9	SSM21N	Short rods	Azotobacter
10	SSM2NP	Short rods	Klebsiella
11	SSM4N	Short rods	Azotobacter
12	SSM18N	Short rods	Bacillus subtilis
13	SSM15N	Bacillus	-
14	SSM11N	Large oval	-
15	SSM06N	Oval	-

Figure 1: GNFM media initially and after 5 ds of incubation. Isolates which were are able to change colour of media are found to be potent ammonia producer

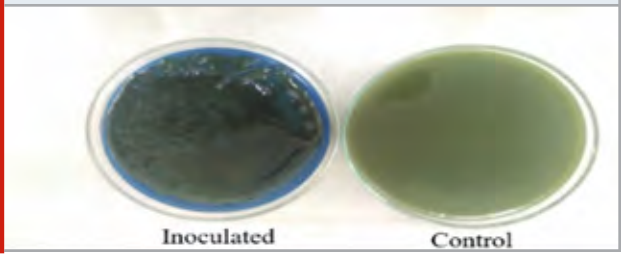

Figure 2: Total nitrogen produce by cell biomass from SSM1N culture which is 0.72ppm compared with standard isolates showing SGM81 0.65ppm and M5a1 0.62ppm.

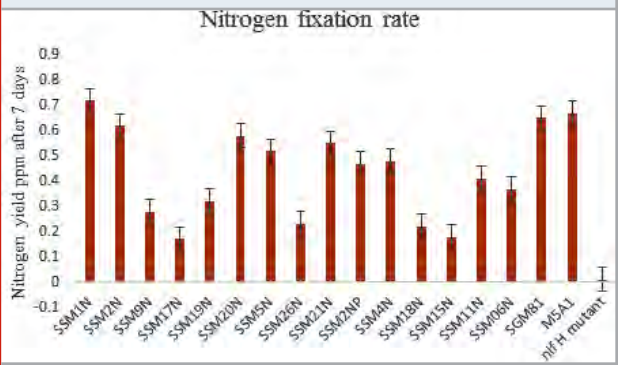

Figure 3: Total nitrogen detection from cell biomass. All the strains were able to develop yellow colour except control and nifH mutant indicating that total nitrate production is absence.

CONCLUSION

The study aimed to isolate potentially dominant diazotrophic bacteria from the rhizosphere of barley, among all the seventy-six isolates, only two were found to be good nitrogen fixer depending upon the total nitrogen produced by bacterial biomass. Based on colour variation isolate producing ammonia in GFNM were screened out. Acetylene reduction assay is a cost-effective assay for detection of nitrogenase enzyme, in substitute to acetylene reduction assay method total

nitrogen production by cell biomass is a good alternative for the screening of nitrogen fixer bacteria.

Conflict of interest: The author has no conflict of interest in preparing this article.

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L-Glutaminase Biosynthesis from Marine Bacteria

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ABSTRACT

L-glutaminase belongs to the superfamily of serine-dependent β-lactamases & penicillin-binding proteins. L-glutaminase amidohydrolase (EC 3.5.1.2) has unique catalytic property to form glutamic acid and ammonia by deaminating L-glutamine. L-glutaminase finds its application in food i.e. flavor enhancer & imparts umami taste. It also finds its application in therapeutics such as cancer therapy, especially for acute lymphocytic leukemia, radical scavenging ability. Lastly it finds suitable application in analytical chemistry as well, wherein glutamine/ glutamic acid biosensors are developed to assess various biological fluids. Marine environment harbors a huge pool of novel enzymes and is expected to have very unique L-glutaminases i.e. salt-tolerant and thermo-stable as needed by food industries. Present study give insights into L-glutaminase biosynthesis from marine bacteria along Gujarat coastal regions i.e. Khambhat, Somnath, Bhavnagar, Dhuvaran, Diu. A total 115 different marine bacterial isolates were obtained which were screened onto minimal glutamine medium supplemented with phenol red. Amongst them 87 bacterial isolates with varying ability to secrete L-glutaminase with a marked color change were selected for further studies. Salt tolerance (5-30%) in minimal glutamine broth were studied for few isolates. Furthermore the optimization, scale up, purification, characterization and application studies will be attempted for few promising isolates..

KEY WORDS: L-GLUTAMINASE, MARINE BACTERIA, MARINE ENZYMES, ANTI-CANCER, ANTIOXIDANTS.

INTRODUCTION

The isolation of novel microbial enzymes from marine resources is increasing in past few years. Moreover, the increasing interest on marine enzymes is related mainly to the catalytic features and robustness in nature which make them attractive for industrial exploration. Catalytic activity and stable under multitude of extremities have been of prime interest for researchers (Raval et al., 2014). All these properties of pH, temperature, pressure, salinity, cold adaptability etc. make marine enzymes the foremost choice purposes (Raval et al., 2013). Exploration of marine resources is followed by highly worked upon disease globally i.e. cancer which is characterized by uncontrolled growth of cells and tissue proliferation leading to further clinical manifestation of the deadly disease. Majority of cancer therapy includes chemotherapy which is effective in most cases but is equally detrimental for the patient and is having serious side effects. The ill-effects of such treatments include serious side effects, immunity suppression, and poor treatment outcomes. There has been a constant surge for novel drugs molecules for

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cancer therapy and marine resources have been explored for the same.

Natural products have been a major source of new drug candidates since ages. Nature has been an in-exhaustible source with a remarkable chemical diversity of organisms living on earth (Demain and Sanchez, 2009; Gupta et al., 2013; Prabhu et al., 2015). Oceans occupy almost 80% of the world's biota, and it is believed that life originated from the ocean. Marine derived enzyme has unique activity that differs from enzyme produced by other sources because marine species have evolved mechanisms to survive in an extremely hostile environment in terms of light, salinity, and pressure as compared with land (Jaspars et al., 2016). Marine ecosystem represents a wide and extraordinary resource of biologically active metabolites because of the chemical and biological diversity of the marine environment (Wang et al., 2009).

Recently improvements in deep-sea collection and aquaculture technology are responsible for the growing interest of the enormous biodiversity present in the marine world which has led to shift of focus toward oceans as being potential source of new anticancer candidates. This has resulted in marine derived compounds entering preclinical and early clinical development phases (Schwartsmann et al., 2003; Mayer et al., 2010). Marine-derived compounds are more bioactive in terms of cytotoxicity than those of terrestrial origin. The fact that many marine compounds have entered into the market proves that marine natural products have their stronghold in the area of anticancer chemotherapy. The research work on L-glutaminase enzyme was initially started in the year 1956 Alexander B. Gutman and Tsai–Fan, which accidentally studied in primary gout was a deficiency of metabolizing glutamine via L-glutaminase enzyme (Gutman, 1963). Since past few years, L-glutaminase as an enzyme has seeked much of attention owing to its characteristic feature of synthesizing glutamic acid and ammonia from L-glutamine by deamination reaction. Hence, it also makes it a very useful candidate for various industrial applications.

L-Glutaminase (EC 3.5.1.2) is an amido-hydrolase a member of serine-dependent β-lactamases and penicillin-binding proteins (Irajie et al., 2016). L-glutaminase is cellular proteolytic endopeptidase that acts on the peptide bonds in the presence of water to yield glutamic acid. Recently L-glutaminase has gained importance, because of its commercial importance in both therapeutic and food industry. It plays an important role in nitrogen metabolism and ammonia detoxification (Binod et al., 2017). In Therapeutics L-glutaminase is the most potent antitumor agent used in treatment of cancer. For the biosynthesis of a potent in-vivo antioxidant i.e. glutathione glutamic acid acts as an important precursor (Nahar et al., 2017). With the advancement of recombinant technology it is now feasible to alter naïve glutaminase and synthesize a tailor made recombinant glutaminase with improved yields and properties targeted for cancer therapy. Deamination reaction eliminates a key metabolite essential for growth of cancerous cells which otherwise is not required for host cell functions. In food industry and related sectors its reduce acrylamide formation at high temperatures. L-glutaminase used to enhance taste and aroma of fermented foods. The oriental tastes in foods such as soy sauce, sufu, miso is due to varying contents of glutamic acid (Rastogi and Bhatia, 2019).

While one of the most sought-after applications in food industries is the increased salt tolerance and L-glutaminases are widely studied for the same. L-glutaminase found to be involve in Synthesis of vitamin B6 viz. vital metabolite for all living organisms and work as cofactor. Lastly, the solubility of glutein (emulsifier) is increased after L-glutaminase treatment (Liu et al., 2011). Analytical use involves use of biosensors for monitoring glutamine and glutamate levels of biological fluids (Unissa et al., 2014). Theanine (N-ethyl-L-glutamine) is water-soluble non-protein major amino acids components present in Japanese green tea is a well-studied nutraceutical and a unique taste-enhancing amino acid of infused green tea. It has been shown to improve the immune system (Zhang et al., 2019). Reduction in fat accumulation and protect nerve cells. Theanine which is a key ingredient and commercially used in many foods (soft drinks, chocolates and desserts, etc.) is produced by enzymatic synthesis of glutamine from glutamic acid via L- glutaminase action and ethylamine.

L-theanine also finds wide applications because of its favorable physiological and pharmacological activities including the inducing sleep and relaxation, improvement in learning ability, lowering of blood pressure, ability to relieve depression, and antitumor and neuroprotective effects (Adhikary and Mandal, 2017). Thus, large-scale production of L-theanine is necessary and can be met by employing L-glutaminase for precursor synthesis. Microbial L-glutaminase has diverse applications, ranging from food flavoring agent to treatment of cancer. Lastly L-glutaminase is found to enrich the growth of health beneficial probiotic bacteria. Various microorganisms such as bacteria (Kumar et al., 2019) fungi (Bülbül & Karakus, 2013), yeasts (Aryuman et al., 2015), and actinomycetes (Desai et al., 2016). As compared to higher organisms glutaminases from microbes possess higher stability (Dutt et al., 2014). Yet there is a continued surge for isolating novel microbes that have improved properties and higher yield for glutaminases.

L-Glutaminase is ubiquitously produced by plants, E.coli, Pseudomonas, Bacillus spp. and others. Two types of Glutaminases have been reported in E.coli based on their cellular nature. Kidney type L-Glutaminase is membrane bound whereas liver type L-Glutaminase is extracellular (Vijayan et al., 2017). Both types have applications in medicinal industry to treat cancer. Thus marine environment had vast bacterial diversity and high fluctuations in their salinities and temperatures become source for salt tolerant; thermo-stable L-glutaminase

(Renu and Chandrasekaran, 1992). Reports on the synthesis of extracellular L-glutaminase by marine microorganisms are very limited to marine bacteria including *Pseudomonas fluorescens, Vibrio costicola* (Prabhu and Chandrasekaran, 1997), *Vibrio cholerae,* and *Micrococcus* luteus-K 3 (Moriguchi et al.,1994). Due to anti-retroviral nature this enzyme is used to treat HIV. Marine environment harbors a huge pool of novel enzymes and is expected to have very unique L-glutaminases i.e. salt-tolerant and thermo-stable as needed by food and pharmaceutical industries.

MATERIALS AND METHODS

1) Sample collection: Marine soil/ water samples were collected in sterile bag and glass bottle from Gujarat coastal regions viz. Dhuvaran, Khambhat, Somnath, Diu, and Bhavnagar and refrigerated till further processing.

2) Isolation of marine bacteria: Further marine samples were processed using enrichment culture technique in Zobell marine broth (ZMB) medium containing (gm/L): Peptone 5.0; Sodium chloride 19.45; Sodium sulphate 3.24; Magnesium chloride 8.80; Calcium chloride anhydrous 1.80; Potassium chloride 0.55; Sodium bicarbonate 0.16; Potassium bromide 0.08; Strontium chloride 0.034; Boric acid 0.022; Sodium silicate 0.004; Yeast extract 1.0; Ferric citrate 0.10; Sodium fluorate 0.0024; Ammonium nitrate 0.0016; Disodium hydrogen phosphate 0.008; Final pH 7.6 ± 0.2. The inoculation i.e. 5% (w/v for soil ; v/v for water) samples were done in ZMB media supplemented with 5-25% (w/v) NaCl concentration and flasks were incubated on rotary shaker at 150 rpm for up to 72 hrs at 37°C. The marine soil / water samples were enriched by serial dilution & later were isolated by spread plate method. Plates were incubated at 37°C and observed for cfu/ml.

3) Screening of L-glutaminase producing marine bacteria: The isolated marine bacterial colonies were screened further for biosynthesis of L-glutaminase in Minimal glutamine broth medium (MGB) containing (gm/L) L-glutamine 5; NaCl 50; KH_2PO_4 1.0; $MgSO_4.7H_2O$ 0.5; $ZnSO_4.7H_2O$ 0.1; $FeSO_4.7H_2O$ 0.1; Phenol red 0.0012 and pH 6.5 adjusted using 20% w/v Na_2CO_3. In minimal glutamine medium L-glutamine act as a sole carbon and nitrogen source, phenol red as an pH indicator. For screening 24 hours active culture of each isolate were used for inoculation in minimal glutamine broth pH-6.5 and incubated at 37°C for 24-48 hours. A marked color change of the medium from yellow to pink indicate a positive result for L-glutaminase production. Breaking down of the amide bond by L-glutaminase releases L-glutamic acid and ammonia into the reaction medium which leads to change in pH from neutral to alkaline and further the color of medium changes to pink.

RESULTS AND DISCUSSION

1)Isolation of marine bacteria: Isolation was done by enrichment culture technique using Zobell marine medium supplemented with 5-25% (w/v) NaCl. Marine samples were enriched by serial dilution & later were isolated by spread plate method (Figure 1&2). A total of 115 distinct marine bacterial isolates were obtained which were further screen out for L-glutaminase activity.

Figure 1: Growth of bacterial isolate on Zobell marine medium from khambhat site

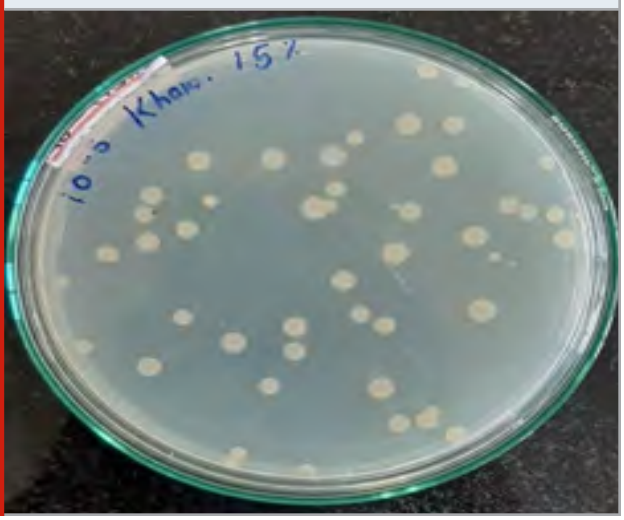
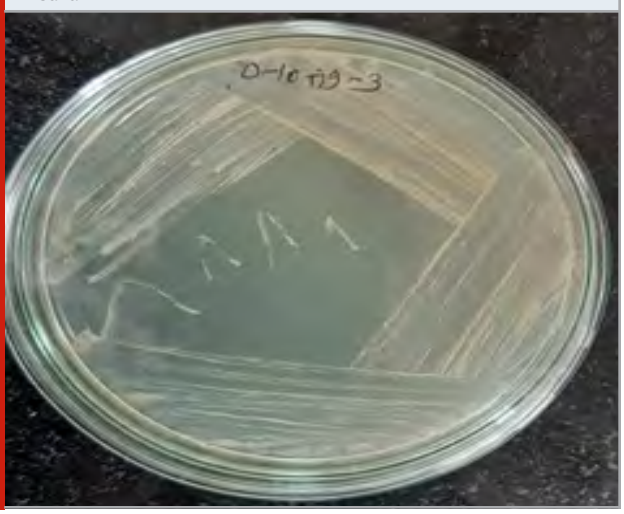


Figure 2: Pure growth of marine bacteria on Zobell marine medium



2) Screening of L-glutaminase producing marine bacteria: A total 115 marine bacterial isolates were screened further for L-glutaminase secretion by broth assay method in Minimal glutamine broth containing phenol red as a pH indicator. A 24 hrs active culture of isolates were inoculated in Minimal glutamine broth of pH 6.5 and incubated at 37±2°C on rotary shaker at 150 rpm for up to 72 hrs. Amongst 115 isolates, 87 isolates were found to secrete L-glutaminase by marked color change from yellow to pink as well as change in pH from 6.5 towards alkaline side indicating positive result for L-glutaminase biosynthesis by broth assay method. (Table 1 ; Figure 3) Out of 87 L-glutaminase producing marine bacteria 30 isolates with higher capability were selected for further study (Figure 4).

Table 1: List of all isolates showing L-glutaminase secretion

Sites	Isolates	L-glutaminase secretion	pH Range/ optimum (6.5-10.60)	Isolates	L-glutaminase secretion	pH Range/ optimum (6.5-10.60)	Isolates	L-glutaminase secretion	pH Range/ optimum (6.5-10.60)
Bhavnagar	B-10-9-3	++++	9.48	B-20-9-4	++++	9.57	B-20-9-7	+++	8.05
	B-15-9-1	++++	9.44	B-25-9-2	++++	10.55	B-10-9-1	++	8.02
	B-15-9-2	++++	9.45	B-25-9-3	++++	9.48	B-10-9-4	++	7.88
	B-15-9-3	++++	9.75	B-25-9-4	++++	9.46	B-10-9-7	++	7.82
	B-15-9-4	++++	10.10	B-25-9-5	++++	9.40	B-10-9-9	++	7.60
	B-15-9-5	++++	9.64	B-25-9-6	++++	10.00	B-20-9-6	++	7.49
	B-15-9-7	++++	10.03	B-10-9-5	+++	8.20	B-20-9-1	++	7.40
	B-15-9-8	++++	9.59	B-15-9-6	+++	8.99	B-10-9-2	+	7.20
	B-15-9-9	++++	9.52	B-15-9-12	+++	8.80	B-10-9-6	+	7.18
	B-15-9-10	++++	9.54	B-15-9-13	+++	8.92	B-10-9-8	+	7.23
	B-15-9-11	++++	9.26	B-20-9-3	+++	8.40			
	B-20-9-1	++++	9.49	B-20-9-5	+++	8.10			
Dhuvaran	D-5-9-4(1)	++++	9.42	D-15-9-6	++++	9.70	D-10-9-2	++	8.08
	D-5-9-4(2)	++++	9.04	D-5-9-1	+++	8.25	D-5-9-4	+	7.70
	D-10-9-3(2)	++++	9.87	D-5-9-2	+++	8.30	D-10-9-3	+	7.00
	D-15-9-3	++++	9.55	D-5-9-3	+++	8.54	D-15-9-2	+	6.87
	D-15-9-4	++++	9.74	D-15-9-*1	+++	8.88	D-15-9-8	+	7.48
	D-15-9-5	++++	9.52	D-15-9-5(1)	+++	8.16			
	D-15-9-5(2)	++++	9.51	D-15-9-3(6)	+++	8.98			
Somnath	S-5-9-3	++++	9.02	S-10-9-2	+++	8.58	S-10-9-1	++	7.49
	S-15-10-1	++++	9.09	S-15-10-3	+++	8.96	S-15-10-2	+	6.85
	S-5-9-2	+++	8.70	S-5-9-1	++	7.23			
Khambhat	K-15-9-7	++++	9.64	K-15-9-K2	+++	8.31	K-10-9-2(1)	+	6.96
	K-15-9*2	++++	9.35	K-20-9-K2	+++	8.65	K-10-9-3	+	7.50
	K-15-9-9	++++	10.04	K-5-9-1	++	7.10	K-15-9-2(1)(3)	+	7.46
	K-15-10-1	++++	10.07	K-15-9-1	++	7.27	K-15-9-4	+	6.95
	K-15-10-1(9)	++++	9.49	K-15-9-3	++	7.60	K-15-9-10	+	6.77
	K-15-10-2	++++	10.40	K-15-9-5	++	8.23	K-15-9*10	+	6.86
	K-20-9-4	++++	10.60	K-15-9-6	++	8.41	K-20-9-1	+	7.08
	K-15-9-6	+++	8.41	K-10-9-2	+	6.66			
Diu	Du-15-9-1	+	6.88	Du-15-9-2	+	7.46	Du-15-9-4	+	7.44

Figure 3 : L-glutaminase biosynthesis by marine bacteria using Minimal glutamine broth

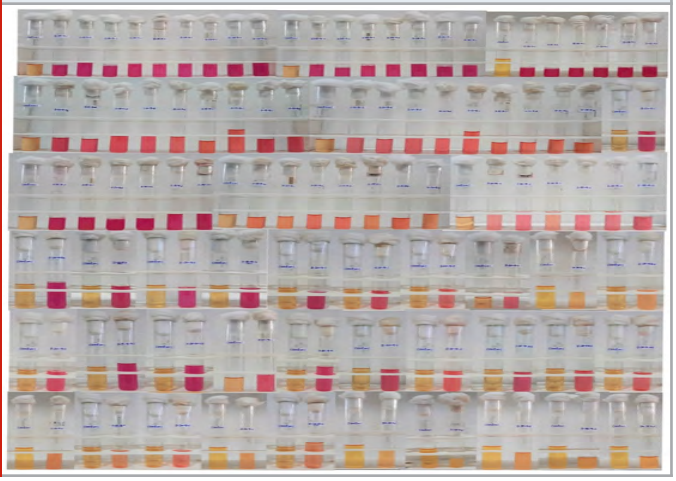
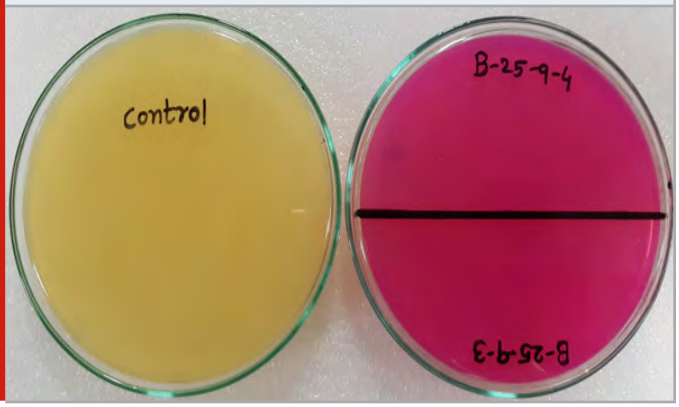


Figure 4: L-glutaminase biosynthesis by marine bacteria in Minimal glutamine agar



CONCLUSION

From this present study it can be concluded that the marine coastal region is unique habitat for salt tolerant bacteria and it is found to be most effective for L-glutaminase biosynthesis by broth assay method. A total of 115 marine isolate were obtained which further screened onto minimal glutamine medium and it is found that 87 isolates have ability to secrete L-glutaminase with varying ability by marked color change. Present study is a step towards L-glutaminase biosynthesis which has various applications in cancer therapy, food industry, pharmaceutical industry, production of neutraceutical i.e., theanine and also it have ability to enrich the growth of probiotic bacteria.

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Screening and Production of Tannase Using Fungal Isolates from Soil

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ABSTRACT

Tannase (tannin acyl hydrolase, EC 3.1.1.20) catalyzes the hydrolysis of ester bonds from gallotannins which are also called complex or hydrolysable tannins producing gallic acid and glucose. Tannase has several important applications in food, feed, chemical and pharmaceutical industries but high-scale use of this enzyme is severely restricted because of its higher production costs. In this study, ten soil samples were collected for primary screening of tannase producing fungi. Total 22 fungal isolates were obtained using potato dextrose agar and mineral salt agar. After primary screening, tannase production of 2.10, 3.99 and 2.10 U/ml was obtained for three fungal isolates named as HP-1, HP-6 and HP-13, respectively at 30°C, 160 rpm for 72 h. Optimization of the culture conditions for tannase production was done for HP-1, HP-6 and HP-13. Effect of different inoculum size was tested and HP-1 (yeast) showed tannase production of 2.45 U/ml with 5% (v/v) inoculum. Two molds, HP-6 and HP-13 showed tannase production of 4.59 U/ml and 2.62 U/ml, respectively with 1×108 number of spores as an inoculum. The 0.75% (w/v) tannic acid concentration was found optimum for HP-1, HP-6, HP-13 giving tannase production of 2.85, 5.04 and 2.70 U/ml, respectively. Among the different nitrogen sources tested, HP-1 gave 3.29 U/ml tannase with peptone (0.5% w/v) after 24 h whereas HP-6 and HP-13 reported 5.56 U/ml and 3.15 U/ml after 48 h.

KEY WORDS: GALLIC ACID, NITROGEN SOURCE, SCREENING, TANNASE, TANNIC ACID.

INTRODUCTION

Tannin-acyl-hydrolase is an important extracellular hydrolytic enzyme also commonly referred to as tannase (E.C. 3.1.1.20). It releases glucose and gallic acid by catalyzing the sequential hydrolysis of depside andester bonds in hydrolysable tannins (Belur and Mugeraya, 2011). Tannins have toxic effects on animals and also inhibits the microbial growths. Some of the microbes have developed mechanisms to degrade tannins in their natural environments and tannase production is one of them.

Tannase production was mainly found with fungal isolates rather than bacteria. Number of tannase producing fungi were reported such as *Aspergillus niger* JMU-TS528 (Wang et al., 2013), *Penicillium atramentosum* KM (Selwal and Selwal, 2012), *Aspergillus niger* HA37 (Aissam et al., 2005), *Penicillium purpurogenum* PAF6 (Jana et al., 2012), *Aspergillus tubingensis* MUT 990, Paecilomyces variotii MUT 1125 (Prigione et al., 2018). Among the fungi, very few yeasts were reported as tannase producers

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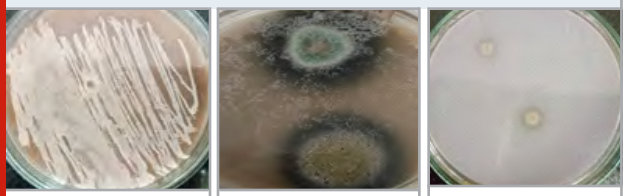
like *Arxula adeninivorans* (Boir et al., 2011), *Rhodotorula glutinis* MP-10 (Taskin, 2013) etc. The bacterial tannase production was reported with Klebsiella pneumoniae KP715242 (Kumar et al., 2015).

Tannase has significant applicability in the field of biotechnology as well as industries like cosmetics, foods, pharmaceuticals, etc. It is commonly used in the degradation of complexes in waste waters (Aissam et al., 2005), biotransformation and bioremediation of tannery wastewaters (Prigione et al., 2018), chromium and tannic acid bioremediation (Chaudhary et al., 2017) etc. In food industries tannse is mainly useful in the preparations of tea, wine and beverages like beer, fruit juices, coffee-flavored drinks etc. It was used in the secondary processing of tea leaves (Li et al., 2017), improve the sweet aftertaste of green tea infusion (Zhang et al., 2016) and biotransformation of green tea (Macedo et al., 2012), detannification of food which ultimately improves its nutritive value. Furthermore, the hydrolytic compound of tannin the gallic acid is a primary anti-inflammatory, cardioprotective agent found in tea, cocoa and wine. Gallic acid is also used for the synthesis of trimethoprim, propyl gallate and photosensitive resin in semiconductor production (Jana et al., 2012). Present study deals with the screening of tannase producing fungal isolates and optimization of its cultural conditions for tannase production.

Figure 1: Growth of HP-1, HP-6 and HP-13 on PDA and mineral salt agar medium



HP 1 - YEAST | HP 6 - Mold | HP 6 - Mold

MATERIALS AND METHODS

Isolation of tannase producing microorganisms: Soil samples were collected from different regions of Gujarat to isolate tannase producing microbes. 1.0 gm of soil samples were added to 10 ml sterile distilled water and solid particles were allowed to settle down. 0.1 ml of appropriately diluted soil suspensions were spread on potato dextrose agar (PDA), nutrient agar supplemented with 0.5% tannic acid and mineral salt agar medium (g/L; 5.0, tannic acid; 3.0, $NaNO_3$; 1.0, KH_2PO_4, 0.5, $MgSO_4 \cdot 7H_2O$; 0.5, KCl and 0.01 $FeSO_4.7H_2O$) and incubated at 30 °C for 72 h. After incubation, plates were observed for fungal growth showing tannic acid hydrolysis by zone of clearance. Shake flask culture method was carried out for secondary screening of tannase producing microbes.

Tannase assay: Tannase activity was assayed by the modified method of Sharma et al. (2000). Firstly, 0.17 gm tannic acid was dissolved in 0.05 M citrate buffer. 0.1 ml of appropriately diluted crude enzyme was added and allowed for incubation at 55 °C in a boiling water bath for 20 min. After incubation period, reaction was stopped immediately by adding 0.2 ml potassium hydroxide. Then, 0.3 ml methyl rhodanine was added and diluted with 4.0 ml distilled water. The control tube was prepared by adding potassium hydroxide first with the substrate methyl gallate. The absorbance was measured at 520 nm. Standard calibration curve of gallic acid (0 – 200 µg) was prepared to measure the amount of gallic acid produced. One unit was defined as the amount of enzyme that produced one µmol of gallic acid per min under assay conditions.

Optimization of tannase production: The isolates HP-1, HP-6 and HP-13 showed the better tannase production were selected for studies. Optimization was carried out using one-factor-at-a-time method and the optimum conditions was provided in the subsequent experimental studies.

Preparation of inoculum: Spore suspension: To produce fungal tannase, young spore suspension of the isolates was prepared and used as an inoculum. The spore suspension was prepared by growing the isolated fungal cultures on potato dextrose agar slants for sporulation at 30 °C upto 4 days. The spores were harvested from slants by adding 10 ml of sterile distilled water containing 0.1% (v/v) Tween 80. The collected slant cultures were vortexed appropriately to obtain fungal spore suspension and then diluted appropriately to count the spores using Neuber's Chamber. The prepared suspension of spores was formerly used as an inoculum for the submerged tannase production.

Yeast culture: To prepare active culture of yeast, potato dextrose broth was inoculated and incubated at 30°C for 24 h. After incubation, absorbance of the culture was measured at 600 nm to get 1 O.D and the production media was inoculated with this freshly prepared inoculum.

Optimization of inoculum size: For mold isolates 1×10^7, 2×10^7 and 1×108 spore suspensions while for yeast isolate 1%, 3% and 5% (v/v) used as an inoculum in 50 ml tannic acid medium and incubated at 30 °C, 160 rpm for 48 h on a rotary shaker. After incubation, filtrate was extracted for tannase production. The inoculum size showing maximum tannase production was selected for additional studies.

Optimization of tannic acid concentration: Tannase production can be induced in the presence of tannic acid. Hence, different concentrations of tannic acid (w/v) viz., 0.25%, 0.5%, 0.75%, 1.0% and 1.5% were used to check the tannase production. All other components were kept same as tannic acid medium. The sterile media flasks were inoculated with fresh spore suspension of molds and activated culture of yeast. The inoculated culture flasks were incubated at 30 °C, 160 rpm on a rotary

shaker for 48 h. After incubation, the fungal biomass was separated and filtrate was collected to analyze the tannase production.

Effect nitrogen sources on Tannase Production: The effect of various organic and inorganic nitrogen sources were tested on tannase production. To check the effect of different nitrogen sources 0.5 %(w/v) of peptone, yeast extract, malt extract, ammonium sulphate and sodium nitrate were used keeping other components constant. The sterile media flasks were inoculated by freshly prepared spore suspension and yeast culture. The production flasks were incubated at 30 °C, 160 rpm on a rotary shaker for 48 h. The filtrate was collected by removing the fungal biomass and analyzed for tannase production.

RESULTS AND DISCUSSION

Isolation of tannase producing microorganisms: Total 22 fungal isolates (1 yeast and 21 molds) were obtained, on the basis of zone of clearance on potato dextrose agar (PDA), nutrient agar and mineral salt agar medium supplemented with 0.5 % (w/v) tannic acid. Zone of clearance produced by the fungal colonies on tannic acid agar plate is shown in Figure 1. The fungal isolates obtained were named as HP-1,....., HP-22. To maintain the isolates they were transferred on the potato dextrose agar slants and preserved at 4 °C by periodic transfer. *Aspergillus niveus* (KM613137.1) was isolated using minimal media supplemented with 0.1% (w/v) tannic acid for tannase production (Chaudhry et al., 2017). Kumar et al. (2014) reported 15 tannase producing fungal isolates by detection of zone of hydrolysis using minimal media supplemented with 1% (v/v) tannic acid.

Tannase production: Further screening of tannase producing fungal isolates was carried out using shake flask culture method. Sterile 50 ml tannic acid medium was inoculated with fungal isolates and incubated at 30°C, 150 rpm on a rotary shaker in 250 ml Erlenmeyer flask. The tannase production was checked after 24 h time interval up to 96 h. To estimate the tannase, filtrate was separated from the fungal biomass using Whatman No.1 filter paper and analyzed (Table 1).

Three isolates HP-1 (yeast), HP-6 (mold) and HP-13 showed maximum tannase production of 2.10, 3.99 and 2.10 U/ml, respectively. These three cultures were selected for further optimization studies. Five yeast strains were isolated and showing the highest tannase production of 8.2 U/mL was found with MP-10 isolated from tannery effluent-contaminated soil was identified as *Rhodotorula glutinis* MP-10 (Taskin, 2013).

Optimization of tannase production: Optimization of culture conditions were carried out for tannase production with one-factor-at-a-time method and the optimum conditions was provided in the subsequent experimental studies.

Optimization of inoculum size: The effect of different inoculum size was carried out with 1×10^7, 2×10^7 and 1×10^8 spore suspensions and 1%, 3% and 5% (v/v) yeast cultures as an inoculum. After incubation, tannase production was checked from the filtrate. The inoculum size showing optimum tannase production was selected further studies (Table 2).

Isolate HP-1 (yeast) showed tannase production of 2.45 U/ml with 5% (v/v) inoculum in 50 ml media incubated at 30 °C, on a rotary shaker (160 rpm) for 48 h. The molds, HP-6 and HP-13 showed highest tannase production of 4.59 U/ml and 2.62 U/ml, respectively with 1×10^8 number of spores as an inoculum in 50 ml media, at 30°C, 160 rpm on a rotary shaker for 48 h. The 20% (v/v) inoculum (1 ml = 3.13×106 spores) volume of Penicillium purpurogenum PAF6 was reported optimum for tannase production (Jana et al., 2012).

Optimization of tannic acid concentration: Effect of tannic acid (w/v) was checked with 0.25%, 0.5%, 0.75%, 1.0% and 1.5% concentrations keeping other components constant. Result of tannase production is shown in Table 3 at 30°C, 160 rpm on a rotary shaker for 48 h. For all the three selected isolates HP-1, HP-6, HP-13 the optimum tannic acid concentration was found as 0.75% (w/v) with 2.85, 5.04 and 2.70 U/ml tannase production, respectively.

Aspergillus niveus (KM613137) was isolated using minimal media supplemented with 0.1% (w/v) tannic

Table 1. Secondary screening of isolates

Isolate Type	No. of Isolates	Tannase Production (U/ml)
Yeast	HP-1	2.10
	HP-2	0.99
	HP-3	0.56
	HP-4	1.88
	HP-5	1.67
	HP-6	3.99
	HP-7	2.24
	HP-8	0.95
	HP-9	1.08
	HP-10	2.08
	HP-11	1.14
Molds	HP-12	0.67
	HP-13	2.10
	HP-14	1.18
	HP-15	0.78
	HP-16	1.89
	HP-17	0.90
	HP-18	1.45
	HP-19	2.07
	HP-20	1.34
	HP-21	1.50
	HP-22	1.89

acid as a sole source of carbon for tannase production (Chaudhry et al., 2017). Kumar et al. (2014) reported tannase producing fungal isolates using minimal media supplemented with 1% to 3% (v/v) tannic acid. *Penicillium purpurogenum* PAF6 showed maximum tannase production of 5.706 U/g with 4% (w/v) tannic acid concentration in solid state fermentation (Jana et al., 2012). *R. glutinis* MP-10 showed highest tannase with 20 g/L tannic acid concentration (Taskin, 2012).

Effect of nitrogen sources on tannase production: Various organic and inorganic nitrogen sources were used to check the tannase production. To check the effect of different nitrogen sources 0.5 %(w/v) of peptone, yeast extract, malt extract, ammonium sulphate and sodium nitrate were used. The culture broths were checked for tannase production after incubation. Among the different nitrogen sources tested, peptone (0.5% w/v) showed the maximum tannase production with all the three isolates. HP-1 showed 3.29 U/ml tannase production after 24 h whereas HP-6 and HP-13 reported 5.56 U/ml and 3.15 U/ml after 48 h, respectively.

Table 2. Effect of inoculum size

No. of spores / % (V/V)	Tannase Production (U/ml)		
	HP-1 (Yeast)	HP-6 (Mold)	HP-13 (Mold)
1×107 / 1%	0.42	2.29	1.44
2×107 / 3%	2.48	2.64	1.78
1×108 / 5%	2.45	4.59	2.62

Table 3. Effect of tannic acid concentration

Tannic acid (%)	Tannase Production (U/ml)		
	HP-1 (Yeast)	HP-6 (Mold)	HP-13 (Mold)
0.25	1.06	3.23	0.48
0.5	1.32	2.18	0.52
0.75	2.85	5.04	2.70
1.0	1.40	3.71	1.80
1.5	0.89	2.79	1.10

Table 3. Effect of nitrogen source

Nitrogen sources	Tannase Production (U/ml)		
	HP-1 (Yeast)	HP-6 (Mold)	HP-13 (Mold)
Peptone	3.29	5.56	3.15
Yeast extract	1.79	1.90	1.82
Malt extract	1.08	0.91	0.98
Ammonium sulphate	1.66	2.29	1.89
Sodium nitrate	1.40	2.01	1.75

Urea (1% w/v) was reported as the best nitrogen source with maximum yield of tannase (2.88 U/g) by Penicillium purpurogenum PAF6 in solid state fermentation (Jana et al., 2012). The maximum tannase production of 28.9 U/ml by P. atramentosum KM was reported with ammonium chloride as a nitrogen source (Selwal and Selwal, 2012).

CONCLUSION

Total 22 fungal isolates were obtained from collected soil samples. Based on their secondary screening three isolates HP-1 (yeast), HP-6 (mold) and HP-13 (mold) were successfully used for tannase production. Optimization of culture conditions like inoculum size, tannic acid concentration and nitrogen source have increased the tannase production to 3.1 U/ml, 5.56 U/ml and 3.15 U/ml for HP-1, HP-6 and HP-13, respectively. The tannase produced may be purified further and can be used in various applications including the production of gallic acid.

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Exploration of Marine Bacteria for their Exopolysaccharide (EPS) Producing Ability Along Various Regions of Gujarat Coast

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ABSTRACT

Marine bacteria are remarkably adapted to diverse environmental conditions because they meet a far greater diversity of habitats and ecological conditions. For adaptation in these conditions marine organisms developed new adaptive approaches like synthesis of secondary metabolites. Exopolysaccharides (EPSs) is one of the important secondary metabolites produced by marine organisms and play vital role in the formation, stabilization of soil aggregates and regulation of nutrients and water flow across plant roots through biofilm formation. With a view of isolation of novel EPS producers, various sites along Gujarat coastline were explored. Initially five marine water samples were collected from various sites such as Khambhat, Dhuvaran, Somnath, Bhavnagar and Diu. The microorganisms were enriched in Zobell Marine Broth supplemented with varying salt concentration 5-25% w/v NaCl. A total of 80 morphologically distinct CFU were obtained, purified and selected for further studies. Exopolysaccharide synthesis is characteristically observed as mucoid appearance of colonies, 16 such bacterial isolates were selected and screened on Congo red agar plates supplemented with 5% sucrose. Out of 16 isolates 4 isolates showed strong blackening on Congo red agar plates. Further the EPS production work is underway. Potent isolates will be further selected for use in various agricultural and biotechnological applications.

KEY WORDS: BIOFILM FORMATION, EPS, HALOPHILES, SECONDARY METABOLITES, ZOBELL MARINE BROTH.

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INTRODUCTION

Marine microbes are being highly explored because of their high diversity, complex physical, biological and metabolic systems. Marine derived enzyme and other bioactive compounds have unique activity that differs from enzyme produced by other bases because marine species have evolved mechanisms to survive in an enormously hostile environment in footings of light, salinity, and pressure as related with land (Jaspars et al., 2016). Marine environments are extreme conditions where organisms have developed new adaptive approaches like synthesis of secondary metabolites for survival in their surroundings. There are various industrially important secondary metabolites such as exopolysaccharides (EPSs), siderophores, biosurfactants, antibacterial, antiviral and anticancer compounds. Salinity stress is one of the key concern for upsetting production of several food plants (Qados and Amira, 2011) as different crop variabilities are extremely procumbent to salinity which affects production (Patel et al., 2018).

The native salt tolerant bacteria obtained during the investigation could be exploited for induced salt tolerance of crop vegetations or production of beneficial metabolites (Hanin et al., 2011). Marine biodiversity is not very known at present because only a minor fraction of the microorganisms can be cultivated (Hugenholtz, 2002; Delong et al., 2014). There are numerous reports on specific marine environments with high microbial diversity; this is the case for deep-sea hydrothermal vents in North Atlantic (Kreft et al., 2007). The next generation high quantity DNA sequencing (NGS) methodologies can offer a way to estimate the biodiversity and can be useful to drive some cultivation experiments when the analysis of metagenomic data sets reveals partial pathway for a given compound crucial for growth, this compound has to be added as an exogenous source (Rocha-Martin et al., 2014).

Exopolysaccharides are one of the vital secondary metabolites which are produced mainly by marine bacteria for survival in extreme marine habitat. These compounds mainly contain 40 - 95% of polysaccharides, proteins, nucleic acid, lipid and humic acid substances. Furthermost polysaccharides used in the industry are extracted from plants, algae and animals. A few of them come from bacteria and have several industrial applications in the paper, food, biotechnological, textile, ecological or health industry (Finore et al., 2014). The most famous examples of microbial used macromolecules are xanthan, alginate, dextran, cellulose, hyaluronic acid, gellan, curdlan, succinoglycan and levan (Kumar et al., 2007; Freitas et al., 2011). Overall, physical and bioactive features of polysaccharides are based on molecular chemical structure: the osidic sequence and linkages in the repeating unit, but also the substituents, affect the conformation and geometry of polysaccharide chains as well as polysaccharide-polysaccharide and polysaccharide-protein interactions (Powell et al., 2004; Pomin, 2009).

The chemical structure regulates the physical properties for example solubility in water or interactions with ions (Geddie and Sutherland, 1993; Kumar et al., 2007). The acetyl content in chitosan sulfate influences the inhibition of propyl endopeptidase which is intricate in cognitive dysfunctions and memory deficits (Je et al., 2005). The sulfate groups in heparin participate in the molecular structure and influence the binding with calcium cations (Chevalier et al., 2004). They also have an excessive effect on the anticoagulant activity (Franz and Alban, 1995; Garg et al., 2002; Huang et al., 2003; Liu and Pedersen, 2007). The antiproliferative activity of the heparin molecule depends on the molecular size but not on 3-O-sulfo group (Garg et al., 2002) and requires both N-acetylation and N-sulfation (Longas et al., 2003).

Exopolysaccharides are used in several industrial fields such as thickeners, stabilizers, in food products and as antitumoral, antioxidant and or prebiotic in pharmacology. A detailed compilation of various macromolecules from diverse microbes and their suitable industrial applications is summarized in (Table 1). A novel alternative to chemical as well as enzymatic modifications to acquire targeted polysaccharide chemical edifice could also be the genetic engineering. This will be possible if supplementary EPS structures are known together with biosynthetic genetic clusters by means of relationship between assembly and bioactivity. EPS biosynthesis pathway reconstruction is feasible with genome sequence and functional footnote and it can be engineered to obtain tailor-made polymers (Schmid et al., 2014).

MATERIALS AND METHODS

Study Site and Sample Collection: Water samples were collected from different sites such as Khambhat, Dhuvaran, Diu, Somnath and Bhavnagar coast, Gujarat India. Water samples were collected in sterile glass bottles. The samples were transferred to the laboratory within 6 hours and stored at 4^{0}C until further analysis.

Enrichment and Isolation: Inoculation of 5% (v/v) marine water sample was proceeded into flask containing Zobell Marine Broth (ZMB) medium supplemented with 5-25% (w/v) NaCl. The medium contained (g/L) peptone 5.0; yeast extract 1.0; $C_6H_5O_7$ 0.100; NaCl 19.450; $MgCl_2$ 8.800; Na_2SO_4 3.240; CaCl2 1.800; KCl 0.550; Na_2CO_3 0.160; KBr 0.080; SrCl2 0.034; H_3BO_3 0.022; Na_2SiO_3 0.004; NaF 0.0024; NH_4NO_3 0.0016; Na_2HPO_4 0.008; Final pH 7.6±0.2. The flasks after inoculation were incubated on rotatory shaker at 180 rpm for 72hrs at 37^{0}C emperature. The enriched culture was consecutively diluted to prepare aliquots ranging from 10^{-1} to 10^{-5} using distilled water as diluent and 100 µl from each aliquot were spread on ZMB agar plates which was supplemented with 5-25% (w/v) NaCl. After incubation at 37^{0}C for 24hrs morphologically diverse colonies were selected and subcultured to acquire pure culture of isolates.

Screening of isolates for EPSs producing Marine Bacteria: Those bacteria which are producing EPS were

screened on the base of colonial characteristic, string test and congo red agar test.

a. Cultural characteristics and string test: Mucoid colonies have a glistening and creepy appearance on agar plates were the primary criteria for assortment of bacteria (Fusconi and Godinho, 2002). The colonies when extended with wire loop form an extended filament (Vescovo et al., 1998). Exopolysaccharide production was established visually or through the string test (Fang et al., 2004) by formation of a string (>5 mm) upon lifting of the loop indicating positive result.

b. Congo red agar plate method: Biofilm formation by the isolates was checked by using CRA medium (Freeman et al., 1989). The isolates were streaked onto Brain Heart Infusion broth (BHI) supplemented with 5% sucrose and Congo red dye. The medium was composed of BHI (37 gm/L), sucrose (50 gm/ L), agar (30 gm/L) and congo red solution (0.8 gm/L). Congo red was prepared as concentrated aqueous solution and autoclaved at 121^{0}C for 15 minutes, separately from other medium constituents and was then added when the agar had cooled to 55^{0}C. Plates were incubated for 24 to 48hrs at 37^{0}C. The positive result confirmed by black and smooth/ mucoid type colony (Darwish and Asfour, 2013).

Table 1. Exopolysaccharides from microbes (adapted from Kumar et al., 2007; Freitas et al., 2011).

Exopolysaccharide	Source	Main applications
Xanthan	*Xanthomonas campestris*	Food industry as texturizing agent, petroleum industry, health care
Alginate	*Pseudomonas aeruginosa*, *Azotobacter vinelandii*	Food hydrocolloid, medicinal, in drug encapsulating agent
Dextran	*Leuconostoc mesenteroides*	In Food industry for food colors, biomedical as plasma volume expander biotechnological supports for separation
Cellulose	*Acetobacter xylinum*	Food industry, biomedical as artificial temporary skin, biotechnological separations as hollow fiber and membranes
Hyaluronic acid	*Streptococcus equi*	In Cosmetic products
Gellan	*Sphingomonas paucimobilis*	Food industry, biotechnology (culture medium gelification)
Curdlan	*Sinorhizobium meliloti*, *Agrobacterium radiobacter*, *Alcaligenes faecalis*	Textile and pharmaceutical industries, bioremediation
Succinoglycan	*Sinorhizobium meliloti*, *Alcaligenes faecalis*	Food and pharmaceutical industries, oil recovery
Levans	*Leuconostoc citreum*	Food industry (prebiotic)

Figure 1: Isolation on ZMB agar medium

RESULTS AND DISCUSSION

Isolation and Enrichment of marine bacteria: A total of 5 water samples were collected from 5 different sites of Gujarat, India. Further dilution and plating on ZMB agar medium supplemented with 5-25% NaCl were carried out. The total bacterial population of water samples as determined via colony forming units (CFU) varied considerably and declined progressively nevertheless of sampling sites with increasing NaCl concentration in the isolation medium. A total 80 different isolates were obtained from marine water samples on the basis of exclusive colonial characteristics on Zobell marine agar plate. Pure culture of isolate S-5-9-1 showed orange colored mucoid colonies (Figure 1).

Screening of isolates for EPS production: The primary screening of biofilm-forming EPS producing isolates

were based on the formation of mucoidal colonies. During the phase of primary screening out of 80 different isolates, 25 isolates producing mucoid colonies on nutrient agar plates supplemented with 5% (w/v) sucrose. The smooth/mucoid colony characteristics serve as the assortment criteria for exopolysaccharide producing bacteria (Fusconi and Godinho, 2002). All 16 isolates (Table 2) were screened for biofilm formation by string test and Congo Red Agar plate assay. In secondary screening formation of black colonies with dry crystalline consistency was observed on Congo red agar considered to be positive for biofilm formation (Figure 2).

Figure 2: Comparison of (a) Biofilm producer, (b) Control and (c) Non biofilm producer

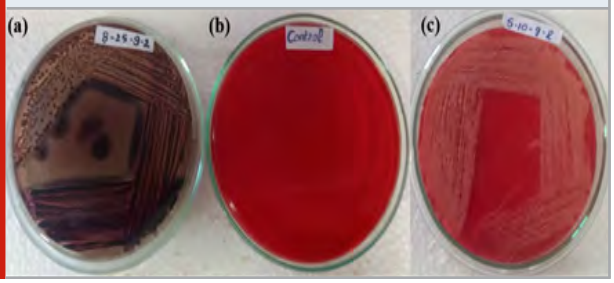
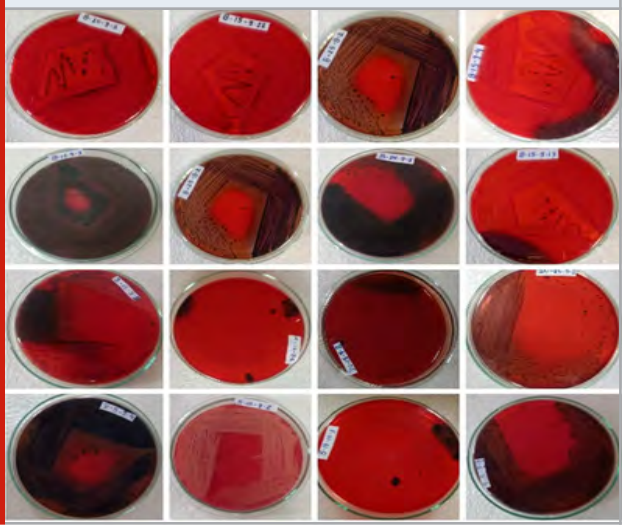


Figure 3: Screening of biofilm producers by Congo red agar medium



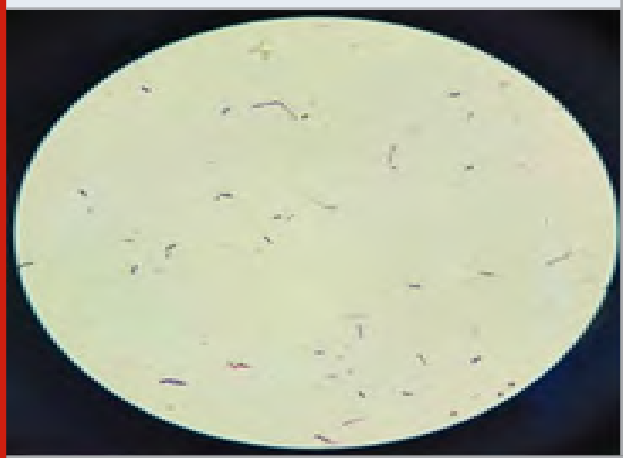
Out of 25 isolates, 16 isolates showed positive for biofilm formation (Figure 3). Among them 4 isolates B-15-9-3, B-25-9-2 and B-25-9-3 are highly potent for EPS production while isolate S-5-9-1 was showing very strong blackening (Table 2) due to biofilm formation within 24hrs and non-biofilm producer mostly produced pink or red colored colonies (Figure 2). Further these isolates were evaluated for their morphological and colonial characterization.

Morphological and Colonial characteristics: Morphological and colonial characteristics helps in partial identification of microorganism. The total 80 isolates were obtained from all the samples at different salt concentration. Maximum number of isolates were

Table 2: Results of screening for biofilm formation

Sites	Isolates	Biofilm formation after 24hrs at 37	Result on Congo Red agar plate	String test
Bhavnagar	B-10-9-5	Slightly Black	+	+
	B-15-9-3	Strong Black	+++	+++
	B-15-9-4	Slightly Black	+	+
	B-15-9-12	Slightly Black	+	+
	B-15-9-13	Black	++	++
	B-25-9-2	Strong Black	+++	+++
	B-25-9-3	Strong Black	+++	+++
Khambhat	K-10-9-2	Slightly Black	+	+
	K-15-9-8	Red	-	-
	K-15-9-2	Red	-	-
Dhuvaran	D-5-9-2	Slightly Black	+	+
	D-5-9-4	Slightly Black	+++	+++
	D-15-9-3	Black	++	++
	D-15-9-3	Red	-	-
Diu	DU-15-9-1	Black	++	++
	DU-15-9-2	Slightly Black	+	+
Somnath	S-5-9-3	Black	++	++
	S-15-10-3	Slightly Black	+	+
	S-5-9-1	Very Strong Black	++++	++++

Figure 4: Gram staining of isolate S-5-9-1



obtained from Bhavnagar and least isolates were obtained from Khambhat. Majority of isolates were colorless to white. Only 20 isolates out of 80 were pigmented. From Somnath two pigmented isolates were obtained showing positive for biofilm formation (Table 3). All four potent isolates B-15-9-3, B-25-9-2, B-25-9-3 and S-5-9-1 for biofilm formation showed Gram positive, short rods and arranged single. A representation of S-5-9-1 is shown in figure below (Figure 4).

Halophiles are isolated from widespread diversity of environments such as solar lakes, Dead Sea, saltern crystallizer and hypersaline lakes. Their enzymatic activity and stablity under assembly of extremities have been of key interest for researchers (Raval et al., 2014). In the Indian context, the manifestation and heterogeneity of the haloalkaliphilic bacteria from the man-made and natural saline habitats along the Coastal Gujarat in India have been investigated over the last several years (Dodia et al., 2006, 2008a, b; Joshi et al., 2008a ; Nowlan et al., 2006; Pandey and Singh 2012; Pandey et al., 2012). From Lonar Lake total 86 halophilic bacteria (Joshi et al., 2008b) screened besides reported *Vagococcus carniphilus* and *Halomonas campisalis* as potent EPS producers. The EPSs producing bacterial isolates were selected essentially on the basis of mucoidness of the colonies (Ruhman et al., 2015). The present study focuses on the distribution of halophilic and halotolerant bacterial communities across various regions of Gujarat coast. Total 05 water samples were analysed by dilution and then plating method and deliver information regarding the accessibility and diversity of halophilic bacteria in the marine habitat. A total of 80 isolates obtained in pure form showing degree of NaCl tolerance. During the course of screening total 16 isolates showed Exopolysaccharide (EPSs) producing ability.

Table 3. Colony characteristics of the isolates

Isolates	Size	Shape	Color	Colony characteristics				
				Margin	Texture	Opacity	Elevation	Appearance
B-10-9-5	Small	Round	White	Entire	Mucoid	Opaque	Raised	Shiny
B-15-9-3	Moderate	Round	White	Entire	Mucoid	Opaque	Raised	Shiny
B-15-9-4	Moderate	Round	Cream	Entire	Mucoid	Opaque	Flat	Shiny
B-15-9-12	Moderate	Round	Cream	Entire	Mucoid	Opaque	Raised	Shiny
B-15-9-13	Large	Round	White	Entire	Mucoid	Opaque	Raised	Shiny
B-25-9-2	Moderate	Irregular	White	Undulated	Mucoid	Opaque	Raised	Shiny
B-25-9-3	Moderate	Irregular	White	Entire	Mucoid	Opaque	Raised	Shiny
K-10-9-2	Small	Round	White	Entire	Smooth	Opaque	Raised	Dull
D-5-9-2	Moderate	Round	White	Undulated	Mucoid	Opaque	Raised	Dull
D-5-9-4	Large	Round	White	Entire	Mucoid	Opaque	Raised	Shiny
D-15-9-3	Moderate	Round	White	Entire	Mucoid	Translucent	Raised	Dull
DU-15-9-1	Small	Round	White	Undulated	Smooth	Transparent	Flat	Shiny
DU-15-9-2	Small	Round	Cream	Undulated	Smooth	Opaque	Flat	Shiny
S-5-9-3	Large	Round	Yellow	Entire	Smooth	Opaque	Flat	Shiny
S-5-9-1	Small	Round	Orange	Entire	Smooth	Opaque	Raised	Shiny
S-15-10-3	Large	Round	Cream	Undulated	Smooth	Translucent	Flat	Shiny

CONCLUSIONS

The research findings about the marine bacteria gave insight into the ecology of microbes, production and identification of bioactive compounds. This study was undertaken to screen halophiles to produce EPS. Sixteen isolates conferred a highly mucoid/smooth colony and potent for biofilm formation. Thus, further studies are needed to evaluate the potential application of the biofilm exopolysaccharides.

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Isolation, Production and Applications of Alkaline Protease from Hot Springs Bacterial Isolates

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ABSTRACT

Microbial proteases contribute 60% of the total enzymes of the world market. Present study was aimed to isolate and optimize the protease production from bacterial isolates of hot springs located in Tuwa, Gujarat, India. Total 12 bacterial isolates were screened on skim milk agar medium for proteolytic activity. Based on relative enzyme activity MNK-1, MNK-2, and MNK-3 were selected for submerged fermentation (SmF) and characterized by cultural, biochemical and microscopic features. Optimization of protease production with respect to production time, carbon source, nitrogen source, inoculum volume, pH and temperature were studied. Protease production of 0.49, 0.5 and 0.49 U/mL were observed with pH 8 at 37°C in shaking condition, respectively by MNK-1, MNK-2 and MNK-3 within 48 h. Highest protease production 0.55 U/mL was observed in casein as compared to other nitrogen sources such as yeast extract, meet extract, tryptone, sodium nitrate and peptone by MNK-2. Various carbon sources like starch, carboxyl methyl cellulose (CMC), glucose, Na-citrate and sucrose were tested for protease production among them CMC produced 0.55, 0.58 and 0.61 U/mL by MNK-1, MNK-2 and MNK-3. The protease production 0.9 U/mL was detected by inoculating 5% v/v inoculum of MNK-2. These three bacteria were able to produce protease in the pH range 6-10 and at pH 8.0 highest 0.9 U/mL protease produced by MNK-3 compared to MNK-1 and MNK-2. Proteases production were studied in range 20 to 65°C and at 45°C MNK-2 produced 1.1 U/mL protease after 48 h of incubation period. We also tested the crude enzyme for gelatin hydrolysis and silver extraction from X-ray films and as detergent additive in laundry.

KEY WORDS: ALKALINE PROTEASE, APPLICATIONS, HOT SPRINGS, OPTIMIZATION.

INTRODUCTION

Proteases are the essential constituents of all forms of life on earth including prokaryotes, fungi, plant and animal. Proteases have a high reactivity and stability at the alkaline pH range comprising approximately 60% of the total enzyme production throughout the world (Elumalai et al., 2020) and catalyze the hydrolysis of the peptide bonds within proteins. Overall, microbial extracellular proteases have rewards over intracellular one in terms of cost and availability. Increasing demand of proteases had led biotechnologist to explore novel source of proteases. Due to fast growth rate and ease of genetic engineering microbes have been chosen for large scale production of proteases and to generate novel enzymes with desired properties (Wilson and Remigio, 2012). Accordingly, the demand for highly active preparation of proteases with appropriate specificity and stability with a wide range of pH, temperature, organic solvents, presence of ions,

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etc. stimulates the continual searching novel enzymes from novel sources (Raval et al., 2014). Proteases are very important group of industrial enzymes and are used extensively in various industries including leather, textiles, detergents, cheese, meat tenderization, baking, dehairing, brewery, organic synthesis and waste water treatment (Sun et al., 2019).

Presently, the worldwide auctions of industrial enzymes are assessed at about $4.2 billion in value (Suberu et al., 2016). Proteases signify one of the 3 largest groups of enzymes and is projected to reach a global market of approximately $ 2.21 billion in terms of value by 2021 at a compound annual growth rate (CAGR) of 6% from 2016 to 2021. Out of proteases produced, microbial proteases account for the largest share in the market in terms of value, next the animal and plant source one to one (Proteases Market by Source, 2019).

MATERIALS AND METHODS

Materials: Casein was purchased from SRL, India. Glucose, sucrose, starch, tryptone, yeast extract, and peptone were obtained from Hi-Media, India. CMC was purchased from Merck India Ltd. All other chemicals used were of analytical grade.

Sample collection and enrichment: The samples were collected using sterile 1-liter glass bottles from 3 different hot Kunds of Tuwa, Panchmahal, Gujarat. The temperature and pH of the sites were measured at time of sampling by thermometer and pH strip, respectively and labeled it. The sample was enriched by using skim milk broth and incubated at 35°C for 48 h.

Screening for proteolytic activity of isolates: After enrichment, samples were serially diluted up to 10-9 times and 100 µL of sample was spreaded on casein agar media and incubated at 37 °C for 4-5 days. At time interval of 24 h, zone of casein hydrolysis and diameter of colony were measured and relative enzyme activity (REA) was calculated. Three isolates MNK-1, MNK-2 and MNK-3 showing more than 3.5 REA were selected for further experimental studies.

(REA= Diameter of zone of casein hydrolysis/Diameter of colony in mm). Based on REA, organisms were categorized into three groups showing excellent (REA>5), good (REA>2.0 to, 5.0) and poor (REA<2) producer of protease (Jani et al., 2016).

Cultural, morphological and biochemical characteristics: Based on zone of hydrolysis on casein agar plates, isolates were marked, labelled and observed to note down their colony characters. Colony characters taken in consideration were colony size, shape, elevation, margins, opacity, pigmentation, texture etc. and also studied morphological characteristics. The growth curve and optimum growth conditions like pH (6-10), temperature (20-70°C) and salt tolerance (0.5-5%) for isolates also have been checked.

The isolates were characterized for their biochemical activities. The biochemical tests carried out for the isolates were: catalase test, urea utilization test, lipid utilization test, starch hydrolysis test, H_2S production test, methyl red test, Voges-Proskauer test, citrate utilization test, indole production test, salt tolerance test, gelatin hydrolysis test, growth pattern in N-broth and carbohydrate (galactose, mannitol, dextrose, sucrose, arabinose, fructose, maltose, lactose) utilization test (Bergay et al., 1994).

Inoculum preparation: From their respective slants 100 µL of bacterial culture were inoculated in nutrient broth under aseptic condition and incubated on a shaker at 37°C, 120 rpm for 24 h. After incubation, optical density of the culture was measured at 600 nm and 1.0 ml of 1.0 OD cells were considered and inoculated as 1 % (v/v) inoculum.

Protease production: The following medium has been used throughout the study for protease production. The composition of production media – glucose 150 mg%, K_2HPO_4 20 mg%, KH_2PO_4 20 mg%, $MgSO_4$ 10 mg%, $CaCl_2$ 10 mg%, casein 200 mg%, $NaNO_3$ 100 mg%, Peptone 200 mg% (Rao and Narasu, 2007). The culture was grown at 37°C 100 rpm on shaking incubator for 48 h. The pH of the medium was adjusted to 8. At the end of fermentation, the broth was centrifuged at 10,000 rpm for 15 min and the supernatant was used for detection of protease.

Protease assay: Protease activity was determined by incubating 1 mL of the crude extract with 2 mL of 0.65% (w/v) casein in 0.2 M Glycine NaOH buffer (pH 8.6) at 55°C for 30 min by a modified Anson -Hagihara (1958) method of Folin-ciocalteu (Lowry et al., 1951). The reaction was stopped by adding 5 mL of 10% trichloroacetic acid (TCA), followed by centrifugation at 10000 rpm for 10 min. After the addition, 5 mL of alkaline copper reagent to 1 mL of the supernatant, 0.5 mL of Folin reagent was added to the mixture and left for 30 mins, after that the tyrosine liberated was measured at 750 nm. One unit of protease activity was defined as the amount of enzyme that released 1 µg tyrosine per min. A control was run in the same way, except the protease was added after the addition of 5% (w/v) TCA.

Optimization of fermentation conditions for protease production in SmF: Various culture condition like incubation time, carbon source, nitrogen source, inoculum size, pH and temperature were optimized for protease production of MNK-1, MNK-2 and MNK-3.

Incubation time: The production medium was inoculated with 3 ml of fresh culture suspension of selected isolates of optical density 1.0 at 600 nm and put at different incubation time 24, 48, 72, 96 and 120 hrs. The supernatant was collected after centrifugation at 10000 rpm for 15 min and used as crude enzyme for measurement of protease productivity.

Carbon source: The effect of various carbon sources on alkaline protease production by the isolates, glucose in the production medium was substituted with other carbon sources, including 1%(w/v) starch, carboxy methyl cellulose, sucrose, sodium citrate. The protease production was monitored after 48 h incubation at 35°C, under shaking conditions (100 rpm).

Nitrogen source: The effect of different nitrogen sources on the bacterial growth and alkaline protease production was investigated by substituting different organic and inorganic nitrogen source such as yeast extract, meat extract, tryptone, peptone and $NaNO_3$ in the production medium. The growth and protease production were monitored after 48 h incubation at 35±2°C, under shaking conditions (100 rpm).

Inoculum size: The effect of different inoculum size on alkaline protease production was investigated by adding 1%, 3% and 5% inoculum having optical density 1.0 at 600 nm and incubated at 35±2°C, 100 rpm on an environmental shaker for 48 h. Protease production was measured from the samples withdrawn periodically.

pH: Effect of pH like 6.0, 7.0, 8.0, 9.0 and 10.0 were inoculated with 5.0 ml fresh suspension of MNK-1, MNK-2 and MNK-3 of optical density 1.0 at 600 nm put on environmental shaker at 35±2°C, 100 rpm for 48 h. The supernatant was collected after centrifugation at 10000 rpm for 15 min and protease production was

Temperature: 5 ml of fresh culture suspension of MNK-1, MNK-2 and MNK-3 of optical density 1.0 at 600 nm were inoculated in production medium and incubated for 48 h at different temperature like 20°C, 30°C, 45°C and 65°C. Subsequently after 48 h supernatant was collected after centrifugation at 10,000 rpm for 15 min and protease production were measured.

Applications Compatibility of crude enzyme with detergent: Application of protease from isolates as a detergent additive was studied to check washing performance of protease on white cotton cloth pieces (10×10 cm) stained with dal fry. The following sets were prepared for the study.

A. Control prepared by cloth stain by dal fry with 300 mL water.
B. Cloth stain by dal fry plus 5 mL crude enzyme with 300 mL water.
C. Cloth stain by dal fry plus 5 mL crude enzyme plus 5 mL 1% detergent with 300 mL water.
D. Cloth stain by dal fry plus 5 mL 1% detergent with 300 mL water.

The systems were kept at room temperature for 30 min. After incubation, cloths pieces were taken out, rinsed with water and dried. Visual examination of various pieces were done and results were note down.

Table 1. Cultural and morphological characters of protease producing isolates

Isolate	Size	Shape	Margin	Elevation	Texture	Opacity	Pigment	Motility	Gram staining
MNK-1	Small	Irregular	Uneven	Flat	Smooth	Opaque	Creamy pink	Motile	Positive
MNK-2	Big	Irregular	Uneven	Flat	Smooth	Opaque	Creamy yellow	Motile	Positive
MNK-3	Big	Round	Even	Flat	Smooth	Opaque	Orange	Motile	Positive
MNK-4	Big	Irregular	Uneven	Flat	Smooth	Opaque	Off white	Motile	Positive
MNK-5	Medium	Round	Uneven	Raised	Smooth	Opaque	white	Motile	Positive
MNK-6	Small	Round	Even	Slightly raised	Smooth	Opaque	Yellow	Motile	Positive
MNK-7	Big	Round	Uneven	Raised	Rough	Opaque	White	Motile	Positive
MNK-8	Small	Round	Uneven	Flat	Rough	Opaque	White	Motile	Positive
MNK-9	Small	Round	Uneven	Slightly raised	Rough	Opaque	Off white	Motile	Positive
MNK-10	Big	Irregular	Uneven	Flat	Rough	Opaque	Off white	Motile	Positive
MNK-11	Medium	Round	Even	Flat	Rough	Opaque	White	Motile	Positive
MNK-12	Big	Round	Uneven	Slightly raised	Smooth	Opaque	White	Motile	Positive

Gelatin hydrolysis and recovery of silver from X-ray film: Used X-ray films were washed with distilled water and wiped with cotton impregnated with ethanol. The washed film was dried in an oven at 45°C for 30 min. X-ray film (2×2 cm pieces) was incubated at 65°C with 1% (1.1 U/mL) of crude protease in different petri plates in glycine NaOH buffer of pH 8.6 for 60 min such that the film is completely immersed in the solution (total volume 20 ml). The results were recorded at an interval of 20 min. Control was prepared using buffer without enzyme addition (Nakiboglu et al., 2003).

RESULTS AND DISCUSSION

Isolation of bacteria: Screening of alkaline protease producing bacteria from hot springs of Tuwa were carried out using alkaline skim milk agar medium. Out of twenty isolates, total twelve potent Bacilli and

Cocci were selected showing zone of casein hydrolysis surrounding their colonies. All were named as MNK-1 to MNK-12, alkaliphilic and having diverse morphological characters (Table-1). The metagenomics study indicate that proteolytic bacteria were present in hot springs, Tuwa (Mangrola et al., 2015).

Screening for proteolytic activity of isolates: Based on morphology and cultural characteristics, it was confirmed that the isolates MNK-1 to MNK-12 were Bacilli and cocci that producing good amount of alkaline protease on solid media (Table-2). This was confirmed by performing REA. On the basis of REA size of colonies were marked and colony characteristics were noted and Gram staining of all 12 isolates were performed and presented in Table-1. Most of them were having REA more than 2.5 except MNK-5 and highest REA 7.5 followed by 4.75 and 4.1 were observed respectively, for MNK-2, MNK-1 and MNK-3.

Table 2. Relative enzyme activity of isolates

Isolates	Zone of hydrolysis (mm)	Diameter of colony (mm)	Relative enzyme activity (24 h)
MNK-1	1.9	0.4	4.75
MNK-2	1.5	0.2	7.5
MNK-3	2.9	0.7	4.14
MNK-4	2.4	0.95	2.52
MNK-5	2.5	1.8	1.38
MNK-6	2.2	0.6	3..66
MNK-7	2.0	0.9	2.22
MNK-8	1.5	0.45	3.33
MNK-9	2.4	1.1	2.18
MNK-10	1.67	0.6	2.78
MNK-11	1.7	0.75	2.26
MNK-12	1.9	0.5	3.8

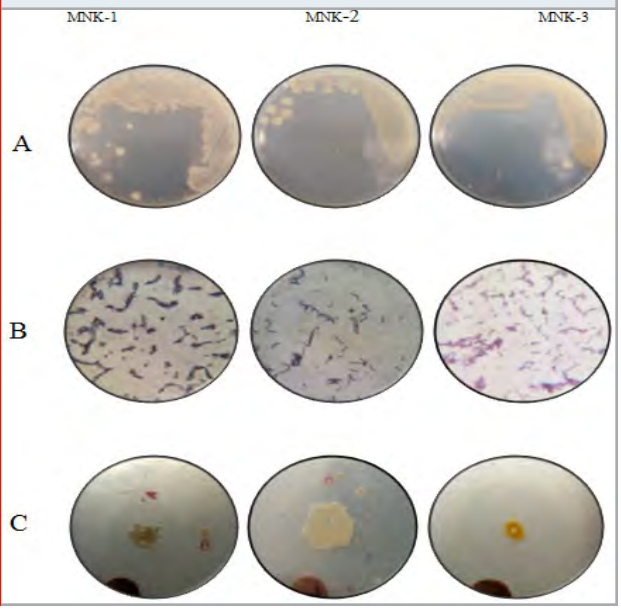
Cultural, morphological and biochemical characteristics: Results of Gram staining and biochemical tests carried out for selected isolates MNK-1, MNK-2 and MNK-3 are presented in figure:1 Table: 3. The isolates MNK-1 was Big, irregular, uneven, flat, smooth, opaque, creamy pink, motile, Gram positive, non-capsulated, spore former, MNK-2 was big, irregular, uneven, flat, smooth, opaque, creamy yellow, motile, Gram positive, non-capsulated, spore former where as MNK-3 was Big, round, even, flat, smooth, opaque, orange, motile, Gram positive, capsulated and non-spore forming bacteria that able to utilize various sugars as carbon source and all were metabolically diverse in nature.

Various growth parameters of isolates were studied like, growth curve, effect of pH and osmotic pressure on the growth. Growth curve indicated that all isolates grow best within 20 h and produce protease in its late stationary phase of life cycle and isolates grow best at pH 8 with range of pH 6 to 10 indicating that isolates were alkaliphilic. When effect of osmotic pressure on growth of isolates were studied, it was found that all grow maximum in presence of 1.0% NaCl with the range of 0.5% to 5.0% of NaCl. Results indicate that isolates produced protease in stationary phase of growth, similar kind of results were reported for *Bacillus* SN1, *Bacillus* SN2 and *Bacillus licheniformis* by Agrahari and Wadhwa, 2010 (Jani et al., 2016).

Table 3. Biochemical tests

Biochemical Test	MNK-1	MNK-2	MNK-3
Starch hydrolysis	Positive	Positive	Positive
Gelatine hydrolysis	Positive	Positive	Positive
Cellulose hydrolysis	Positive	Positive	Positive
Lipid hydrolysis	Positive	Positive	Positive
MR test	Negative	Negative	Negative
VP test	Negative	Negative	Negative
Indole test	Negative	Negative	Negative
Citrate utilization	Positive	Positive	Positive
H2S production test	Negative	Negative	Negative
Catalase test	Positive	Positive	Positive
Urea utilization	Negative	Negative	Negative
6 % Salt tolerance	Negative	Negative	Negative
Carbohydrate fermentation Test			
Sucrose	Negative	Positive	Negative
Dextrose	Negative	Positive	Positive
Mannitol	Positive	Positive	Positive
Arabinose	Negative	Negative	Negative
Maltose	Positive	Positive	Positive
Lactose	Negative	Positive	Positive
Galactose	Negative	Negative	Positive
Fructose	Positive	Positive	Positive

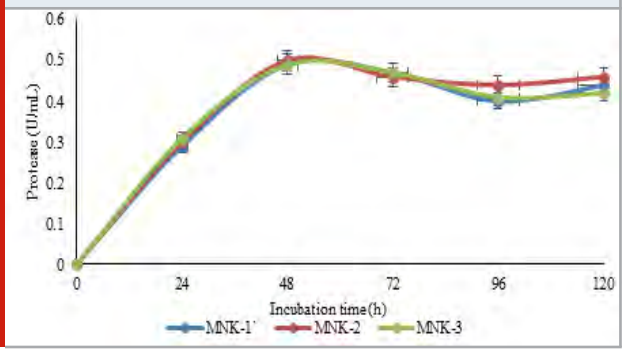
Figure 1: A. Pure culture of isolates after 24 h B. Gram staining C. Spot test on Casein agar plate after 48 h



Optimization of fermentation conditions for protease production in SmF

Incubation time: The cell growth and alkaline protease production were studied at various incubation period. The results indicated that the maximum production of enzyme was observed till 48 h. The results of enzyme production is presented in Figure: 2 in which it was indicated that incubation period for best production was 48h for all three isolates. The maximum protease production 0.5 U/ml was observed for MNK-2 and MNK-1

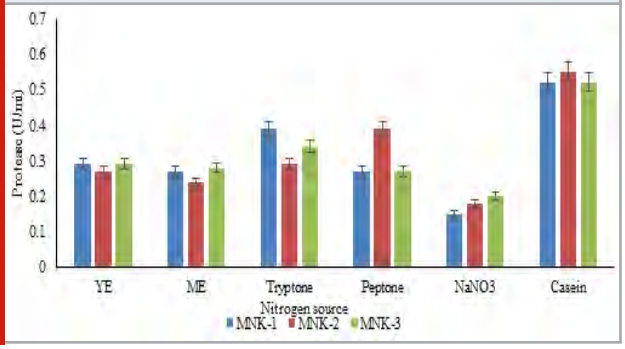
Figure 2: Effect of incubation time



and MNK-3 showed 0.49 U/mL protease production after 48h incubation at 37°C.

Nitrogen source: Nitrogen sources in the production medium regulate enzyme synthesis in the bacteria (Patel et al., 2005). Among the nitrogen sources tested in the medium, several organic sources supported both growth and enzyme production, with maximum yield in medium containing casein, followed by yeast extract, and other organic nitrogen sources, respectively. Tryptone and casein also serve as excellent nitrogen sources by many microorganisms for maximum protease production (Jayasree et al., 2009). Casein was used by MNK-1, MNK-2

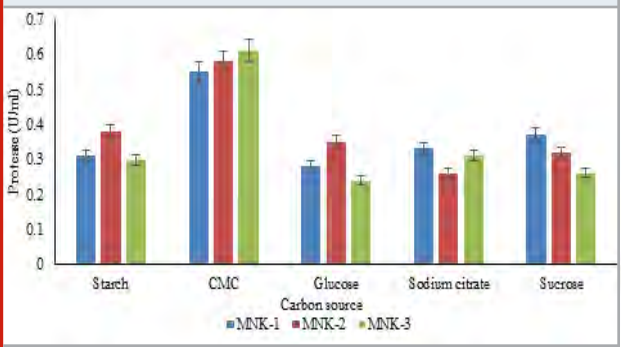
Figure 3: Effect of Nitrogen source



and MNK-3 giving maximum protease production 0.52, 0.55 and 0.52 U/mL respectively (Figure: 3).

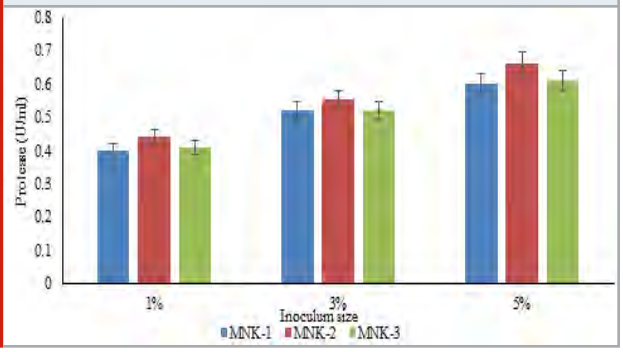
Carbon source: The influence of different carbon sources, including monosaccharides, disaccharides and polysaccharides, on the cell's growth and production of alkaline protease was investigated. We are reporting first time that carboxy methyl cellulose (CMC) was found to support maximum protease production with an activity

Figure 4: Effect of Carbon source



of MNK-1, MNK-2 and MNK-3 0.55, 0.58 and 0.61 U/mL, respectively (Figure: 4).

Figure 5: Effect of Inoculum volume



Inoculum size: To determine the effect of inoculation size, 100 ml of the production media were inoculated, with 1%, 3% and 5% (v/v) of the first subculture of each isolates. Isolates MNK-1, MNK-2 and MNK-3 produced 0.7, 0.9 and 0.75 U/mL by 0.60, 0.66 and 0.61 U/mL protease by 5% of inoculum volume in production medium (Figure: 5). An inoculation size of 2–5% range was an optimum value for *Bacillus* type of species reported in the literature, which supported our finding (Gençkal & Tari, 2006). Based on Agrawal et al., 2004 production medium with inoculating 5% culture fungi produced less amount of protease compared to bacteria.

pH: Generally commercial proteases from microorganisms have maximum activities in the alkaline pH range of 8.0 to 12.0 (Genckal and Tari, 2006). The protease enzyme was most stable at pH 8.0 where residual activity was nearly 90% after 24 h of incubation (Wilson & Remigio, 2012). In the present study, maximum protease production was observed in pH range 6 to 10, and found that pH 8 was optimum and produced 0.8, 0.8 and 0.9 U/mL protease by MNK-1, MNK-2 and MNK-3, respectively (Figure: 6). This protease production ability is also found similar in *Bacillus subtilis* B22 (Elumalai et al., 2020).

Temperature: Temperature is one of the most critical parameters for the production of extracellular enzyme (Chi et al., 2007). The effect of different incubation temperatures on protease production were evaluated and results showed that 45°C was the most favorable temperature for alkaline protease production. The

Figure 6: Effect of pH

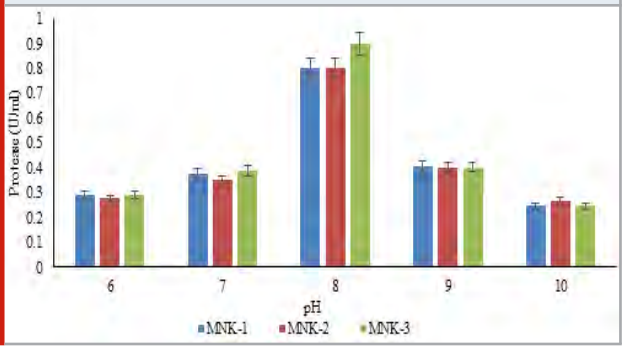
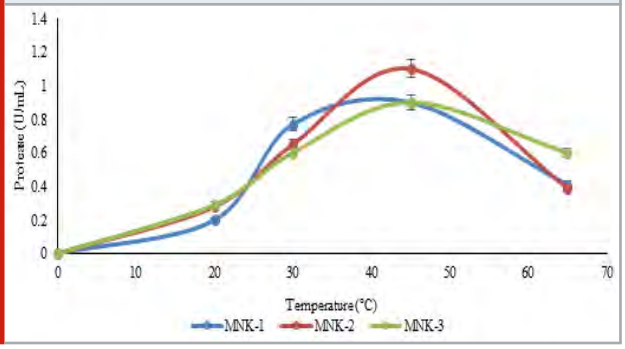


Figure 7: Effect of Temperature



maximum protease production was observed 1.1 U/mL in MNK-2 followed by 0.9 U/mL for both MNK-1 and MNK-3 (Figure: 7).

Applications Compatibility of crude enzyme with detergent: According to the literature, some bacterial based protease enzymes are also efficient in the removal of stain from cotton cloths (Hammami et al., 2020). Protease from *Bacillus* strain B13 was also reported for removal of chocolate stain and compatibility with commercial detergent (Gupta et al., 2002). Washing performance of protease of MNK-2 was studied on white cotton cloth pieces (10×10 cm) stained with Dal fry. After 30 min of incubation at room temperature the detergent solution supplemented with the enzyme was able to remove the stains completely, while the detergent solution only could not remove it. The best washing performance was recorded by washing with only enzyme, followed by washing with detergent, washing with enzyme plus detergent and washing with only water. Same kinds of studies were also reported for proteases from *Siplosoma obliqua, Bacillus brevis, Bacillus cereus* (Kanmani et al., 2011).

Gelatin hydrolysis and recovery of silver from x-ray film: To determine the efficiency to hydrolyze the gelatinous coating on X-ray film, 1.1 U/ml of MNK-1, MNK-2 and MNK-3 crude protease were incubated at 65°C with used X-ray films. Hydrolysis was complete within 2 h (Figure: 8). It was observed that enzyme started hydrolyzing gelatinous coating within 30 min incubation at 65°C. Finally, after 2h, it was found that 100% gelatin coating was hydrolyzed and the film was transparent

Figure 8 Hydrolysis of gelatin coating at time interval: X-ray film after, 2h incubation with crude enzyme at 65°C by MNK-1, MNK-2 and MNK-3

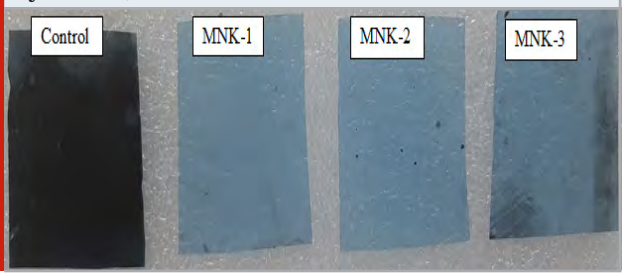
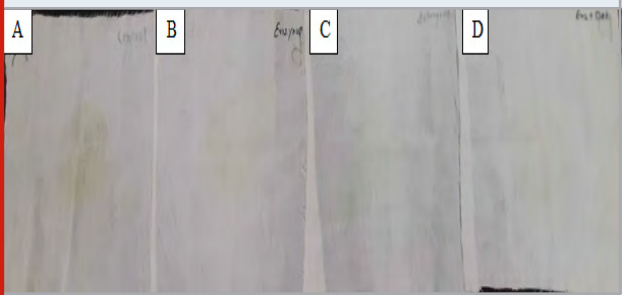


Figure 9: Washing performance of MNK-2 protease on white cotton cloth pieces (10X10 cm) stained with food at 37°C after 30 min incubation

A. Control B. Crude enzyme only
C. Detergent D. Crude enzyme plus Detergent



and clean. The waste X-ray/photographic films contain 1.5 – 2.0 % (w/w) black metallic silver which can be recovered and reused (Jani et al., 2016).

CONCLUSIONS

Present study represent that efficient thermostable alkaline protease was produced by thermotolerant bacteria. Production was optimized with respect to biotic and abiotic parameters. Organisms were utilized carboxy methyl cellulose for the maximum protease production. All this information could be used to design media for large scale protease production. Thus, protease from isolates can be used as detergent additive in commercially available detergent and it has been ability to hydrolyze gelatin and extract silver from used X-ray film.

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Screening, Production and Characterization of Fungal Laccases

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ABSTRACT

Laccases are copper containing enzymes involved in the degradation of lignocellulosic materials and used in the treatment of phenol containing wastewater. In present research work total 6 fungal isolates producing laccases were screened on Potato Dextrose Agar medium enriched with 4 mM tannic acid and 3mM ABTS from soil as well as decaying wood samples collected from various sites of Ahmedabad. Confirmation of laccase production was done by Bavendam test which is a plate assay technique using 3 mM guaiacol. Isolates giving best results were processed further for production of laccase using Solid State Fermentation and Submerged fermentation. Initial laccase production was done using liquid culture medium consisting 2% wood chip and pH 5.5. The highest enzyme activity of 2.82 U/ml was given by isolate SM3. Effect of different natural carbon source were examined using saw dust, wood chip, wheat straw on laccase production. Characterization of fungal isolates was done by morphological analysis. This study showed that enzyme production can be increased by media manipulation in the fungal cultures. Optimization of the media condition can maximize the desired enzyme production. Dye decolorization assay was been performed using 100 mg/l of phenol red and methylene blue, where dye decolorization efficiency showed the ability of laccase to decolorize synthetic dyes.

KEY WORDS: LACCASE, ABTS, GUAIACOL, TANNIC ACID, BAVENDAM TEST.

INTRODUCTION

Lignin, cellulose, and hemicellulose are major compounds present in plant residues. Lignin having high molecular mass, three-dimensional macromolecule, it consists of phenylpropane units (having vanillyl, syringyl and cinnamyl alcohols) in variable proportions specific to plant groups (Adler, 1977; Hedges and Mann, 1979; Kögel, 1986; Kögel-Knabner et al. 2002).It has complex cross-linked structure and so its resistance against chemical and biological decomposition (Hofrichter and Steinbüchel, 2001; Martinez et al., 2005), this enzyme has large diversity are involved in the degradation, of which ligninolytic exoenzymes, namely lignin and manganese peroxidases and laccases, are to date predominantly investigated (Allison et al., 2007).

The use of enzyme in the diverse field of industrial application exhibit greater importance in recent years. Many potential enzymes are distributed in nature; laccase is one of them which is oldest and most studied enzymatic system. Laccase is currently the focus due to its attention because of its diverse applications such as dye

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decolourization, waste detoxifications and bioremediation applications. Laccases catalyse the oxidation of a broad range of substrates such as ortho and para-diphenols, methoxy-substituted phenols, aromatic amines, phenolic acids and several other compounds. Laccase coupled to the reduction of molecular oxygen to water with one electron oxidation mechanism (Atallah et al., 2013).

Laccase is widely distributed in higher plants, fungi and bacteria (Benfiled et al., 1964; Diamantidis et al., 2000). Laccases are secreted out in the medium extracellularly by several fungi during the secondary metabolism Fungi belong to Deuteromycetes, Ascomycetes as well as Basidiomycetes are known producers of laccase (Gochev and Krastanov et al., 2007; Sadhasivam et al., 2008). The role of laccases in lignin and phenolic compound degradation has been evaluated in a large number of biotechnological applications such as dye degradation, bioremediation of some toxic xenobiotics (e.g. chlorinated aromatic compounds, polycyclic aromatic hydrocarbons, nitroaromatics, and pesticides), and biosensor developments (Gunne M et al, Shrestha et al.). In addition, laccases have been used in food industry for wine and beer stabilization, fruit juice processing, and different food-related biosensors (Gunne M et al). Use of industrial and agricultural wastes for laccase production by white-rot fungi is an effective way to reduce the cost of production (Y. Ferry et al, R. Mustafa et al).

In addition, laccase-mediated delignification allows to increase the nutritional value of agro industrial by-products for animal feed or soil fertilizer. To accomplish the transformation of these agro-industrial wastes, submerged and solid state cultures have been conducted. Submerged fermentations are used in industrial process; although they have drawbacks due to physical space, and energetic and water requirements (V.S. Valeriano et al). Also, solid state cultures present several advantages. Like mimicking the natural habitat in which the microorganism grows, reduced water activity that reduce microbial contaminations, and limited water consumption and equipment size. Also, it has been reported that solid-state cultures present higher volumetric yield, less energy requirements, and higher end-product stability and concentration than submerged cultures (R.C. Minussi et al, A.I. Yarpolov et al). Nevertheless, there are important issues related to heat and mass transfer that must be overcome in order to scale up a solid-state culture, in addition to accrued estimation of the biomass and recovery of the end product (S. Chawla et al). This study focus on the screening of fungal laccases collected from the local region, which also includes the low-cost media formulation and process parameters optimization of media components. The experiment was designed to evaluate screened media components and other parameters for optimized laccase production and its application in decolorization of dyes.

MATERIALS AND METHODS

Sample collection: Decayed wood, bark and tree samples for isolation of fungal laccases producers were collected from various site of Ahmedabad. The samples were collected in sterile plastic bags swapped using alcohol and were sealed and brought to the lab aseptically for further processed inside laminar air flow.

Isolation and screening of fungi for laccase production: The samples were homogenized and suspensions were made with dilution up to 10^{-5} in sterile distilled water. The prepared suspensions were used for inoculating onto the plates. Plate assay method was used for primary screening of fungal laccases using Potato Dextrose Agar medium consisting 4 mM tannic acid as substrate and incubated at room temperature for 3 days. The production of laccase enzyme was indicated by the formation of dark brown coloured halo around the fungal colonies. The isolates showing positive results were used for further studies. Another plate assay method was used for primary screening of fungal laccases using Potato Dextrose Agar medium consisting 3 mM ABTS (2-2'-Azino-bis-[3-ethyl benzthiazoline-6-sulfonic acid]) as substrate and incubated at room temperature for 3 days. The production of laccase enzyme was indicated by the formation of green coloured halo around the fungal colonies.

Bavendam test: Confirmation of laccase production was done by Plate assay technique using 10 mM guaiacol as a substrate. The colour change from colour less to reddish brown colour was to be observed in fungal culture plate for presence of laccase (Soponsathien, 1998).

Production of laccase enzyme: Laccase production was done using submerged fermentation and solid state fermentation.

Submerged fermentation: All experiments were carried out in 250-ml Erlenmeyer flasks containing 100 ml liquid culture medium for submerged fermentation. The liquid culture medium contained (g/1 distilled water): 4.5 wood chip, 1.5 yeast extract, 1 glucose, 0.5 ammonium sulphate and 100 ml salt solution. The salt solution contained 2 g KH_2PO_4, 0.5 g $MgSO_4$.$7H_2O$, 0.1 g $CaCl_2$. 2H2O and 0.5 g KCl dissolved in 1 l tap water and with pH 5.0. The medium was autoclaved at 121°C for 15 min. Each flasks were inoculated using an agar disc of 10 mm diameter cut from an actively growing fungal culture of 4 days incubation at 30 °C. The flasks were incubated at 30 °C on a rotary shaker (130 rpm) and laccase activity was measured at interval of 24 h for 12 days.

Solid state fermentation: Solid state fermentation was done using 1 L Erlenmeyer flask consisting 50 g wheat straw, and 100ml of basal media. The basal medium contained mineral salt solution that is 2 g KH_2PO_4, 0.5 g $MgSO_4$. 7H2O, 0.1 g $CaCl_2$. $_2H_2O$ and 0.5 g KCl dissolved in 1 l tap water. The pH was adjusted to 5.0. The flasks were autoclaved at 121°C for 30 min. After sterilization and cooling, agar plugs (10 mm diameter) were taken from the PDA plates containing 7 days old cultures of fungal isolate inoculated in the solid media. Incubation was carried out at 30°C in a static condition for 21 days. After cultivation for 21 days the flasks were harvested by adding 50 ml sterilized distilled water and keeping

on shaker for one hour. After one hour the content was filtered then centrifuged and the supernatant was analysed for laccase activity.

Laccase activity: The laccase activity was measured by spectrophotometer using guaiacol as a substrate .Crude enzyme extract from the liquid culture medium was collected under aseptic condition. The reaction mixture consists of 3 ml of 100 mM of guaiacol dissolved in 10% acetone in sodium acetate buffer and 1 ml of culture filtrate (crude laccase). The mixture was incubated at 28°C for 60 min and the absorbance was recorded at 470 nm. One unit of laccase activity was defined as the amount of enzyme catalyzing the substrate (guaiacol) for the production of 1 µl of colored product per min per ml (Collins and Dobson, 1997). Laccase activity was measured as decrease in absorbance of Guaiacol reagent (substrate) due to laccase enzyme (1ml) in 60 min (Hadri, S.H et al). It was calculated by the following formula;

$$IU/ml = \frac{\text{Decrease in the absorbance of guaiacol reagent}}{\text{Incubation period}} \times \text{Dilution factor}$$

Characterization of fungal laccase: The fungal isolate SM3 showed highest enzyme activity, was processed for enzyme characterization. Enzyme characterization was done using temperature (30°C, 40°C, and 50°C) and incubation time of (1h, 2h, 3h). Enzyme activity was measured at the interval of 24 h.

Cultural and morphological characterization of fungal isolates: The fungal isolate was further subjected to the colony characteristics and morphological characteristics using staining technique using bromophenol blue as stain and observation under low power and high dry objective lens.

Optimization of media components: Optimization was done using different natural carbon sources which include sawdust, wood chip and wheat straw. Mineral salt medium with 2% of carbon source were used for optimization. All the flasks with different carbon sources were inoculated with fungal culture and kept on rotary shaker at 150 rpm at room temperature. Enzyme activity was measured at the interval of 24 h till 11 days.

Dye decolorization experiments: Phenol red and methylene blue were used for dye decolorization assay, 100 mg/l dye was added in 100 ml mineral salt medium and was sterilized using autoclave. The flasks were inoculated by 10 mm of agar plug of fungal isolate and control was kept un inoculated. Fungal isolate was grown for 4 days. The flasks were incubated at 28 °C for 72 h. Dye content was monitored photometrically at 540 nm and 665 nm for phenol red and methylene blue respectively using spectrophotometer, which are the maximum visible absorbance of this dyes. Decolorization efficiency was been measured further. Percent decolorization of dyes was calculated by considering the optical density of control flasks as 100 % (0 % decolorization).

Figure 1: Screened fungal isolates

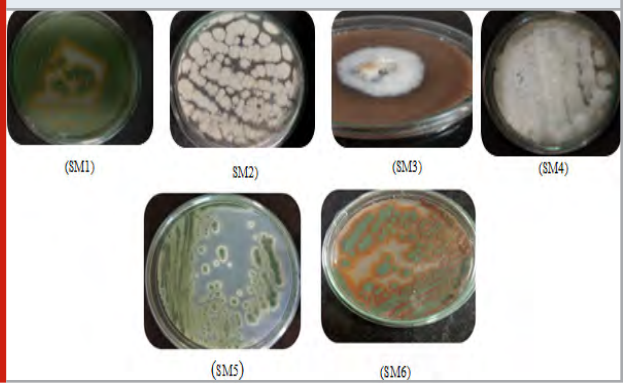
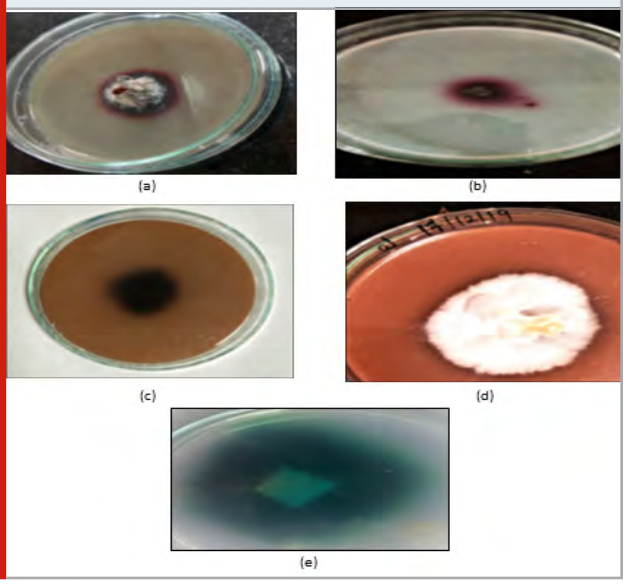


Figure 2: Various substrates used for screening of laccases, a,b) front and back morphology of guaiacol screening-reddish brown oxidation zone c , d) front and back morphology of tannic acid screening-dark brown oxidation zone , e) back morphology of ABTS screening dark green zone



RESULTS AND DISCUSSIONS

Isolation and screening of laccase producing fungi: The laccase producing fungi were isolated from various samples of decayed wood and bark of trees. It was inoculated on PDA medium supplemented with guaiacol having concentration 4 mM as a substrate. The reddish brown oxidation zone was developed around 6 fungal colonies shown in figure-1 namely SM1, SM2, SM3, SM4, SM5, SM6. Based on the initial higher laccase activity, 3 fungal isolates SM1, SM2, SM3 were selected for the further study. The fungus SM1, SM2, SM3 showing potential oxidation zone were further screened for laccase production using 3 mM ABTS (2-2'-Azino-bis-[3-ethyl benzthiazoline-6-sulfonic acid]) and 4mM tannic acid as a substrate. In plate assay method the dark green color was developed by all the three isolates in medium containing ABTS as a substrate and dark brown color was

developed for plate containing tannic acid as a substrate as shown in fig (2). The screening results confirm that isolated fungus are able to produce laccase enzyme.

Figure 3: (a) (b) - Laccase production using submerged fermentation and solid state fermentation

Figure 4: Laccase production using submerged fermentation

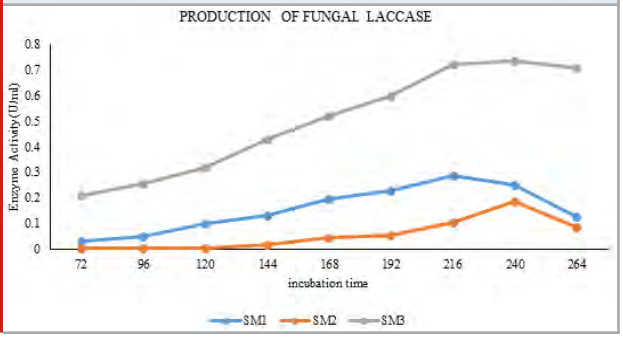
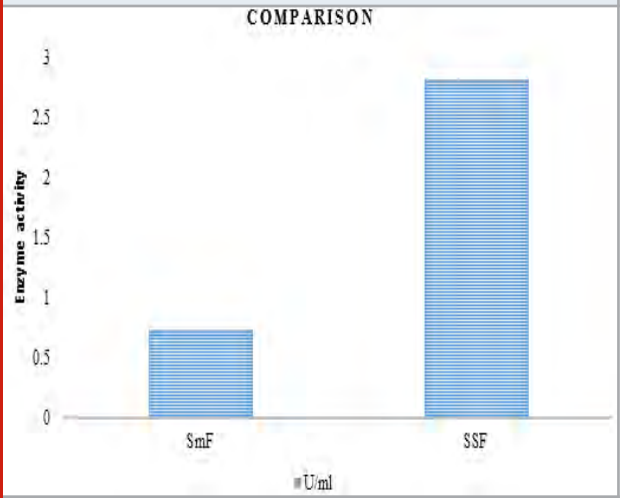
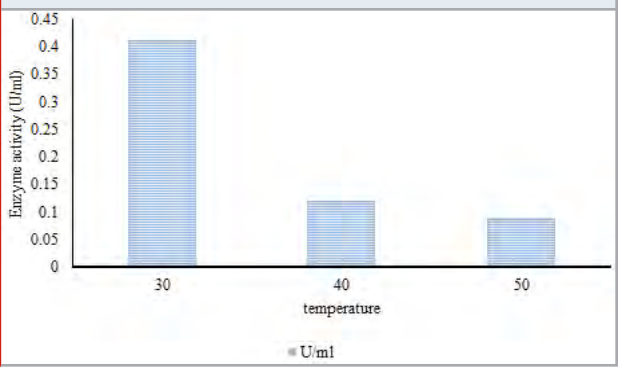


Figure 5: Comparison between SSF and SmF where highest production of 2.78 U/ml was given SM3 SSF



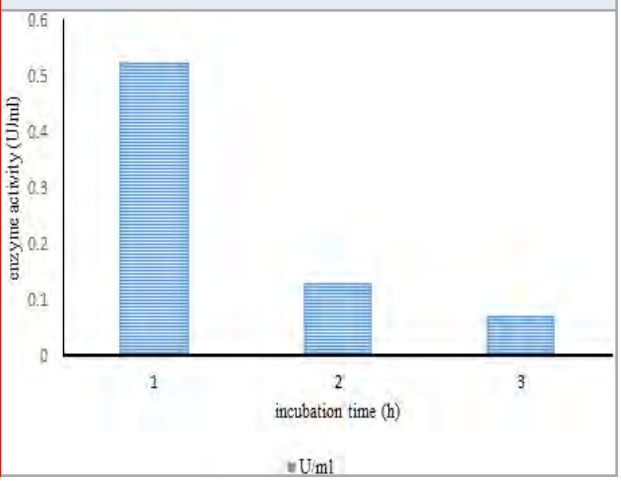
Laccase production: The fungal isolates showing highest oxidation zone SM1, SM2 SM3 were evaluated for laccase production. The laccase producing fungi that were screened and selected for laccase production, was grown on PDA slants for 5 days at 28°C preserved and stored at 4°C. Production was done initially by submerged fermentation (Fig 3.a), supernatant of the culture containing the SM3 showed highest enzyme activity of 0.712 U/ ml after 240 h of incubation (fig.4) indicating the extracellular nature of the laccase. Solid state fermentation was done of these fungal laccase (Fig3.b), which showed enzyme activity of 2.78 U/ml after the incubation of 21 days. Comparison between both SSF and SmF was shown in Fig (5) where SSF showed higher enzyme activity. Other reports shows the highest enzyme activity of 1.479 U/ml by R.A. Abd El Monssef et.al (2016). Where the initial activity of 3.2 U/ml from the liquid culture medium by white rot fungus was given reported by T. Senthivelan et al. (2019).

Figure 6: Effect of various temperature on fungal laccase



Characterization of fungal laccases: Characterization of enzymatic activity was carried out and presented in the Fig. 3 with various temperature like 30°C, 40°C, 50 °C and incubation time of 1h, 2h, 3h . Where highest activity of 0.411 U/ml was observed at 30 °C (Fig.6) and 0.512 U/ml at incubation time of 1h (Fig.7).

Figure 7: Effect of incubation time activity fungal laccase



Characterization of fungal isolates: Fungal isolates with maximum enzyme activity that is SM3 was characterized by colonial characteristics and morphological analysis (Fig.8a) and inferred that the isolated fungus belongs to the class of basidiomycetes white rot fungus shown in Table 1.

Figure 8: (a) pure slant of fungal isolate SM3 (b) microscopic image of fungal isolate SM3

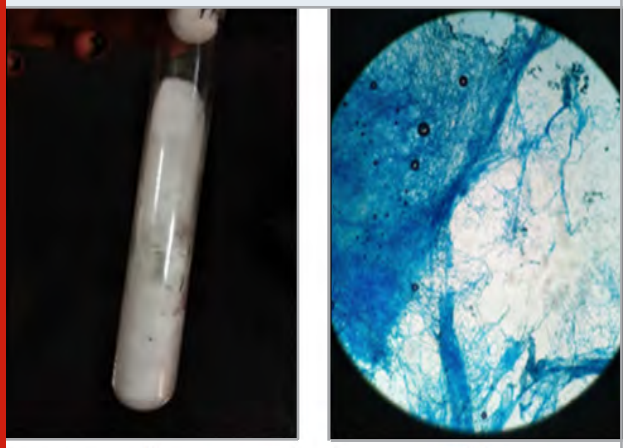
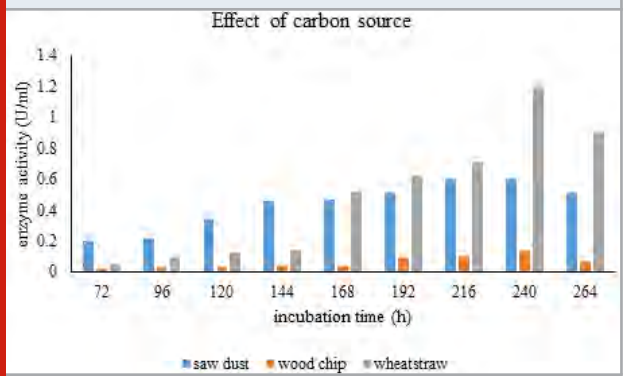


Table 1. Characterization of fungal isolate SM3

Organism	Cultural Characteristics	Morphological characteristics	Identified as
SM3	White cottony, fluffy colony with aerial hyphae.	Filamentous, spore forming, Septate mycelia, Clamp connection.	*Pleurotus spp.*

Figure 9: Effect of carbon sources on laccase production



Optimization of media components: Optimization of carbon source was done for fungal isolates showing highest enzyme activity. Effect of different natural carbon source like saw dust, wheat straw, and wood chip was tested at shake flask level and incubated at 28°C for 11 days. Enzyme production was measured at the interval of 24 h using guaiacol as substrate and highest enzyme activity was given by SM3 using wheat straw that is 1.20 U/ml after 240 h of incubation as shown in fig.7. Maximum laccase activities were found to be 2.795 U/ml, 2.595 U/ml and 2.38 U/ml using corn cobs, sugarcane bagasse and rice straw as substrates in report of Saqib et al (2014).

Dye decolorization experiments: Phenol red and methylene blue were used for dye decolorization assay, 100 mg/L dye were used for the experiment. Dye content was monitored photometrically at 540 nm and 665 nm for Phenol red and methylene blue respectively using spectrophotometer, which is the maximum visible absorbance of these dyes. A control was kept un inoculated. Decolorization efficiency was been measured after 72 h of incubation (figure.10). Maximum dye decolorization efficiency of 80 % of Phenol Red and 68% of Methylene Blue were observed that is shown in (Table 2) Where another report shows dye decolorization with efficiency of 43 % Rehan et al. 2016.

Figure 10: Dye decolorization assay of SM3 using phenol red and methylene blue

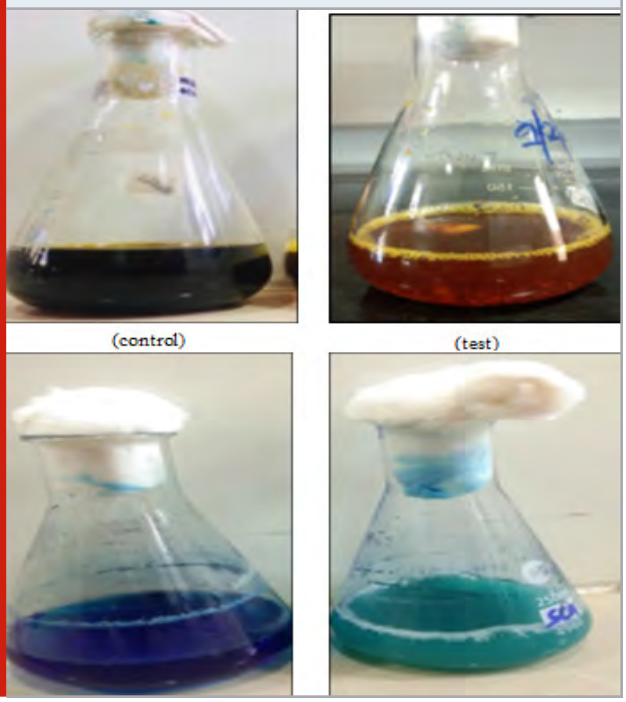


Table 2. Dye decolorization control using phenol red and methylene blue

Dye	OD of Control	Decolorization of Control	OD of test	Decolourization of Test
Phenol red	1.25	0%	0.255	80%
Methylene blue	1.56	0%	0.470	68%

CONCLUSIONS

The laccase producing potential isolate SM3 was isolated from bark of tree and used for enzyme production.

Based on the screening results using various substrates of ABTS, guaiacol and tannic acid, laccase positive test were confirmed and production was carried out .Where fungal laccase production was done using solid state fermentation and submerged fermentation where maximum enzyme activity was 2.78 U/ml and 0.736 U/ml respectively at 30°C . Enzyme characterization showed that enzyme work at 30 °C as optimum temperature and incubation time of 1h. Medium optimization of carbon source was done by classical one-factor- at-a-time method .The optimum laccase activity of 1.280 U/ml after the incubation of 11 days was obtained by classical method. Laccase exists in decolorization mechanism could be concluded using dye decolorization mechanism. Results showed that laccase enzyme was responsible for 80 % decolorization of phenol red and 68% decolorization of methylene blue in time interval of 72 h. The use of the laccase may conceivably be extended to other anthraquinone-type textile dyes, indeed suggesting a potential application field for the removal of dyes from industrial effluents.

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Effect of Microbial Induced Calcite Precipitation on Strength Improvement of Marine Clays

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ABSTRACT

The challenges encountered to strengthen the weak soils or marine clays always prompted the need for further research investigation to develop a new, eco-friendly and sustainable method of soil stabilization. Soil being a natural material composed of certain chemical constituents which are favorable for many aqua and non-aqua bacteria's. Among all the available techniques, Microbial-Induced Calcite Precipitation (MICP) is now gaining more attention as a sustainable method for soil improvement. This study focuses on bacterial calcium carbonate precipitation and its effect on the compressibility and strength of two different types of marine soils (high plastic clay and low plastic clay). A species of Bacillus group B.megaterium (MTCC-428) was used to activate and catalyze the calcite precipitation caused by reaction between urea and calcium chloride. MICP uses bacteria to hydrolyse urea and give carbonate ions which react with a calcium chloride solution to produce calcium carbonate (calcite) that binds the soil particles together which leads to increase in soil strength and stiffness. A special nutrient delivery system was use to induce cementation reagents. In the present study, effect of variable parameters such as concentration of cementation reagent (0.25M, 0.50M, 0.75M, 1M) and curing period (1day, 3day, 7day) were studied. These parameters were applied on both the type of soils for specified duration and range to utilize the effect of MICP. From the results, it is observed that unconfined compressive strength has improved (1.15-2 times) in both the type of soils. Further with increase in treatment duration, strength increases significantly for both types of soils. The experimental results were validated using Scanning Electron Microscope (SEM) analysis..

KEY WORDS: B.MEGATERIUM; CALCITE PRECIPITATION; SOIL STABILIZATION; UNCONFINED COMPRESSIVE STRENGTH.

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INTRODUCTION

The need for stabilizing soils becomes necessary mainly because of poor soil properties and need for urbanization especially in areas having problematic soils. It is believed that the demand for new and sustainable soil stabilization techniques is increasing rapidly. The artificial cementation of soil particles due to soil stabilization is often achieved through the use of chemical stabilizers via shallow and deep mixing or injecting chemical grouts that can permeate through soils. Physical properties of soil can be modified by the use of mechanical compaction or grouting while chemical properties of soil can be modified by the use of chemical stabilizers such as Portland cement, lime and fly ash. Mechanical compaction is recommended for sandy soils which is effective and economical to a depth less than 10 m (Chu. & Ivanonv., 2012). Chemical stabilization is typically recommended for expansive soils (Petry & Little, 2002).

Environmentally safe techniques such as pre-wetting and moisture barriers are only possible for small confined spaces, and are not suitable for larger construction projects such as highways and railways which spread for miles. Traditional ground improvement techniques have many limitations. High pressure is required for injecting the grout due to its fast hardening time or high viscosity. Freezing is not a permanent solution during construction. Most of these methods are expensive, disturbing urban infrastructure, require heavy machinery, and involve chemicals with significant environmental impact. Consequently, these conventional methods are not suitable for treating large volumes of soil. Artificial cementation techniques are never feasible and environment friendly. However, decrease in the use of artificial cementation techniques can be done by addition of environmental friendly methods or materials.

One such method of soil stabilization technique is, Microbial Induced Calcite Precipitation (MICP). This technique is a better and more environmental friendly alternative to the conventional technologies (Animesh & Ramkrishnan, 2016). In this technique, microbes employs as a primary factor for stabilization. Calcium carbonate precipitation has been induced inside the soil structure by microorganism through their metabolic process to improve the engineering properties of soil. Hence, this technique is called as microbial induced carbonate precipitation or MICP. Successful implementation of MICP will have its application in a wide variety of civil engineering fields such as stability of retaining walls, embankments and dams, controlling soil erosion, stabilizing cohesion less soils to facilitate the stability of underground constructions, increasing bearing capacity of shallow and piled foundation and reducing the liquefaction potential of soil.

Theoretical Background: Lee Min Lee et al. (2012) studied the effect of MICP (Lee Min Lee et al., 2012) on shear strength and reducing hydraulic conductivity of soils. The results showed that MICP could effectively increase the shear strength and reduce hydraulic conductivity of soils. In general, MICP can be achieved by urea hydrolysis, aerobic oxidation, de-nitrification, sulphate reduction, etc. Urea hydrolysis refers to a chemical reaction where urea ($CO(NH_2)_2$) is decomposed by Urease enzyme that can be either supplied externally (Greene et al., 2003), or produced in situ by Urease producing microorganisms (DeJong et al., 2006). Urea hydrolysis processes the highest calcite conversion rate compared to other studied processes (Harkes et al., 2010; Whiffin et al., 2007). The latter process requires Urease positive type bacteria, i.e. genera *Bacillus*, *Sporosarcina*. 1 mole of urea decomposes into 2 moles of ammonium according to following reaction:

$$CO(NH_2)2 + 2H_2O \rightarrow 2NH^{4+} + CO_3^{2-} \quad (1)$$

Table 1. Properties of soil

Sr No.	Test	Symbol	Result Soil 1	Result Soil 2	IS Code
1	Liquid Limit	LL	54%	35%	IS 2720(Part 5)-1985
2	Plastic Limit	PL	26.57%	26.19%	IS 2720(Part 5)-1985
3	Plasticity Index	PI	27.43%	8.81%	IS 2720(Part 5)-1985
4	Soil Classification	-	CH	CL	IS 1498-1970
5	Shrinkage Limit	SL	22.59%	10.76%	IS 2720(Part 6)-1978
6	Specific Gravity	G	2.71	2.64	IS 2720(Part 3)-1980
7	Free Swell Index	FSI	14.5%	10.5%	IS 2720(Part 40)-1977
8	Hydrometer	Clay	40%	17%	IS 2720(Part 4)-1985
	Silt		56%	61%	
9	Standard	OMC	23.0 %	19.0%	IS 2720(Part 7)-1980
	Proctor Test	MDD	15.9kN/m^3	17.3kN/m^3	
10	PH	_	8.83	8.54	IS 2720(Part 26)-1987
11	Coefficient of	Kh	1.03 x	0.82	IS 2720(Part 17)-1986
	permeability		10-9 m/sec	x 10-9 m/sec	

The release of ammonium (NH4+) cause increase in pH, which in due course, creates a perfect condition for calcite precipitation with the availability of calcium ion(Ca^{2+}) from the supplied calcium chloride:

$$Ca^{2+} + CO_3^{2-} \rightarrow CaCO_3 \quad (2)$$

The $CaCO_3$ precipitates formed are gelatinous in nature and thus helps in bonding the soil particles together (Wei-Soon Ng et al., 2012).

MATERIAL AND METHODOLOGY

1.1. Soil Characteristics: Two types of soils were considered for this study. Both soil samples were collected from Dholera (Gujarat, India).The basic properties of the collected samples and soil classification are in Table 1. These samples were treated with bacteria and cementation reagent and then tested for strength incremen.

Figure 1: (a) Freeze dried culture (b) & (c) Activated culture on Petri plate

A B C

Figure 2: (a) Diagram of Laboratory Setup

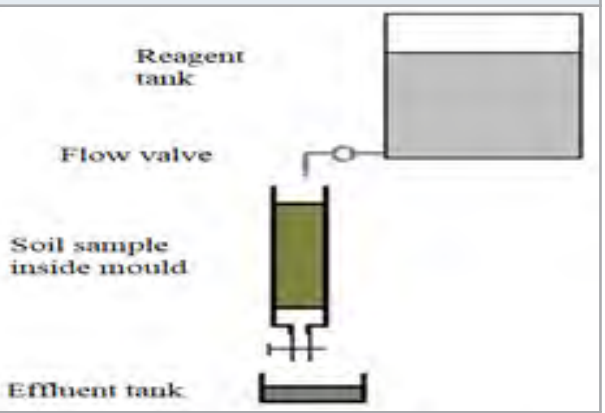
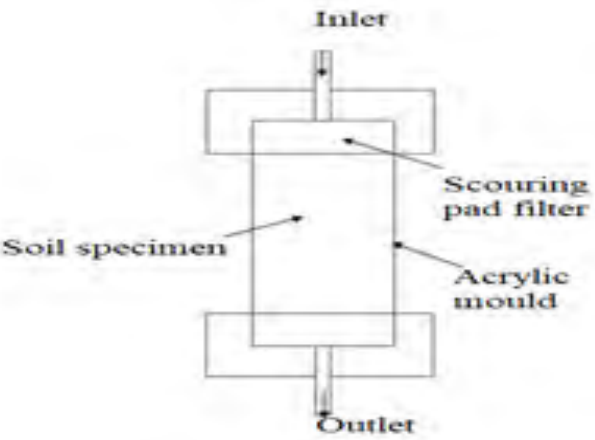


Figure 2(b): Soil Specimen Mould



1.2. Activation and Cultivation of Bacteria: In the present study, Bacterial strain bacillus megaterium (MTCC-426) is used and procured from microbial type culture collection and gene bank (MTCC) Chandigarh in freeze dried condition. This freeze-dried culture was taken in a petri plate which was previously solidified with agar and incubated at -4˚c temperature under condition recommended for the culture. The growth media used to grow the microorganisms was primarily nutrient broth (NB). Microbial concentration tests were used in this research to determine the impact of variable concentration in evaluating the effect of MICP in soils. In order to maintain the consistency of microbial concentration throughout the research, colony formation unit (CFU) method was adopted to determine the concentration of microbes in a given solution. After 48 hours of incubation, the optical density (OD) of these cultured microbes was measured.

In this method, by measuring the turbidity of the sample at certain wavelength (usually 600nm) used to determine the concentration of microbes. These cultured microbes were then serially diluted and after serial dilution, 10ml of the serial diluted media was taken and then plated in a NB plate. (NB plate was prepared by mixing 10 g of LB and 6 g of agar in 400 ml of distilled water. The media after autoclaving was poured into the petri dish and solidifies after few hours due to the presence of agar. After 48 hours of plating, the number of colonies were counted. The CFU/ml for each serial dilution is given as per Equation.

$$CFU/ml = \frac{No.of\ colonies\ counted \times Dilution\ factor}{Volume\ of\ culture}$$

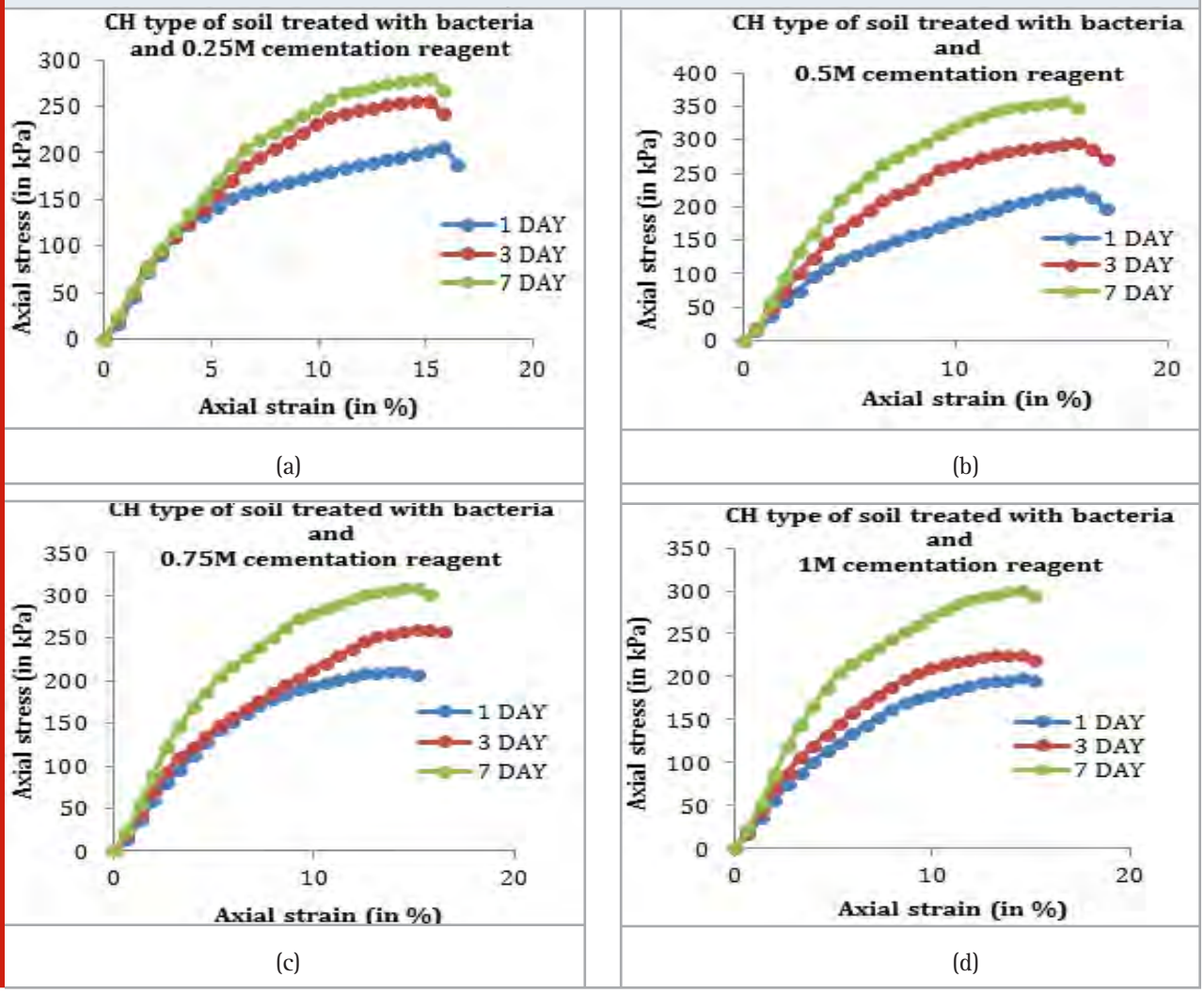
1.3. Cementation reagent: Cementation reagent serves as important ingredients for promoting calcite precipitation. As shown in equations (1) and (2), the ammonium (NH^{4+}) and calcium (Ca^{2+}) ions are decomposed from urea ($CO(NH_2)_2$) and calcium chloride ($CaCl_2$), respectively. It is thus important to supply sufficient amount of urea and calcium chloride into the soil. The cementation reagent also contained 3 gm nutrient broth, 10 gm NH_4Cl, and 2.12 gm $NaHCO_3$ per liter of water.

1.4. Laboratory Setup: Fig. 2 shows the schematic diagram of the laboratory setup. The test is carried out in a prefabricated mould, which has the provision of inlet and outlet and control of flow. The apparatus is of a split type acrylic mould of 90 mm internal diameter (ID) and 160 mm height. The setup consists of a reagent tank, prefabricated mould, inlet and outlet valve, and an effluent collector. Air compressor is used to apply the pressure in reagent tank to initiated the flow through soil specimen.

1.5. Preparation of soil specimen: Initially, bacteria was added to the soil and mixed properly. Soil was compacted at 95% of MDD and respective OMC prior to treatment in the prefabricated mould. Since treatment duration was also one of the parameters, so soil samples of given molar concentrations were kept for curing duration of 1, 3 and 7 days. For everyday treatment, 1litre of nutrients are allowed to pass through the sample. Upon the completion of the treatment, the soil was extruded from the acrylic mould and tested for its shear strength characteristics via. unconfined compression testing machine.

1.6. Experimental Variables: Two different types of soils were used for this study. The main variable in this was the concentration of cementation reagent and curing period. Four concentrations were adopted, i.e. 0.25 M, 0.5 M, 0.75M, and 1 M. Three parameters were selected for curing period, i.e. 1 day, 3day and 7 day.

Figure 3: Soil S1 (CH) treated with bacteria and (a) 0.25M (b) 0.5M (c) 0.75M (d) 1.0M cementation reagent



RESULTS AND DISCUSSION

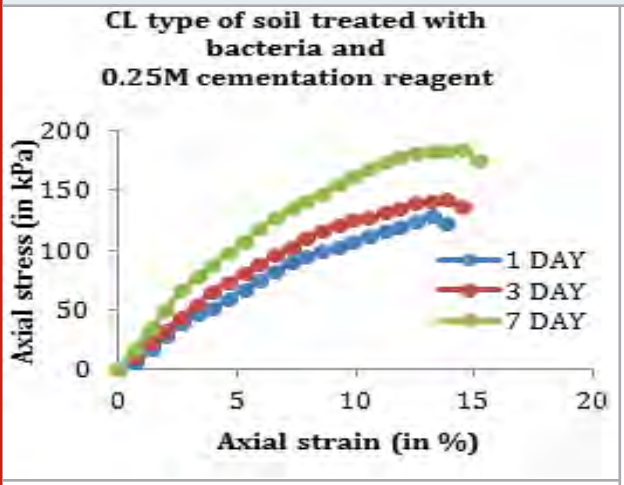
1.7. Unconfined compressive strength: The stress strain curves of all the soil specimens tested in the unconfined compression testing machine. The UCS value for virgin soil sample 1 (CH Soil) was 178.63 kPa, which on treatment with MICP was found to increase further. The test results of soil sample 1 (CH Soil) are tabulated in Table 2. It was observed from the test results that by increasing the treatment duration, UCS values was increased. For soil sample 1 (CH Soil), highest increment was observed for molar concentration of 0.5 M of cementation reagent. UCS value further increased on increasing the treatment duration. The test results of soil sample 2 (CL Soil) are tabulated in Table 3.

The optimum strength achieved at 0.5M concentration and the stress strain curves for that optimum value are shown in figure 3 and figure 4. For 0.50M, it shows highest increment in both CH and CL type of soil compared to other molar concentration because at 0.50M concentration gives the best favorable condition for bacteria to perform and produce maximum amount of urease enzyme for calcite precipitation. Higher bond formation in soil then lead to increase in cohesion of soil which is one of the parameters for the soil's shear strength and hence increase in shear strength.

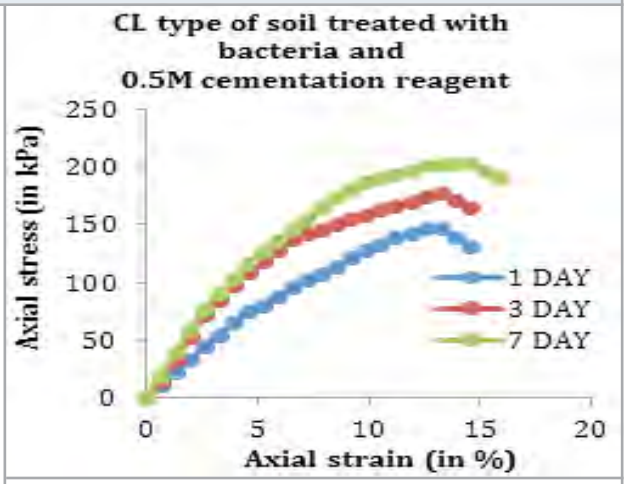
Table 2. Strength of Soil 1 (CH) soil after treatment

Bacteria	Cementation reagents	Soil 1 (CH) Unconfined Compressive Strength (kPa)			
		Untreated	1 day	3 day	7 day
1×108 CFU/ml	0.25 M	178.63	205.66	255.47	279.59
			(1.15 times)	(1.43 times)	(1.56 times)
	0.50 M	178.63	224.79	296.54	356.70
			(1.25 times)	(1.66 times)	(1.99 times)
Bacillus	0.75 M	178.63	211.31	260.67	308.50
Megaterium			(1.18 times)	(1.46 times)	(1.25 times)
	1.0 M	178.63	199.16	225.88	301.78
			(1.11 times)	(1.26 times)	(1.69 times)

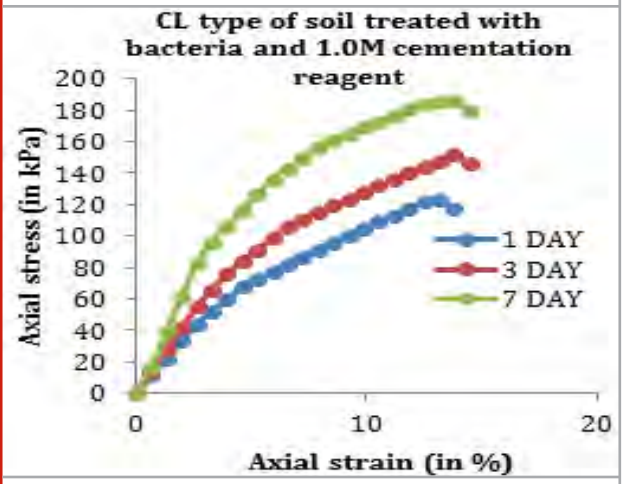
Figure 4:Soil S2 (CL) treated with bacteria and (a) 0.25M (b) 0.5M (c) 0.75M (d) 1.0M cementation reagent



(a)



(b)



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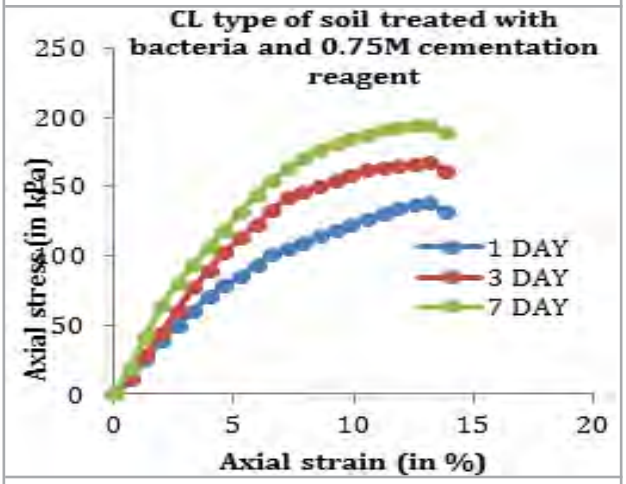
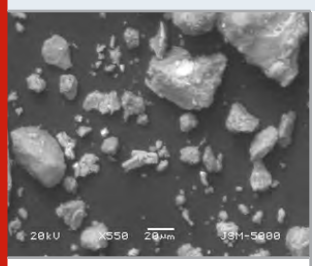


(d)

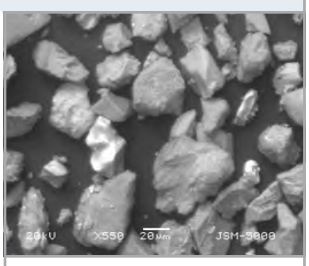
Table 3. Strength of Soil 2 (CL) soil after treatment

Soil 2 (CL) Unconfined Compressive Strength (kPa)					
Bacteria	Cementation reagents	Untreated	1 day	3 day	7 day
	0.25 M	104.05	128.24	141.96	184.59
			(1.23 times)	(1.36 times)	(1.77 times)
Bacillus Megaterium	0.50 M	104.05	147.97	177.57	204.02
1×108 CFU/ml			(1.42 times)	(1.71 times)	(1.96 times)
	0.75 M	104.05	138.11	167.70	194.83
			(1.33 times)	(1.61 times)	(1.87 times)
	1.0 M	104.05	123.3	151.75	186.01
			(1.19 times)	(1.46 times)	(1.78 times)

Figure 5: SEM of untreated and treated CH soil

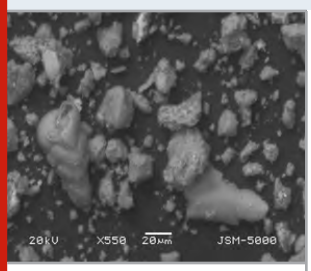


(a) Untreated

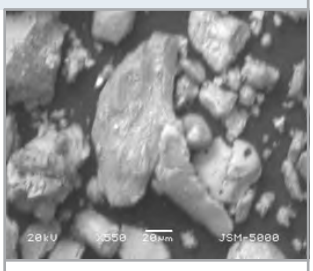


(b) Treated

Figure 6: SEM of untreated and treated CL soil



(a) Untreated



(b) Treated

CONCLUSIONS

MICP was found to increased the unconfined compressive strength of both the CH (1.15 times to 1.99 times) and CL type of soils (1.19 to 1.96 times).It was also observed that strength increased with increase in curing duration. As the curing time increases to 7 days the highest value for unconfined compressive strength was achieved.MICP was found to increase the cohesion of soil. Improve strength characteristics and it can be match up with other properties and possible indication will guide to reduced permeability and increased bearing capacity of soil.MICP can be effective in cost also, as it can be produced in huge quantity at a very minimal price. Bacterial solution can be prepared in huge amounts at very low costs and cementing reagents are very economical compared to other soil improvement techniques.

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Enhancement of Biological Production of Chitooligosaccharides from Chitin Waste Through Media Optimizations

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ABSTRACT

Chitin generated annually as an aquaculture wastes. Chitin is found in abundant amount from the aquaculture wastes on every year. Chitin is also present in the cell walls of most fungi and exoskeleton of arthropods. In most situations these substances are discarded as wastes with serious disposal problems leading to environmental pollution. We made a consortium of microorganisms capable to degrade chitin and standardized the degradation and conversion rate. The proposed research work was aimed at depiction of fermentation medium optimization for the production of chitooligosaccharides. The chitinolytic bacteria were isolated from the marine soil, marine animal dumping site and grown on the minimal salt medium. We have optimized the cultural condition to enhance the production of chitinolytic enzyme by altering the different parameters like pH, chitin concentration and yeast extract concentration in media. Taguchi DOE methodology was applied for media optimization. A total of 18 experimental runs with different conditions were studied where three pH 3.5, 6.0 and 8.5, two different temperatures 30°C and 40°C three chitin concentrations of 0.25%, 0.75% and 1.25%, yeast extract concentrations 0.25%, 0.50%, 0.75%. The optimized cultural conditions, pH 6.0, temperature 30°C chitin 1.25%, yeast extract 0.25% have shown more than 90% degradation of chitin in just 9 days.

KEY WORDS: CHITIN, CHITO-OLIGOSACCHARIDES, DEGRADATION OF CHITIN, POLLUTION.

INTRODUCTION

Chitin is linear amino polysaccharide that has N-acetylglucosamine (GlcNAC) as monomer and they are linked by β1, 4- glycosidic bonds. After cellulose, chitin is the second most abundant substance in the planet. Approximately, 10^{12} – 10^{14} tons of chitin is produced annually by living organisms in ocean (Dhillon et al., 2013).Chitin is white inelastic polysaccharide which contributes heavily to coastal area pollution .Chitin can be found in fungi, shrimp, crab, lobster, exoskeleton of insect and many other invertebrates. Among all, highest percentage (nearly 90%) of chitin is found in shrimps and crabs. Chitin exists in three different forms α- chitin, β- chitin, γ- chitin. α- chitin is the most plenteous form of chitin (Kaya et al., 2015). Chitinases (E.C 3.2.2.14) are glycosyl hydrolases which means they hydrolase β-1,4 glycosidic bonds. The chitinase enzymes are divided into two groups i) Exochitinase and ii) Endochitinases (E.C

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3.2.1.14). Chitin is cleaved at internal position randomly by endochitinase whereas; exochitinase progressively removes two sub units either from reducing or non-reducing ends of chitin (Sahai and Manocha, 1993).

Many organisms like bacteria, insects, fungi, plants, mammals synthesize chitinase. The exochitinases have been further divided into 2 subcategories: Chitobiosidases (E.C. 3.2.1.29) that are involved in cleaving the progressive release of di-acetylchitobiose beginning at the non-reducing end of chitin, and also 1-4-β-glucosaminidases (E.C. 3.2.1.30) catalysing the oligomeric products of chitobiosidases as well as endochitinases thus generating monomers of N-acetylglucosamine. Chitinases are divided into three families 18,19 and 20 of glycosyl hydrolases and into five classes (Henrissat and Bairoch, 1993). Depending upon the source chitinase can be bacterial chitinase, fungal chitinase, plant chitinase, insect chitinase and mammalian chitinase. Chitinases are a diverse and large group of enzymes that exhibit differences in their catalytic mechanism, substrate specificity and molecular structure. It is important to do background research about the substrate specificity of chitinases because it reveals the relationship between the substrate specificity and physiological roles, in addition to that it permits one to degrade chitin into products having commercial applications (Matsumiya et al., 2008).

The chitin binding domain can either be located in the carboxyl terminal or amino terminal domains of the enzyme in bacterial chitinases (Watanabe et al., 1994). Majority of the bacterial chitinases that have been isolated and sequenced till now, are part of family 18 of the glycosyl hydrolases with few exceptions (Ohno et al., 1996). Some bacterial strains and plants are part of family 19 chitinases (Watanabe et al., 1999). The amino terminal region of bacterial chitinase has sequence similarity with non-catalytic domains of many other bacterial lytic enzymes which includes proteases, cellulases (Suzuki et al., 2002).Bacteria produce chitinases is because of the chitin degradation and to utilize it as an important energy source and by this they supply carbon and nitrogen for nutritional source (Cohen and Chet, 1998). Bacterial chitinases have molecular weight in the range of 20-60 kDa. Most of the bacterial chitinases sequenced and characterized so far are included in group A (Svitil and Kirchman, 1998). It is being found that chitinase genes which belong to group A are more profuse compare to group B and C enzymes (Metcalfe et al., 2002).

Chitin degradation is a highly regulated process. Chitin can be degraded by biological and chemical methods. Biological methods possess many advantages over chemical methods as chemical methods are costly and environmentally harsh. There are basically two pathways for chitin degradation. Chitin can be converted to N-acetylglucosamine directly by the chitinase and N-acetylglucosaminidase. There is another way also where chitin is first converted to chitosan by enzyme named chitin deacetylase after that, it is further degraded to N-glucosamines (GlcpN) by chitosanase and glucosaminidase enzymes. Chitosan is commercially important compound and offers a range of applications in the fields of agriculture, pharmaceutical, cosmetics, food, textile, wastewater treatment and biomedical

Table 1: Details of taguchi orthogonal array design of experiments for chitin degradation

Sr. No.	Temperature (°C)	Chitin Concentration (gm)	pH	Yeast extract Concentration (%)	Shaking Speed (RPM)
1	30	0.25	3.5	0.25	0
2	30	0.25	6	0.5	0
3	30	0.25	8.5	0.75	180
4	30	0.75	3.5	0.25	0
5	30	0.75	6	0.5	180
6	30	0.75	8.5	0.75	0
7	30	1.25	3.5	0.5	0
8	30	1.25	6	0.75	0
9	30	1.25	8.5	0.25	180
10	40	0.25	3.5	0.75	180
11	40	0.25	6	0.25	0
12	40	0.25	8.5	0.5	0
13	40	0.75	3.5	0.5	180
14	40	0.75	6	0.75	0
15	40	0.75	8.5	0.25	0
16	40	1.25	3.5	0.75	0
17	40	1.25	6	0.25	180
18	40	1.25	8.5	0.5	0

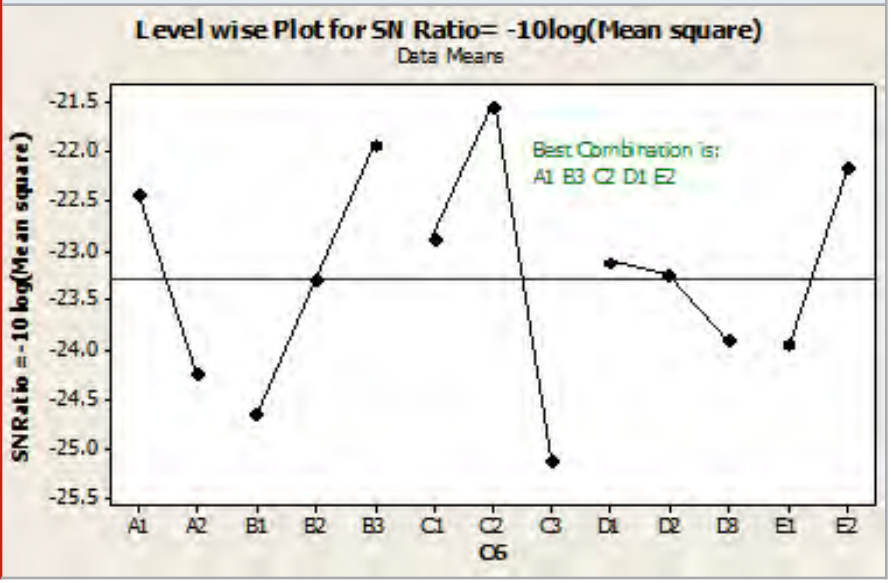
industries (Rinaudo, 2006; Jayakumar et al., 2011; Anitha et al., 2014; Fernando et al., 2016).The production of chitinases in bacteria is extensive among phyla and also the occurrence of multiple chitinolytic enzymes through individual bacterial strains seem to be a common trait (Saito et al., 1999; Shimosaka et al., 2001; Tsujibo et al., 1994).

Optimizing the parameters by statistical method for chitin degradation is aimed to reduce the time and expense. Current study involves the identification of important parameters through OFAT (one factor at a time) and making all the identified important parameters as part of interaction effect through DoE approach. Chitin degradation requires careful control of various

Table 2: results of taguchi array design of experiments for chitin degradation.

Sr. No.	Temperature (°C)	Chitin Concentration (gm)	pH	Yeast extract Concentration (%)	Shaking Speed (RPM)	>70% Chitin Degradation (In Days)
1	30	0.25	3.5	0.25	0	15
2	30	0.25	6	0.5	0	12
3	30	0.25	8.5	0.75	180	21
4	30	0.75	3.5	0.25	0	13
5	30	0.75	6	0.5	180	12
6	30	0.75	8.5	0.75	0	12
7	30	1.25	3.5	0.5	0	13
8	30	1.25	6	0.75	0	12
9	30	1.25	8.5	0.25	180	12
10	40	0.25	3.5	0.75	180	12
11	40	0.25	6	0.25	0	13
12	40	0.25	8.5	0.5	0	26
13	40	0.75	3.5	0.5	180	12
14	40	0.75	6	0.75	0	18
15	40	0.75	8.5	0.25	0	21
16	40	1.25	3.5	0.75	0	19
17	40	1.25	6	0.25	180	12
18	40	1.25	8.5	0.5	0	12

Figure 1: level wise plot for sn ratio for output results



process parameters in order to degrade it and produce value added products. Taguchi methods helps to designs recognize that not all factors that cause variability can be controlled. These uncontrollable factors are called noise factors. Taguchi designs are used to identify factors which are controllable factors (control factors) which minimize the effect of the noise factors.

MATERIALS AND METHODS

Microbial Consortia Preparation by Enrichment Methods: The chitinase-producing bacteria were isolated from the different places such as industrial processing unit, near to ponds, canal and dump places. One percent inoculum used for enrichment techniques with 1% chitin selective medium contains minimal media with yeast extract. Sub-culturing of inoculum was initially done after 30 days and slowly reduces time based on chitin flakes solubilized in medium. Enrichment techniques were used to make microbial consortia capable to degrade chitin.

Fermentation of media: Microbial consortia of chitin degraders were added into medium targeting a) chitin concentration b) pH c) temperature d) yeast extract concentration e) static and shaking speed. During growth phase and stationary phase microbes secreted various enzymes which help in chitin degradation.

Design of the experiment: The entire experiment was carried out in 18 different flasks with different variables. The experimental design has been summarized in triplicates Table 1. Minitab 18 statistical software was be used to analyse the data generated from Taguchi experiments to view the pattern of response and identify significant parameters.

RESULTS AND DISCUSSION

The degradation data from different batches were collected and percent chitin degradation with time (in days) was analyzed in terms of its distribution with minimum and maximum values. As defined in the problem statement, degradation time of chitin to be minimize as much as possible to achieve at least 70% chitin degradation from the initial. It was found that time taken for chitin degradation was very much low (12 days) and some batches were above 20 days with average value of 19.87 in batches (Table 2).

Based on Results obtained from Experiments, Data Analysis was performed using Minitab 18 statistical software for level wise signal to noise ratio (smaller is better). Based on the outcome of the experiments, it was observed that to degrade chitin in minimum time, it is advisable to set process parameter with combination of A1B3C2D1E2 (Figure 1).

Where A1 = 30oC Temperature 30^{0}C;
B3 = 1.25 % Chitin Concentration;
C2= pH 6.0;
D1=0.25% Yeast Extract Concentration;
E2 = 180 Shaking speed in rpm

Table 3: Confirmatory experiment based on level wise signal to noise ratio and degradation of chitin in days

							Chitin % Degradation							
Experiment Number	Temp (^{0}C)	Chitin Conc. (gm)	pH	Yeast Conc. (%)	Shaking Speed (RPM)	Day	1Day	2Day	3Day	4Day	5Day	6Day	7Day8	Day9
Replication 1	30	1.25	6.0	0.25	180	13%	21%	46%	62%	71%	78%	88%	91%	90%
Replication 2	30	1.25	6.0	0.25	180	11%	17%	47%	65%	74%	79%	91%	94%	92%

Figure 2: percent (%) chitin degradation result with optimum process parameters based on levelwise sn analysis.

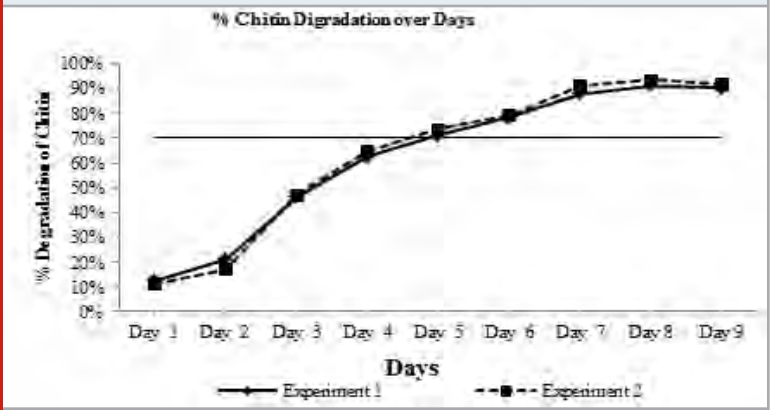


Table 4. variables having direct influence on chitinase production.

Parameter operating Range	Units	Current manufacturing range	Revised Normal operating range
Temperature	°C	30-40	30
Chitin Conc	grams	0.25-1.25	1.25
pH	pH	3.5-8.5	6.0
Yeast Extract Conc	%	0.25-0.75	0.25
Shaking Speed	RPM	0-180	180

We have carried out confirmatory experiment (Table 3) with optimum parameters found in Level wise Signal to Noise ratio results (figure 1) and we have found that 70% chitin degradation can be achieved in span of 5 to 6 days. This study also reveals that more than 90% of chitin degradation can be occurred in 9days. Temperature range 30^{0}C to 40^{0}C was considered as majority of the microorganisms growin this range. Highly acidic (3.5), slightly acidic (6.0) and moderate alkaline (8.5) were taken into account. The chitin concentration form 0.25% to 1.25% and yeast extract concentration of 0.25% to 0.75% also factored in along with static (0 rpm)/ shacking (180rpm). Out of that range we figured out the optimum variables. Based on the confirmatory experiments, parameter range was finalized as mentioned in Table 4.

In both the experiments, more than 70% and 90% chitin degradation were observed at 5 Days and 9 days respectively, which was comparatively much lower compare to of 18-20 days, it is in line with the general expectations and process requirement. Based on the Signal to Noise ration analysis, revised normal operating range was finalized for temperature, chitin concentration, pH, yeast extract concentration and shaking speed and new process condition was tested while running 2 experiments at small scale. As per the expectation, >70% Chitin degradation time was found to be 5 days in both runs which was a great improvement for existing process. This improvement holds a great promise to make the existing process more cost efficient and commercially beneficial.

Temperature is most commonly regarded a critical factor in controlling the rate of chitin degradation. There are reports of variation in the rate of chitin degradation with regards to the highest enzymatic activity during high temperature (Gooday et al., 1991; Boyer, 1994). Many data suggest that temperature and chitin supply are important environmental factors controlling both chitin hydrolysis rates and the chitinolytic community structure. Thus, chitin degradation can be explored as a common model to understand microbial degradation of polysaccharides in the biosphere. The medium composition including pH has significant effect on the chitinase enzyme production. Yeast extract consists of proteins, growth factors so they promote the growth of the microorganisms. For certain strains it is proved better than many other sources like casein, ammonium nitrate, peptone etc. In many study available colloidal chitin was used for chitinase enzyme activity instead of chitin flakes. In *Microbispora sp.* colloidal chitin was proved to a better substrate (Nawani et al., 2002) whereas, for Metarhizium *anisopliaechitin* flakes were considered superior substrate (Leger et al., 1986).

This kind of statistical Taguchi array based designs are more helpful and appropriate while working with multiple variables and the factor which is the most critical can be identified. It is also efficient in process development and to study interactive variables and their quadratic effect (Aliabadi et al., 2016). Apart from the Taguchi method, there other methods like Response Surface Method (RSM), Plakett- Burman (PB) are available. Taguchi method is relatively easy to work with compare to the rest of them.

CONCLUSION

The present study is an attempt to optimize components of culture medium to enhance biological degradation of chitin wastes in least possible time. As suggested in Taguchi method, here we have finalized five variables e.g. temperature, pH, chitin concentration, yeast extract concentration, static/shacking. Based on the results, we can conclude that maximum degradation of chitin achieved at 30^{0}C temperature, 1.25% chitin concentration, 6.0 pH, 0.25% yeast extract concentration in shaking condition. The result also indicated that more than 70% of chitin degradation was found on 5th day after inoculation by using optimized parameters.

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Conflict of interest: Authors declare no conflict of interest.

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Screening of Hydrolytic Enzymes Produced by Bacteria Isolated From Mango Trees Rhizosphere and Mango Fruits (*Mangifera indica*)

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ABSTRACT

Cell wall-degrading enzymes of rhizobacteria are very important due to their ability to damage the structural integrity of the cell wall of phytopathogenes. So, they are more and more employed in biocontrol of fungal phytopathogens. The aim of this study was to screen from bacterial strains isolated from mango trees rhizosphere and healthy mango fruits those have ability to secrete hydrolytic enzymes useful in the biocontrol of fruit fungal diseases. Among the seventy hundred and six bacterial (706) strains isolated from mango trees rhizosphere and healthy mango fruits of Côte d'Ivoire, twenty strains presumptively identified as *Bacillus spp*. and *Pseudomonas spp*. were screened on the basis of their ability to produce hydrolytic enzymes. There were sixteen *Bacillus spp*. and four Pseudomonas spp.. The analysis have been done on agar plate with specific substrates : laminarin for beta 1,3 glucanase activity, carboxymethyl cellulose for cellulase activity, chitin colloidal for chitinase activity, skim milk for protease activity and rhodamine- olive oil for lipase activity. For the different enzymes tested, halo of enzyme activity ranged beetwen 10 and 31.33 mm. Isolates BF68(2), BF24, and BF81 have showed the highest halo for cellulase activity with 31.33 mm. BF68(2) and B236 have showed the highest protease activity with halo of 25.66 mm. For chitinase activity, the highest activity has been shown by B236 with 12 mm of halo. The highest β1,3 Glucanase activity has been shown by BF68(2), BF76, BF75, BF46, B69, B236, B234 and P115 with 11 mm of halo. *Bacillus spp*. and *Pseudomonas spp*. strains isolated from mature and healthy mango fruits and from rhyzospheric soil of mangoes trees have shown promising biocontrol abilities trough the production of cell wall-degrading enzymes.

KEY WORDS: *BACILLUS SPP.,* HYDROLYTIC ENZYMES, PHYTOPATHOGENES, PSEUDOMONAS SPP., RHIZOBACTERIA.

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INTRODUCTION

The rhizosphere soil contain diverse types of microorganisms and can be grouped into bacteria, fungi, actinomycetes, algae and protozoa. These bacteria are able to find their foods from root exudates and at the same time they provide also essential nutrients and protection to the plants. The rhizobacterial strains are widely used in agriculture but also in the industries because of many attributes they have. In the field of agriculture, they are used as biocontrol agents (Kandel et al. 2017). The antagonistic activities of bacterial biocontrol agents can be attributed to (i) synthesis of hydrolytic enzymes they can produce. From those hydrolytic enzymes, antagonistic bacterial are capable to lyse fungal cell walls (such as cellulase, chitinase, glucanase, protease, and lipases) or competition for nutrients and niches (Beneduzi et al., 2012). A number of soil bacterial strains have been exploited for their biocontrol potentials, especialy, the genera *Bacillus* (Lee et al., 2017) and *Pseudomonas* (Priyanka et al., 2017). The genus *Pseudomonas* possesses high biocontrol properties because of their adaptive metabolism and their ability to produce a range of antifungal compounds (Trivedi et al., 2008).

They can produce several antifungal and secondary products including hydrolytic enzymes (Solanki et al., 2014). Bacterial strains belonging to the genera *Bacillus* also are considered to be safe micro-organisms and hold the remarkable abilities of synthesizing a wide variety of beneficial substances for agronomical and industrial purposes. Hydrolytic enzymes produce by rhizosphere bacteria are responsible for the degradation of various components of fungal pathogens. The enzymes of these types are able to breakdown glycosidic linkages present in the polysaccharide of the cell wall of phytopathogens. Among the large quantities of hydrolytic enzymes, chitinase, glucanase, protease and cellulase are of major interest due to their ability to degrade and lyse fungal cell wall. Thus, these hydrolytic enzymes are employed in biocontrol of fungal phytopathogens (Mabood et al., 2014). Cell wall-degrading enzymes of rhizobacteria have been reported by several searchers and proved to be a potential antifungal compounds.

Among the fruits in the world, mango is one of the most important and most traded fruit in the World. But the high moisture content of mango fruits make them highly susceptible to the attack of pathogens. Major loss of harvested mango fruits is due to fungi. The most important postharvest diseases of mango fruits are Anthracnose, Stem end rot, *Aspergillus niger* rot, soft rot, *Alternaria* rot. For reducing post-harvest losses, treatment with synthetic fungicides is the primary mean. However, the development of fungicide-resistant strains of pathogens, the detection of undesirable chemical residues in the food chain and the deregistration of some of the most effective fungicides have intensified the search for safer approaches to efficiently control post-harvest decay caused by microbial infections (Arya 1993). The aim of this study was to isolate and select local rhizosphere soil bacteria but also bacterial isolates from healthy fruits for their ability to produce hydrolytic enzymes which could be used as biocontrol agents for fruits diseases, particularly mango fruits.

MATERIALS AND METHODS

Isolation and Identification of bacterial isolates: Seven hundred and six (706) bacterial isolates were isolated from mature and healthy mango fruits and from rhyzospheric soil of mangos trees from three different localities of Côte d'Ivoire. Bacterial strains were isolated from the samples by serial dilution technique. Briefly, the soil samples were homogenized into sterile distilled water and mango fruits samples were dropped in sterile distillated water, shaken and then kept for 20 min. For *Bacillus* isolation, solution obtained was heated to 80°C for 12 min to eliminate vegetative form. The medium used was Yeast Potato Dextrose Agar (YPDA). So sterile molten medium in Petri dishes was subsequently inoculated with 0.1 ml of different dilutions of the homogenized solution. Inoculated plates were incubated at 30°C for 24 h. For *Pseudomonas* isolation, solution obtained after homogenization was directly diluted. Then, sterile molten of King B medium in Petri dishes was subsequently inoculated with 0.1 ml of different dilutions of the homogenized solution. Inoculated plates were incubated at 30°C for 24 h. Then, bacterial isolates were presumptively identified by means of morphological examination and some biochemical characterizations. The parameters investigated included colonial morphology, Gram reaction, endospore formation, catalase production, oxydase production, mobility test, starch hydrolysis.

Ezyme activities: Capacity of bacterial strains to produce antifungal enzymes (chitinase, cellulase, protease, β- 1,3 glucanase, lipase) was tested qualitatively.

β-1 ,3 Glucanase production: Glucanase activity was detected qualitatively as described by Sadfi-Zouaoui et al. (2008) on an agar medium containing 0.1% laminarin from *Laminaria* digitata (Sigma L9634) as the sole carbon source. The medium contained (g/l): 7 g NH4)2SO4, 1 g K2HPO4, 1 g NaCl, 0.1 g MgSO4.7H2O, 0.5 g yeast extract, 15 g agar and 5%NaCl (w/v). Medium was poured into Petri dishes and then inoculated with each isolate. After incubation in the room temperature for 5 days, 10 ml of congo red solution were poured on the top media surface and it was left for 10 minutes. The solution was discarded and replaced with 10 ml of 1 M NaCl solution for 15 minutes. β -1 ,3 Glucanase activity was noticed by a clear zone around the growing colony.

Chitinase production: For qualitative detection of chitinase activity, cells were grown on colloidal chitin agar plates containing a semi-minimal medium (Sampson et Gooday, 1998). The medium consisted of 1 :1 mixture of minimal medium (containing 0.1 % NH4)2SO4, 0.03 % MgSO4 7H2O, 0.6 % K2HPO4, 1 % de KH2PO4, pH 7) and nutrient broth supplemented with colloidal chitin (1 %) and solidified with 1.5 % agar. After incubation of the plates at 30° C for 5 days, they were fushed with 1

% congo red solution for 10 min, followed by washing with 1 M NaCl. The zone of chitin clearing became visible translucent halos around the colonies.

Cellulase production: Cellulase activity was determined as described by Teather and Wood, (1982) with some modifications. Bacterial strains were inoculated in carboxymethyl cellulose (CMC) agar medium. The medium consisted of mixture of 1.0 g KH2PO4, 0.5 g MgSO4.7H2O, 0.5 g NaCl, 0.01 g FeSO4.7H2O, 0.01 g MnSO4.H2O, 0.3 g NH4NO3, 10 g CMC and 13.0 g of nutrient broth plus 15 g agar in 1 L distilled water. After incubation for 4 days at 30° C, plates were flooded with 0.1% Congo red for 10 min and then with 1M NaCl for 10 min. After staining, plates were observed for zone formation around the test strains as an indication of cellulase production.

Protease production: Skim milk was used as a substrate to refer protease activity. That medium contained only 0.5 % of skimmed milk and 1.5 % of Agar. Medium was poured into Petri dish and then inoculated with each isolate, and incubated at 30 ° C for 3 days. Protease activity was produced by creating a clear zone surround the culture growth.

Lipase production: Lipase activity was determined as described by Bornscheuer et al., (2002) with some modifications. For lipase detection, bacterial strains were inoculated on rhodamine-olive oil-agar medium. The medium contained (g/L): 13.0 g nutrient broth,4.0 g NaCl, 10 ml olive oil and 20 g agar. The medium was adjusted to pH 7.0, autoclaved. Then, 10 ml of rhodamine B solution (1.0 mg/ml distilled water and sterilized by filtration) was added. It was then poured into Petri plates and bacterial cultures were inoculated for 3 days at 37°C. The formation of orange fluorescent halos around bacterial colonies visible upon UV irradiation showed lipase activity.

Enzymatique activity calculation: Cellulase, glucanase, protease, chitinase activities were measured by the following formula : EA= D – d where D was the diameter of colony plus clear zone ; d the diameter of colony.

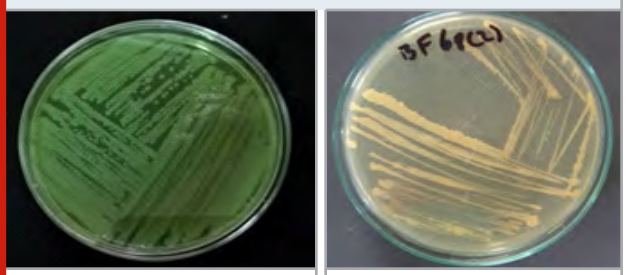
RESULTS AND DISCUSSION

Isolation and Identification: From seven hundred and six bacterial strains isolated from mango fruits and rhyzospheric soil, seventy five isolates were identified as *Pseudomonas spp*. and *Bacillus spp*. (figure 1). Then twenty isolates from them were screened and selected on the basis of they capacity to produce hydrolytic enzymes.

Enzyme activities: It is well known that bacteria commonly produce cell wall-degrading enzymes to hinder the growth of other micro-organisms (Shoda 2000). Further, our results proved that the twenty selected bacterial isolates have shown their ability to produce different cell wall hydrolytic enzymes (figure 2) and each of them produce at least three different enzymes. From them, sixteen Bacillus spp. designated BF68(2) , BF10, BF92, BF47,BF24, BF75, BF89, BF76, BF106, BF81, BF48 , BF46, B137, B69, B236, B234 and four *Pseudomonas spp*. designated P104, P115, P90, P120 (table 1). Isolation and characterization of rhizospheric bacteria like *Pseudomonas* and *Bacillus* producting hydrolytic enzymes have been reported by other reports. The results of this study are similar with those of Carzola et al., (2007).

They have isolated and selected bacterial strains from avocado trees rhyzosphere. They have selected four *Bacillus* strains and analysed them for their ability to produce hydrolytic enzymes. And their results showed that all of them produce hydrolytic enzymes.

Figure 1: *Pseudomonas spp*. (a) and *Bacillus spp*. (b) isolates on Petri dish



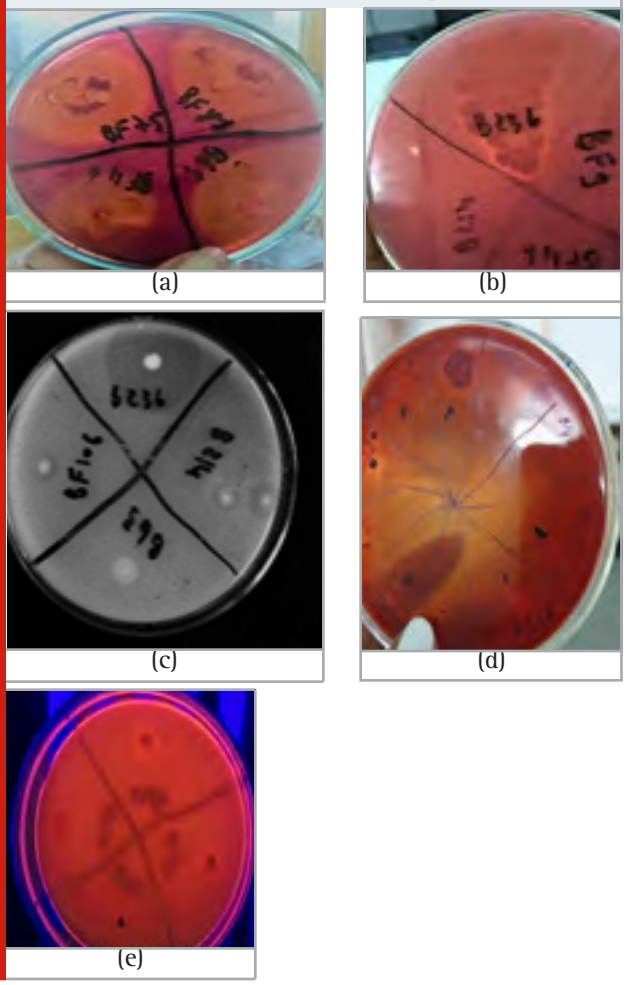
(a) (b)

Table 1. Hydrolytic enzyme activity of bacteria isolated from mango rhizosphere and mango fruits

Isolates	Cellulase	Protease	Lipase	Chitinase	β1,3 Glucanase
BF68(2)	+	+	-	+	+
BF10	+	+	+	-	-
BF92	+	+	+	-	-
BF47	+	+	+	-	-
BF24	+	+	-	+	-
BF75	+	+	+	-	+
BF89	+	+	+	-	-
BF76	+	+	-	-	+
BF106	+	+	+	-	+
BF81	+	+	+	-	+
BF48	+	+	+	-	-
BF46	+	+	-	-	+
BF137	+	+	+	-	+
B69	+	+	+	-	+
B236	-	+	+	+	+
B234	-	-	+	+	+
P104	+	+	+	-	-
P115	-	+	+	-	+
P90	+	-	+	-	+
P120	-	-	+	+	+

Also, Essghaier et al. (2008) have tested differents species of *Bacillus* for their ability to produce hydrolitic enzymes to fight against grey mould of strawberry fruits and the results showed that all the *Bcillus* species were able to produce chitinase, glucanase, protease and cellulase with antifungal activity.

Figure 2: Production of hydrolytic enzyme activity on agar plate
(a): cellulase actuvity ; (b): chitinse activity ; (c): protease activity ; (d): glucanase activity ; (e): lipase activity



The hydrolysis of different substrates: laminarin, colloidal chitin, olive oil, carboxymethyl cellulose, skim milk respectively by β1,3 Glucanase, chitinase, lipase, cellulase, protease induced the appearence of halo around the colony on agar plates. For the different enzymes tested, halo diameter ranged beetwen 10 and 31.33 mm. Isolates BF68(2), BF24, and BF81 have showed the highest halo for cellulase activity with 31.33 mm. BF68(2) and B236 have showed the highest protease activity with halo of 25.66 mm. For chitinase activity, the highest activity has been shown by B236 with 12 mm of halo. The highest β 1,3 Glucanase activity has been shown by BF68(2), BF76, BF75, BF46, B69, B236, B234 and P115 with 11 mm of halo.

Table 2. Diameter of enzyme activity

	Diameter of enzyme activity (mm)			
Isolate	cellulase	Protease	chitinase	glucanase
BF10	10	10	nd	nd
BF68(2)	31.33	25.66	10	11
BF92	23.33	21.66	10	nd
BF47	nd	20	nd	nd
BF24	31.33	16.66	10	nd
BF75	30	10	nd	11
BF89	21.33	19	nd	nd
BF76	22.33	10	nd	11
BF106	24.66	10	nd	10
BF81	31.33	21.66	nd	10
BF48	24.	10	nd	nd
BF46	21.66	18	nd	11
B137	16	14	nd	10
B69	10	11	nd	11
B236	nd	25.66	12	11
B234	nd	Nd	12	11
P104	12	10	nd	nd
P115	nd	10	nd	11
P90	13.33	nd	nd	10
P120	11.66	nd	nd	10

Kye nd : Undetermined

CONCLUSION

Bacillus spp. and *Pseudomonas spp*. strains isolated from mature and healthy mango fruits and from rhyzospheric soil of mangoe trees have shown promising biocontrol abilities because of several cell wall degrading enzymes they produced. All the selected bacterial strains were able to produce at least three enzymes out of five enzymes tested. Also some of them could produce all the five enzymes tested and with the acceptable enzymatic activity.

Conflict of intrest: The authors have no conflict of intrest in preparing of this article.

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Isolation and Optimization of Production Conditions for Fungal Cellulases

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ABSTRACT

Cellulolytic enzymes of microbial origin have great industrial importance because of their wide applications in various industrial sectors. Present study was aimed to isolate and screen fungal strains from soil with a potential to produce cellulase enzyme. Fungi from various type of soils were isolated by dilution plating technique on Martin Rose Bengal agar medium. Total 31 fungal cultures were isolated using CMC agar medium. 12 isolates developed zone of clearance surround colonies upon congored treatment out of which, JRF15 produced largest zone of hydrolysis showing Colony Index (CI) value of 4.2. The positive isolates in the primary screening showing higher CI values were screened quantitatively for the production of cellulases at shake flask level using Mandels medium. Cellulase production was measured in terms of endoglucanase (EG) or 1, 4-β-d-glucan-4-glucanohydrolase (CMCase) and FPase. The best cellulolytic fungal isolate JRF15 produced FPase activity of 0.151 IU/ml after 96 h and CMCase activity of 0.40 IU/ml after 72 h at 28°C and pH 5.5. Optimization of environmental factors like temperature (28°C, 37°C and 45°C), pH (4 to 8) and nitrogen source [(NH_4) SO_4, yeast extract, peptone, urea and combinations of ammonium sulphate and Yeast extract, ammonium sulphate and peptone, ammonium sulphate and urea] were studied under submerged culture condition. After optimization the highest activities of FPase (0.896 IU/ml) after 48 h and endoglucanase (0.680 IU/ml) after 72 h at 28°C and pH 7 with ammonium sulphate and Urea as a nitrogen source were obtained in submerged condition with 10 g/l of CMC.

KEY WORDS: CELLULOSE, CELLULASE, ENDOGLUCANASE, LIGNOCELLULOSIC MATERIALS, SUBMERGED FERMENTATION.

ARTICLE INFORMATION

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INTRODUCTION

The success of exciting applications of microbial cellulase depends on the ability to produce cellulase economically. Optimization of media for high productivity will signify the strain on cost of cellulase production which ultimately allows cost-effective applications in various fields (Mohite et al., 2012). Lignocellulose could be utilized by conversion through cellulolytic enzymes. Cellulose is degraded by combined action of endo-β-1,4-glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). All these three enzymes act in synergistic way on cellulose and broken down into simpler sugar like glucose (Watanabe and Tokuda, 2010).

Fungi can be easily grown in a medium comprising salt as nutrients and a protein source for healthy growth and spore formation (Mandels and Reese, 1999). In recent years, cellulolytic and hemicellulolytic fungal and bacterial cultures are extensively studied for utilizing forest waste, sugarcane bagasse, avicel, xylan, rice straw, CMC, and wheat straw as a source of cellulose. Among these microbial cultures, fungus strains in the genus *Aspergillus, Trichoderma*, Penicillium and Fusarium are extensively studied because of their higher activities (Ahamed and Vermette, 2008). This study was planned to screen and identification of potential novel cellulolytic fungus and further production optimization, purification and characterization of carboxymethyl cellulase in submerged fermentation.

MATERIALS AND METHODS

Sample collection and Isolation of fungi: Samples were taken from soil and rotting wood of different area of Ahmedabad. Rotting wood was taken from botanical garden of Gujarat University. Soil samples were taken from botanical garden soil, farm soil and agriculture waste dumping soil. All samples were collected in sterilized polyethylene bags, labelled and stored at 4°C for further analytical work. One gram of each soil sample was serially diluted ten times in sterile distilled water. 100 µl suspension was spread on potato dextrose agar (PDA) and Martin Rose Bengal agar medium and incubated at 28°C for 7-10 days. To prevent the growth of bacteria, 30 mg/l Streptomycin was added in Sterilized potato dextrose agar (PDA) plates. The plates were used for isolation purpose and after purification all isolates were kept on PDA slants at 4°C.

Primary Screening for Cellulase Production: Primary screening was done by inoculating the fungal isolates on CMC agar medium with 1% (w/v) Carboxy Methyl Cellulose as a carbon source. Plates were incubated at 28°C for three days. After three days of incubation, the plates were kept at 50°C for 30 min. for enzyme activation and then after plates were flooded with 1% Congo red solution for 10 min. followed by washing with 1N NaOH. The plates were observed for the clear zone of hydrolysis surrounding the colony (Bai et al., 2017).

Cellulase Production under Submerged Fermentation: From primary screening, fungal isolates showing positive detection were selected for the quantitative determination of cellulase production by submerged fermentation.

Inoculum Preparation and cellulase production: For preparation of fungal inoculum for fermentation, 2 ml of sterile distilled water containing 1.0 g/l of Tween 80 was introduced into the sporulated slants of each fungus and the spores were dislodged into the liquid by vigorous shaking. Standard amount of inoculum (1ml) containing 107 spores/ml counted by hemocytometer was transferred into a 250 ml Erlenmeyer flask containing 100 ml of Mandels medium (Mandels and Reese, 1957) with an initial pH of 5.5, supplemented with 10 g/l CMC (Carboxy methyl cellulose) as carbon source and maintained at a constant agitation of 120 rpm at 28°C. The composition of production media was KH_2PO_4: 2 g/l, $CaCl_2 \bullet H_2O$: 0.3 g/l, $MnSO_4 \bullet H_2O$: 0.0016 g/l, $(NH_4)\ SO_4$: 1.4 g/l, $FeSO_4 \bullet 7H_2O$: 0.005 g/l, $ZnSO_4 \bullet 7H_2O$: 0.0014 g/l, Yeast extract: 1 g/l, Peptone: 1 g/l, $MgSO_4 \bullet 7H_2O$: 0.3 g/l. The flasks were autoclaved at 121°C for 30 min. and cooled at room temperature, inoculated and incubated at 28°C on a shaker at 120 rpm for 7 days.

Enzymatic Assays: Culture broth was harvested at every 24 hours interval and it was centrifuged at 10,000 rpm at 4°C for 20 min. The supernatant was used as the crude extracellular enzyme source for enzyme assay. Enzyme activity was examined according to the DNSA (3, 5- dinitrosalicylic acid) methods and it was recommended by the International Union of Pure and Applied Chemistry (IUPAC) commission of Biotechnology. The DNS method was followed for the incubated samples and optical density was read at 540 nm against blank. A standard curve of glucose (1mg/ml) was developed under identical conditions to determine the reducing sugars formed. One unit of cellulase activity of filtrate was expressed as Unit per ml (U/ml), which is defined as the amount of enzyme which liberates 1 µM of reducing sugar per ml per minute under assay conditions (Saxena et al 2015). Both endoglucanase and filter paper activity measurement were adapted from the procedure developed by Ghose (Ghose, 1987).

Endoglucanase activity was measured against 1% Carboxy methyl cellulose (CMC) solution prepared in 0.05 M sodium citrate buffer (pH 4.8). 0.5 ml of CMC solution was incubated with 0.5 ml of the test enzyme solution at 50 °C for 30 min. And after that 1000 µl of DNSA reagent was added to terminate the reaction and incubated at 100°C for 5 min. and further diluted with 10 ml distilled water. The reducing sugar concentration produced from the enzymatic reaction was then measured at 540 nm. Filter paper activity (FPase) was measured by using 50 mg of Whatman No. 1 filter paper, used as a substrate. 500 µl test enzyme solution and 1000 µl of citrate buffer (0.05 M, pH 4.8) was incubated at 50 °C for 1 h. The released sugar was measured by DNS method same as endoglucanase estimation. The enzyme activity was measured as U/ml and defined as the amount of

reducing sugar released in µmole per min. per ml of culture supernatant.

Optimization of Parameters for Cellulase Production: Various process parameters such as, incubation temperature (28 °C, 37 °C and 45 °C), medium pH (4 to 8), and different nitrogen sources [Urea, (NH_4) SO_4, peptone, yeast extract and combination of (NH_4) SO_4 and Yeast extract, (NH_4) SO_4 and peptone, (NH_4) SO_4 and urea] were optimized for maximum production of cellulases by JRF15 in submerged fermentation.

Effect of pH on Enzyme Production: Production media containing CMC (Carboxy methyl cellulose- 1%, w/v) was used for enzyme production with different pH varying from 4 to 8 in shake flask maintained at a constant agitation of 120 rpm at 28 °C. pH was adjusted to required value manually on daily basis (one time in a day) by adding sterilized HCl/NaOH inside laminar hood using a sterile pH electrode connected with a pH meter (Bai et al 2017). Samples were taken at every 24 h interval for estimation of enzyme activity.

Effect of temperature on Enzyme Production: Production media containing CMC (Carboxy methyl cellulose- 1%, w/v) was used for enzyme production at different temperature viz. 28°C, 37°C and 45°C at shake flask maintained at a constant agitation of 120 rpm. Samples were taken at every 24 h interval for estimation of enzyme activity.

Effect of nitrogen source on Enzyme Production: Production media containing CMC (Carboxy methyl cellulose- 1%, w/v) were used for enzyme production with different nitrogen source as Urea, $(NH_4)SO_4$, peptone, yeast extract and combinations of $(NH_4)SO_4$ and Yeast extract, $(NH_4)SO_4$ and peptone, $(NH_4)SO_4$ and urea in shake flask maintained at a constant agitation of 120 rpm at 28°C. Samples were taken at every 24 h interval for estimation of enzyme activity.

RESULTS AND DISCUSSIONS

Isolation of Fungi from different samples: Potato Dextrose Agar medium and Martin's Rose Bengal agar medium were used for isolation of fungi which was implemented by Serial dilution method. Many colonies of fungi were developed on the medium. Individual colonies of Fungi were shown in Figure 1.

Sub-culturing and Preservation: Thirty-one isolates JRF1 to JRF31 (from farm soil, garden soil, rotting wood and agricultural waste dumping soil) were randomly selected on the basis of difference in their plate morphology. The pure fungal isolates were subcultured and preserved on slants and petriplates of Potato Dextrose Agar Medium (PDA).

Primary screening of isolates for Cellulase Production: For Cellulase Production, the fungal isolates were screened by visual detection of the clear zone around the fungal colonies, which formed on plates containing CMC agar media in presence of congored dye (Figure 2). Colony Index (CI) has been studied in CMC agar plates. After observation of fungal isolates, total twelve fungal isolates (Table-1 and Figure 2) gave clear zone for cellulolytic activity and these all fungal isolates were selected for cellulase production. Among all fungal isolates, JRF15 produced largest zone of hydrolysis showing CI value of 4.2. The positive isolates in the primary screening showing higher CI values were screened quantitatively for the production of cellulases at shake flask level using Mandels medium.

Secondary Screening for cellulase Production by Submerged Fermentation: Cellulase production was measured in terms of endoglucanase (EG) or 1, 4-β-d-glucan-4-glucanohydrolase (CMCase) and FPase (filter paper) activity. The uniform spore suspension of selected fungal isolates was inoculated into production media under controlled conditions. Samples were taken at every 24 h and growth of fungal biomass and cellulase activities were observed. JRF15 showed a peak in the volumetric endoglucanase CMCase) activity at 72 h (0.68 U/ml) and FPase activity (0.896 U/ml). This result was similar to that observed for T. reesei QM9414 but with a slightly higher activity value of CMCase (0.86 U/ml) and FPase (0.083U/ml). For both species, the volumetric enzyme activity decreased after 96 h. (Santa-Rosa 2018).

Effect of Initial pH on Cellulase Production: The optimal pH varies with different microorganisms and enzymes, for fungal cellulase in most cases it ranges from 3.0 to 6.0. Moreover, it was found that when the pH level increased or decreased than the optimum pH, the enzyme production was reduced (Bai et al 2017). In the present study, the optimum pH for maximum cellulase production was reported at pH 7 (Figure. 3 to 8). Initial medium pH of 5.0 was favorable for CMCase production by *Penicillium sp*. (Prasanna et al., 2016). Kathiresan and Manivannan (Kathiresan and Manivannan, 2010) reported initial medium pH of 6.5 was good for *cellulase production* by *Penicillium fellutanum*. Karthikeyan (Karthikeyan et al., 2010) suggested that some strains of *Pencillium sp*. produced cellulase under acidic pH (3.0).

Effect of Temperature on Cellulase Production: Temperature is a crucial parameter that affects both growth and cellulase production. The temperature normally employed in SFP is in the range of 25°C –35°C (El Bergadi et al 2014; Sajith et al., 2014). In the present study, optimal temperature for maximum cellulase production by the JRF15 was reported at 28°C (Figure. 3 to 8) and reduced at higher or lower temperature. In accordance with our research, Prasanna (Prasanna et al., 2016) stated that incubation temperature of 30°C was optimum for maximum production of CMCase by Penicillium sp in submerged fermentation.

Effect of Nitrogen Source on Cellulase Production: The results stated that strain exhibit ability to utilize various organic and inorganic nitrogen sources efficiently, and the maximum enzyme activity FPase (0.896 IU/ml) after 48 h and endoglucanase (0.680 IU/ml) after 72 h at 28°C

and pH 7 was observed when combination of ammonium sulphate and urea both were used as nitrogen source. However, the enzyme activity was very low when organic nitrogen or inorganic nitrogen sources were used as the sole nitrogen sources (Figure 9). This suggested that medium composition and environmental conditions had strong influence on enzyme production by various strains (Rajoka, 2004). Inorganic nitrogen sources can fulfil the demand of cellulase production by *T. reesei* (Rodriguez-Gomez and Hobley., 2013). Yeast extract was proved to a suitable source of nitrogen for cellulase production by *Penicillium sp*. (Prasanna et al., 2016) and *Penicillium fellutanum* (Kathiresan and Manivannan, 2010). Some studies suggested that combination of peptone and ammonium nitrate was best nitrogen sources for cellulase production by *Penicillium waksmanii* (Han et al., 2009) and Penicillium K-p strain (Karthikeyan et al., 2010).

Figure 1: Microscopic morphology of isolated fungal strains

Figure 2: Visualization of cellulase activity with 1% Congo red treatment

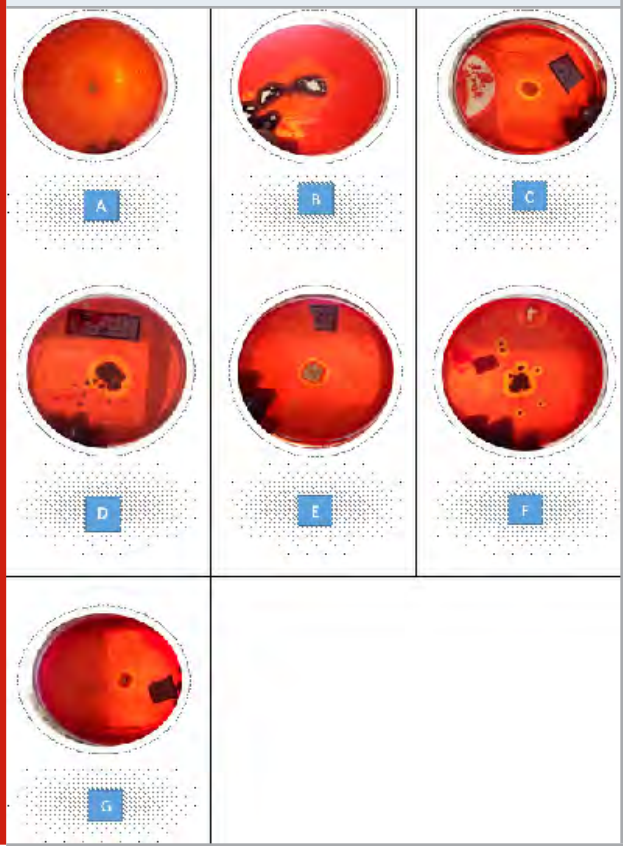


Figure 3: Effect of temperature at 28°C and pH 6.5 on cellulase production by JRF15

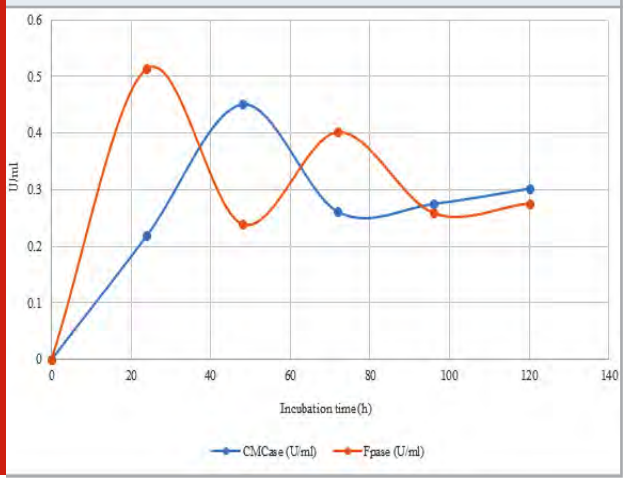


Figure 4: Effect of temperature at 28°C and pH 7 on cellulase production by JRF15

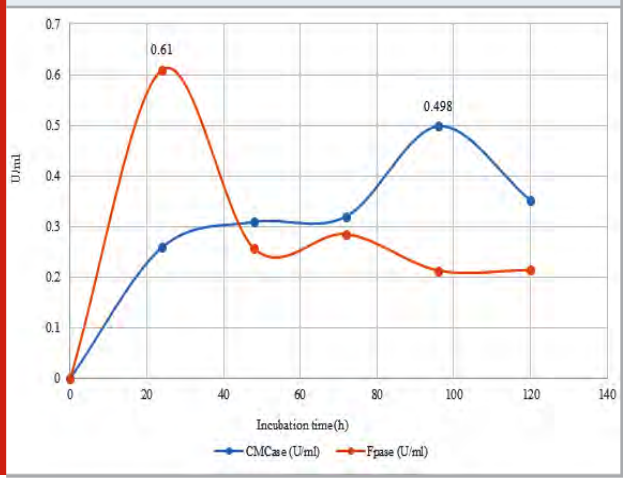


Table 1 Qualitative Screening of Cellulolytic Fungi and their CI (Colony Index) values

S. No.	Isolate	Source	Colony diameter (cm) (B)	Zone of hydrolysis (cm) (A)	CI value CI= A/B
1	JRF5	Rotting Wood	3	3.5	1.2
2	JRF7	Rotting Wood	3	3.5	1.2
3	JRF11	Soil	0,9	1.5	1.6
4	JRF12	Soil	1	4	4
5	JRF15	Rotting Wood	1.2	5	4.2
6	JRF20	Soil	0.9	1.5	1.6
7	JRF21	Rotting Wood	1	2	2
8	JRF23	Soil	1	1.7	1.7
9	JRF26	Soil	0.8	1	1.3
10	JRF27	Soil	0.9	1.2	1.33
11	JRF29	Rotting Wood	0.9	1.9	2.1
12	JRF31	Soil	0.7	1.1	1.6

Figure 5: Effect of temperature at 37°C and pH 6.5 on cellulase production by JRF15

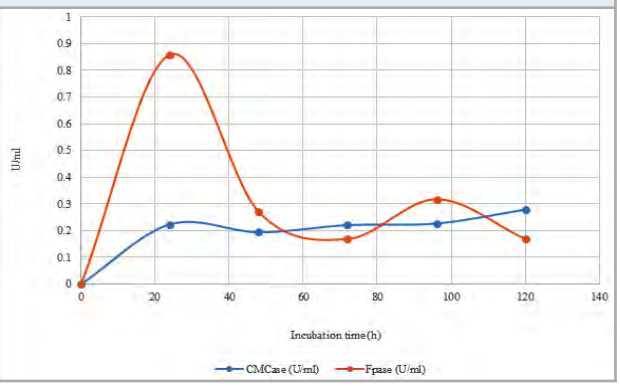


Figure 6: Effect of temperature at 37°C and pH 7 on cellulase production by JRF15

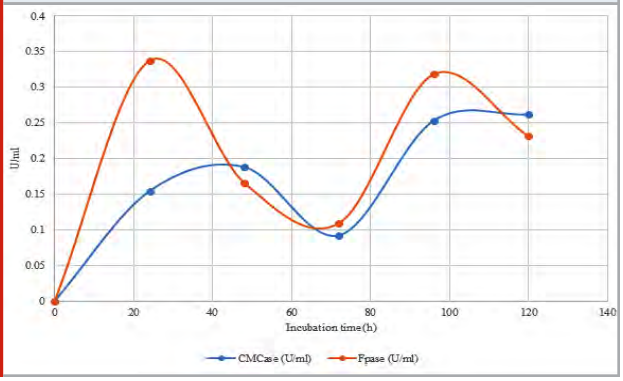


Figure 7: Effect of temperature at 45°C and pH 6.5 on cellulase production by JRF15

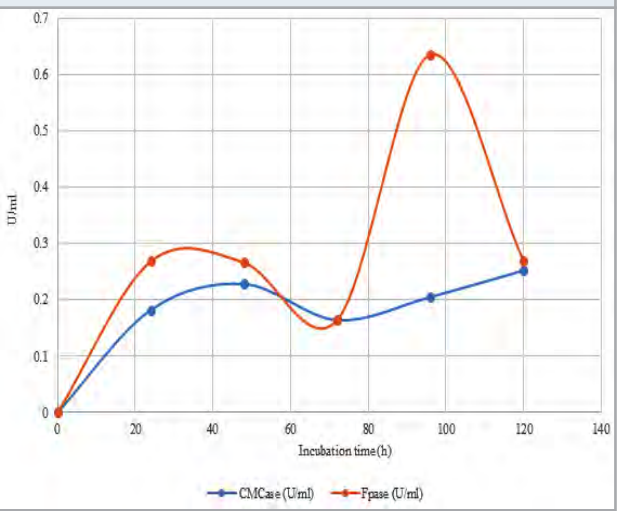


Figure 8: Effect of temperature at 45°C and pH 7 on cellulase production by JRF15

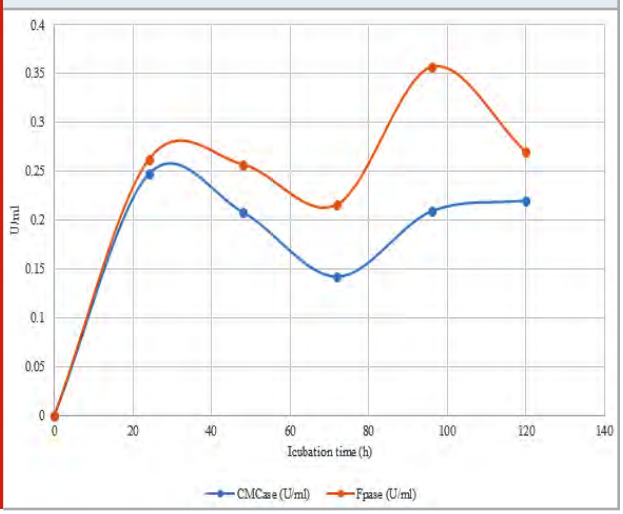
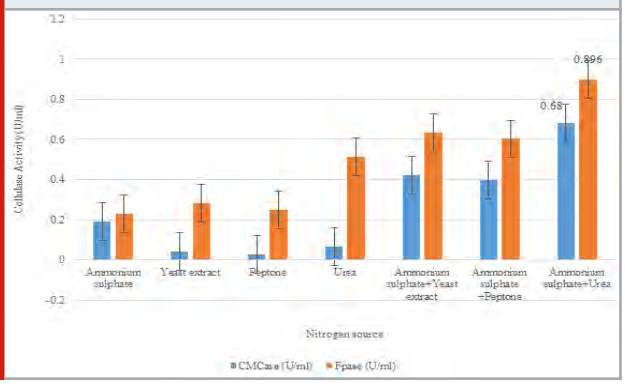


Figure 9: Effect of nitrogen source on cellulase production by JRF15



CONCLUSIONS

Fungal cellulases have excellent industrial applications. The present study was carried out to evaluate the cellulolytic activity of fungi isolated from the fungal flora of different soil samples and rotting wood. Screening and determination of cellulase enzyme activity revealed that isolate JRF15 is promising candidate for large scale cellulase production. Molecular Characterization of fungal isolate and cellulase production from agricultural waste like wheat straw, rice straw and sugarcane bagasse needs further experimentation in order to improve enzyme secretion, purity and activity. Results of this study demonstrate hydrolytic potential of produced cellulases and make this fungal a potential candidate for further improvements and scale-up studies.

Conflict of interest statement: On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Computational Ligand Receptor Interaction Studies of Antibiotics and Phytochemicals with Penicillin Binding Proteins of Beta Lactam Resistant Microbes Towards New Drugs For MRSA Via Molecular Docking

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ABSTRACT

For several years, bacteria have been developing resistance to antibiotics, such as, Methicillin Resistant Staphylococcus aureus (MRSA), as most antibiotics are not as efficient in curbing the infections making them difficult to control. Therefore, to overcome this problem, the present study involves use of various phytochemicals and in silico docking studies were performed using AutoDock Vina in Chimera and were analysed in Discovery Studio. Penicillin binding proteins (PBP) are thought to be essential for synthesis of cell wall, therefore PBP were integral to the study. Protein preparation and ligand preparation was done to make it suitable for in silico docking and results were analysed. On the basis of binding affinity and hydrogen bonding of antibiotics with phytochemicals at the active site of protein, phytochemicals were compared with antibiotics and the most effective phytochemicals out of all twenty five taken for study were deduced, to propose as a natural alternative to the use of antibiotics. Such study can pave a path to discover phytochemicals as the next generation of antibiotics.

KEY WORDS: MRSA (METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS), PENICILLIN BINDING PROTEINS (PBP).

INTRODUCTION

The multiple antibiotic resistance of methicillin-resistant strains of Staphylococcus aureus (MRSA) has become a major clinical problem worldwide. The life threatening infections of MRSA such as skin and respiratory infections, osteomyelitis, Ritter's disease, endocarditis, and bacteraemia in the developed world has become a global clinical problem and remains a major cause of morbidity and mortality among bacterial diseases (Siddiqui and Koirala, 2018). Staphylococcus aureus have developed resistance against some β-lactams by expressing drug-insensitive Penicillin-Binding Proteins (PBPs) and through the action of β-lactamase which is hydrolysing β-lactam drugs preventing them from acting on their target. Penicillin-binding proteins play a paramount role in bacterial cell cycle, by catalysing the transpeptidation reaction in cell wall construction. In addition to the 3 types of PBP (PBP2A, PBP3, and PBP4) (Figure 4.) MRSA has the potential to express PBP2a, which is resistant to the action of methicillin and some other β-lactams (Rani et al., 2018). Among the structural

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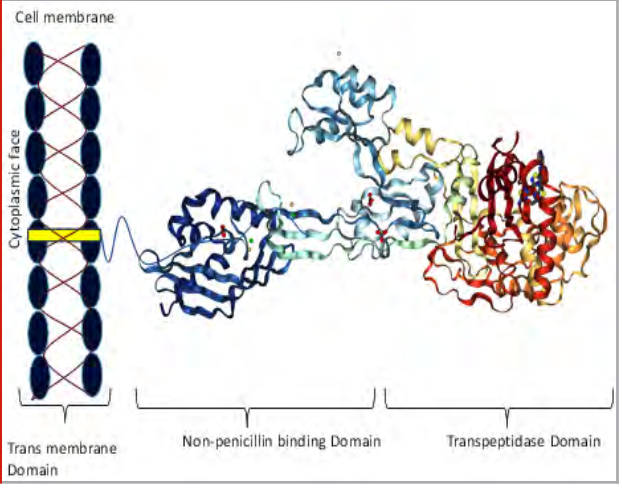
domains, the transpeptidase (TPase) domain plays a crucial role in bacterial life (Figure 1.).

Figure 1: Structure of PBP2a

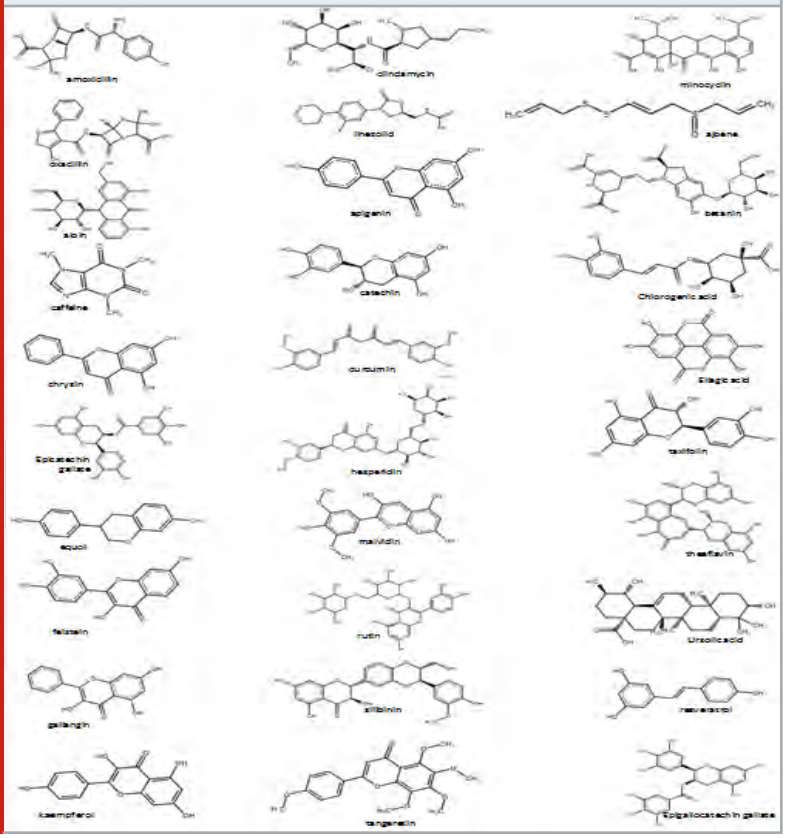


PBP2a is a product of the resistance gene mecA, which functions as a surrogate transpeptidase when other PBPs are inhibited. Owing to the phenomenon of horizontal gene transfer, the menace of multi drug resistance is prevalent, even for the recent antibiotics and thus, the need to find new anti-MRSA therapies is the need of the hour. In the present research, we have set our goal towards studying the possibilities of finding potential PBP2a inhibitors and to predict the 3-dimensional (3D) structure of PBP2a as well as using computational tools. Moreover, to discover potential inhibitors for PBP2a, some phytochemicals were screened in silico using molecular docking, simulations were employed to position the inhibitors into protein active site to find out the most probable binding mode with most reliable conformation. Developed models and docking methods provide insights to design molecules with enhanced activity. Findings from this study indicates that, theaflavin (3,4,6-trihydroxy-1,8-bis[(2R,3R)-3,5,7-trihydroxy-3,4-dihydro-2H-chromen-2yl]benzo[7] annulen-5-one), a phytochemical from black tea, exhibits bioavailability and favourable molecular interaction with amino acids present at the active site of selected bacterial sub-cellular proteins.

MATERIALS AND METHODS

Protein preparation: Selecting a suitable 3D conformation of the molecular target, i.e., the spatial coordinates that define the relative position of each atom within the structure, is a critical step in performing a molecular docking study. One of the most commonly used sources of 3D structural data for biological macromolecules, the Protein Data Bank (PDB), incorporates structures determined by biophysical methods such as X-ray crystallography, NMR spectroscopy, and cryo-electron

Figure 2: Representation of 30 ligands (25 phytochemicals and 5 antibiotics)



microscopy. PDB is freely accessible at http://www.rcsb.org/ and it was used to obtain the 3D structure of the molecular target. 3D crystal structure of PBP2A, PBP3, PBP4 with RCSB PDB code: 4DKI, 3VSL, 5TW8 respectively was downloaded from PDB (http://www.rcsb.com). Dock prep performs several tasks to prepare structures for docking or for other calculations, such as; by (1) removing water molecules, (2) repairing truncated side chains, (3) adding hydrogens, (4) assigning partial charges, (5) writing files in Mol2 format.

Ligand Preparation: The next critical step is the selection of the set of compounds that will be used in the molecular docking studies. Usually, freely available virtual databases that encompasses a wide chemical diversity are used. In general, these compounds repositories are interactive interfaces that allow the application of chemical filters to search and select focused subsets, representing a specific chemical space. Molecules in these databases are stored as line notations (e.g., SMILES, SMARTS, or InChI files), which are automatically converted into 3D structures with appropriate ionization states, partial charges, and stereochemistry once downloaded (Figure .2)

When no information is available about known active ligands or when the investigated target is known for interacting with different chemical classes, general libraries containing widely diverse chemotypes can be used. This type of collection generally contains hundreds of thousands (or millions) of entries. Some examples of these general and large repositories are the ChemSpider (http:// www.chemspider.com/) and ZINC (http://zinc.docking.org/) databases. In present study, a total of 25 phytochemicals (Table. 1) from different plant sources were retrieved from the SMILES or CID ID and were screened against the active site pocket of PBP2a. Ligand minimization wizard in Chimera was used to prepare the ligands for docking that included, addition of hydrogen, optimization, and minimization of binding energy. Finally, for the 30 ligands (25 phytochemicals and 5 antibiotics), a total of 180 conformations were generated and used for docking.

Docking of ligand molecules with PBP2a protein: The next phase consists of docking the selected compounds into the target binding site. The conformational search routine probes the energy landscape of each compound, and those compounds ranked as promising hits are selected for post-docking analysis, the next stage of this process. This procedure involves visualizing the predicted ligand–receptor complexes and enables the examination of critical features, such as binding conformations and intermolecular interactions. The analysis of these elements is useful for deciding the top-scoring compounds that have to be prioritized for experimental studies. Molecular docking studies are extensively used to study the interaction between two molecules, in addition to finding the suitable orientation of ligands, which would form a complex with overall minimum energy.The amino acid residues at active site of the receptor protein undergoes conformational changes upon docking with its respective ligand.

Figure 3: Multiple sequence alignment of protein sequences of 4dki, 3vsl, 5tw8

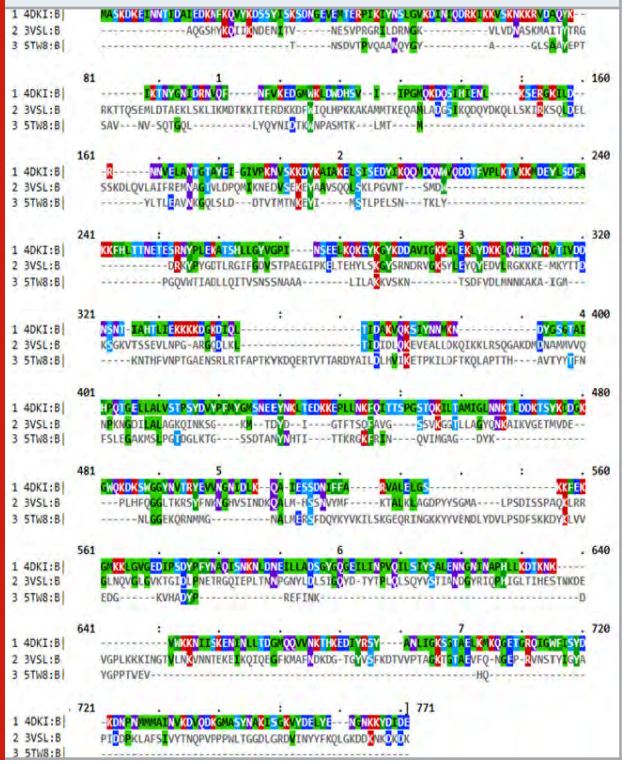
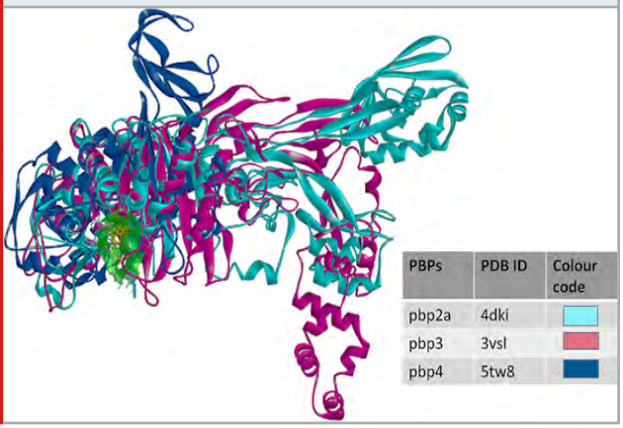


Figure 4: Representation of the active site of penicillin binding proteins.



The ligand, along with active site residues, has to be provided flexibility in order to find optimum fit at the active site thereby, increasing the amount of data to be analysed. The active site is, therefore assumed to be rigid and flexibility is only provided for the ligand, allowing prompt and efficient screening of ligands. Post docking, the binding affinity between receptor and ligand can be determined using molecular mechanics force fields. The various components such as; solvent effects, conformational changes in the protein and ligand, free energy due to protein-ligand interactions, internal rotations, association energy of ligand and receptor to form a single complex and free energy due to changes in

vibrational modes, contribute to the binding free energy (ΔG) (Shen et al. 2013).

Figure 5: Hydrophobic cavity showing ligand interaction and their comparative orientation of superimpose at the active site of the protein 4dki.

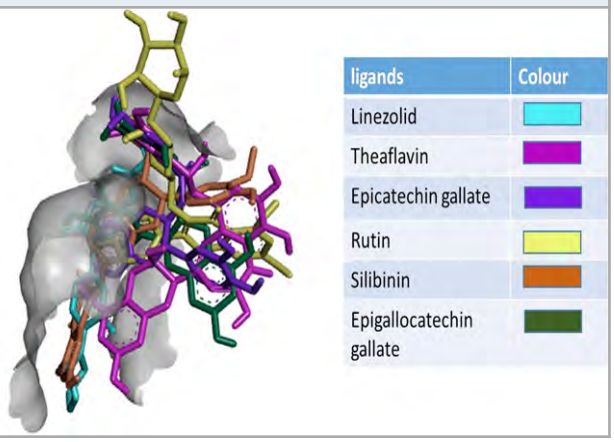


Figure 6: Representation of top 2 phytochemicals (Theaflavin and Epicatechin gallate) compared with antibiotic linezolid

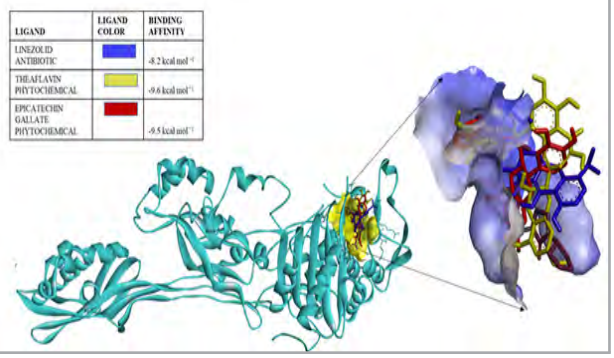
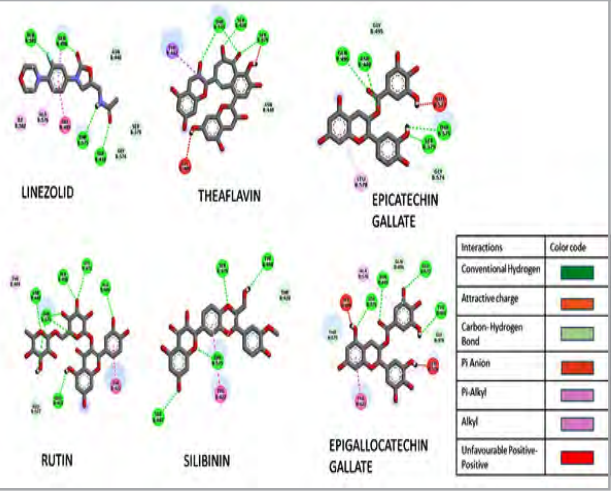


Figure 7: 2D interaction diagram of hydrophobic interaction between PBP2A (4DKI) and ligands using discovery studio program



The sum of energies such as; lipophilic, hydrogen bonding, metal interaction, rotatable bond counts and salvation contribute to the docking score whereas, the glide energy is binding free energy. The best conformation as well as ligand were chosen based on the docking score and glide energy. Molecular docking analysis was performed by using Chimera v1.11.

Table 1. Different sources and classification of phytochemicals

Phytochemicals	Classification	Source
Ajoene	Organosulfur	Garlic
Aloin	Anthraquinone	Aloe Vera
Apigenin	Flavone	Red Wine
Betanin	Betalains	Beet Juice
Caffeine	Methylxanthine	Espresso
Catechin	Flavanol	Mimosa Catechu
Chlorogenic Acid	Phenolic Acid	Potato
Chrysin	Flavone	Passiflora Incarnata
Curcumin	Flavanonoe	Turmeric
Ellagic Acid	Phenolic Acid	Strawberry
Epicatechin Gallate	Flavanoid	Green Tea
Epigallocatechin Gallate	Polyphenol	Apple Skin
Equol	Isoflavone	Soybean
Fisetin	Flavanoid	Cucumber
Galangin	Hydroxyflavonol	Alpinia Galanga Plant
Hesperidin	Flavanone	Lemon
Kaempferol	Flavonol	Broccoli
Malvidin	Anthocyanidin	Blueberries
Resveratrol	Stilbenoid	Peanuts
Rutin	Flavonoid	Fig
Silibinin	Flavanolignan	Silybum Plant
Taxifolin	Flavanonols	Cocoa
Tangeretin	Flavone	Mandarin Orange
Theaflavin	Polyphenol	Black Tea
Ursolic Acid	Terpenoid	Lavender

Docking Parameters: Having prepared the ligand and receptor input files, ligands will be docked into the binding cavity of β-chain of 4dki. Using Chimera, open the 3D structure of ligands (ligand.pdb) and go to Tools → Surface/Binding Analysis → AutoDock Vina. A new window will pop up with several options describing the parameters that will be used for docking. In the Output file section, enter a name for the file that will record the final docking results in the previously created Docking folder, and click on Set Output Location. In the Receptor and Ligand options, choose the receptor and ligand to be used (these are 4dki and ligand.pdb, respectively).

Table 2. Virtual screening results of interactions of various ligands (antibiotics and phytochemicals) with 4dki.

Ligands Antibiotics	H Bonds	Hydrophobic Interactions	Energy Kcal/mol
Amoxicillin	Thr:420, Asn:440, Ser:438, Ser:379, His:558	Thr:575, Ala:606, Lys:382	-7.8
Clindamycin	Ser:379, Tyr:494, Asn:440, Gln:496	Ala:576, Glu:577, Tyr:422, Thr:575	-6.8
Linezolid	Gln:581, Gln:496, Thr:575, Ser:438	Ile:582, Ala:576, Gly:495, Asn:440, Ser:379, Gly:574	-8.2
Minocycline	Thr:575, Asn:440, Ser:379, Tyr:494, Gln:496	Tyr:422, Leu:578, Thr:420, Lys:382	-8.0
Oxacillin	Tyr:422, Asn:440, Thr:575	Tyr:494, Lys:382	-8
Ligands Phytochemicals	H Bonds	Hydrophobic Interactions	Energy Kcal/mol
Aloin	Thr:420, Asn:418, Tyr:417, Gln:496, Lys:579, Leu:578	-	-6.9
Apigenin	Lys:382, Ser:438, Ser:379, Asn:440	His:558, Tyr:422, Thr:575	-7.8
Betanin	Thr:420, Tyr:494, Asn:418, Gln:496, Tyr:417, Asn:440	Thr:575, Lys:382, Ser:438, Tyr:422	-8.8
Caffeine	Thr:420, Tyr:494, Asn:418, Gln:496, Tyr:417, Asn:440	Tyr:494, Asn:440, Thr:575	-5.7
Catechin	Gln:496, Tyr:494, Asn:440	Gly:378, Gly:494, Tyr:422	-7.8
Chlorogenic Acid	Ser:437, Thr:420, Gln:496, Thr:575, Asn:440, Ser:379, Lys:382	-	-7.6
Chrysin	Asn:440, Lys:382, Ser:379, Ser:438	Thr:575, Tyr:422, His:558	-7.9
Curcumin	His:558, Ser:573, Gln:496	Tyr:422, Gly:574	-7.5
Ellagic Acid	Asn:440, Ser:379, Ser:438, His:558, Ser:573	Tyr:422, Thr:575	-8.7
Epicatechin Gallate	Gln:496, Asn:440, Thr:575, Ser:379	Gly:495, Glu:577, Gly:574, Leu:578	-9.5
Epigallocatechin	Ser:379, Asn;440, Glu:577, Tyr;494	Thr:575, Ser:438, Ala:576,	-9.1
Gallate Equol	 Lys:382	Gln:496, Gly:378, Leu:578, Tyr:422 Tyr:422, Ser:438, Thr:575	 -6.6
Fisetin	Asn:440, Ser:438, Ser:379, His:558, Ser:573	-	-7.8
Galangin	Asn:440, Ser:379, Thr:575	Tyr:422	-7.6
Hesperidin	Ser:438, Ser:379, Lys:382, Asn:440	Tyr:422, Thr:575	-7.2
Kaempferol	Tyr:494, Ser:379, Glu:577	Ser:576, Ala:576	-7.8
Malvidin	Ser:573, His;558, Ser:379	Tyr:422, Thr:575, Asn:440	-7.5
Resveratrol	Ser:379, Ser:438, Tyr:422	Thr:575	-7.3
Rutin	Asn:440, Thr:575, Ser:438, Lys:572, Ala:606, Glu:423	Tyr:494, Glu:577, Tyr:422	-9.4
Silibinin	Ser:379, Tyr:494, Thr:575, Ser;607	Tyr:422, Thr:420	-9
Taxifolin	Ser:379, Asn:440, Thr:420, Gln:496	Gly:378, Gly:495, Ala:576, Gln:581, Thr:575	-7.8
Tangeretin	Ser:438, Ser:379, Gln:496	Glu:577, Thr:575, Tyr:422	-7.1
Theaflavin	Thr:575, Ser:438, Ser:379	Tyr:422, Asn:440, Lys:406	-9.6
Ursolic Acid	Thr:575, Gln:496	Leu:578, Tyr:422	-8.4

Over the blank fields in the Receptor search area, enter the X, Y, Z cartesian coordinates. The next field, named Size, defines the length in A˚ of each side of the box that will delimitate the binding site. The optimal value for this parameter changes according to the volume and geometry of the binding site of the system under study.

In general, this parameter should include all residues that could engage in intermolecular interactions with the ligand; however, it should not be excessively large because that could cause staggering of the conformational search. After filling the parameters for the binding site, a wire-frame box showing the selected binding site region will appear. Next, we should consider the parameters for receptor and ligand handling. First, the option add hydrogen in Chimera should be ignored, as we already set up protonation states during receptor preparation. Furthermore, because Autodock Vina considers hydrogen atoms bound exclusively to polar atoms to compute the molecular interactions, enable Merge charges and remove non-polar atoms and Merge charges and remove lone pairs in both receptor and ligand options. The protein backbone is in cartoon representation. The ligand, water and residues in the binding site are depicted as sticks crystallographic water molecules in the binding site of 4dki by setting the option Ignore waters to false.

Table 3. Comparing binding affinity of PBP with ligands

LIGANDS	PBP2A	PBP3	PBP4
ANTIBIOTICS			
Amoxicillin	-7.8	-6.7	-6.8
Clindamycin	-6.8	-7.5	-6.4
Linezolid	-7.7	-7.3	-7.3
Minocycline	-8.2	-6.8	-9.7
Oxacillin	-8	-7.5	-5.8
PHYTOCHEMICALS			
Ajoene	-4.5	-4.1	-4.5
Aloin	-6.9	-7.1	-5.9
Apigenin	-7.8	-6.7	-7.1
Betanin	-8.8	-8.8	-7.1
Caffeine	-5.7	-5.5	-5.4
Catechin	-7.8	-7.4	-6.6
Chlorogenic acid	-7.6	-7.5	-7.5
Chrysin	-7.9	-7.2	-6
Curcumin	-7.5	-6.8	-6.7
Ellagic acid	-8.7	-8.1	-7.6
Epicatechin Gallate	-9.5	-7.3	-8
Epigallocatechin Gallate	-9.1	-8.1	-7.5
Equol	-6.6	-6.6	-6.2
Fisetin	-7.8	-7	-6.7
Galangin	-7.6	-7.5	-6.8
Hesperidin	-7.2	-7.5	-6.9
Kaempferol	-7.8	-7.2	-7.5
Malvidin	-7.5	-7.1	-7.5
Resveratrol	-7.3	-5.9	-6.6
Rutin	-9.4	-8.6	-7.3
Silibinin	-9	-8.3	-7.5
Taxifolin	-7.8	-7.4	-7.1
Tangeterin	-7.1	-6.3	-6.6
Theaflavin	-9.6	-9.1	-6.7
Ursolic acid	-8.4	-8.1	-6.1

Finally, the section Advanced options is used to specify output parameters, such as; (1) maximum number of binding modes generated for each docking analysis, (2) the extension of the conformational search procedures and (3) the energy gap between the accepted solutions. In general, setting higher values increase the probability of finding likely solutions; however, it also increases the computational time. Therefore, we should always consider the available computational resources and the desired duration of the simulation to set those parameters appropriately. Dealing with only one compound with low structural complexity enables the use of maximum allowed values. Chimera interface to AutoDock Vina allows the molecular docking to be run locally or to use a remote web server.

Analysis of Docking: After running the molecular docking procedure, the ViewDock window containing the scores of the predicted binding solutions will open. Each 3D conformation can be observed by clicking on the corresponding score. Each value corresponds to the predicted binding energy, in kcal/mol, calculated by the AutoDock Vina scoring function. Therefore, more negative the value, higher will be the probability of the ligand–receptor interaction occurring in experimental tests. Additionally, the hydrogen bonds for each of the predicted ligand–receptor complexes can be observed in the ViewDock window by selecting the following option: HBonds →Add Count to Entire Receptor. If the simulation led to the correct prediction, you should observe a distinct interaction between the backbone valine 628 amino group and the ligand N3 nitrogen of the 7H–purine ring. Explore other predicted conformations, and look for the interactions in each complex. Having identified all the predicted conformations, we can compare these solutions with the crystallographic binding mode of Molecular Docking and Structure-Based Virtual Screening .To do this, open the original 4dki structure in Chimera by going to File →Fetch by ID and writing its PDB code (4dki) at the blank field. If all steps were performed correctly, the top-scoring conformation should be very similar to the crystallographic one.

RESULTS AND DISCUSSION

MRSA remains a leading cause of bacterial infections worldwide, ranging from minor skin infections to severe conditions such as staphylococcal scalded skin syndrome, infective endocarditis and bacteraemia. It has PBP2a enzyme, which plays a crucial role in the b-lactam resistivity. Due to the low affinity of PBP2a towards the b-lactam antibiotic, it is considered as a prime target for MRSA inhibition. Even though different inhibitors for PBP2a exist, they were reported to have problems such

as side effects and allergy, which necessitate identifying alternatives. (Gould, 2009).

This study may be the first step towards development of more effective drugs before in silico and in vitro studies. The in silico study may demonstrate the potential of ligand and receptor by binding energy score and their activity. Multiple sequence alignment of 3 PBP (4DKI, 3VSL, 5TW8) is performed using Clustal Omega (Figure. 3). The lowest binding energy indicates great potential for bonding between the ligand and its receptor. Therefore, in silico study also provides the bonding information and can be known the potency of its pharmaceutical drugs (Kusuma, and Susilowati, 2018).

Since natural compounds are considered safe, non-toxic and less prone to side effects, in this study (Rani et al., 2018) naturally occurring phytochemicals were selected and validated for PBP2a inhibition. Molecular docking analysis was performed to examine the binding efficiency of phytochemicals and antibiotics towards the PBP2a active site (Rani et al., 2018). On the basis of the AutoDock score, Theaflavin and Epicatechin gallate was predicted as the top ranked compound to bind with the PBP2a active site pocket strongly. Docking results of each of the ligand–PBP2a complexes, including antibiotics, were compared and the variations were observed in the binding affinity of phytochemicals with PBP2a. The binding affinity of phytochemicals was analysed based on the AutoDock score (Rani et al., 2018).

On the basis of the AutoDock score, among 25 ligands a total of five phytochemicals were found to exhibit higher binding affinity (Figure 5.).The protein-ligand binding energy along with AutoDock score was analysesd, where it was found that Theaflavin and Epicatechin gallate intermingled with the active site pocket of PBP2a with a binding energy of -9.6 kcal mol1 and -9.5 kcal/mol (the more negative value the better binding affinity) respectively (Figure 6.) which was highest among other ligands, whereas, the binding energy of linezolid was found to be less (-8.2 kcal/mol) with respect to the Theaflavin and Epicatechin gallate. The binding residues of PBP2a with ligands were also analysed (Rani et al., 2018).

The docking results revealed strong interactions between the PBP2a Theaflavin complex with AutoDock score binding energy of -9.6 kcal mol^{-1}, and 4 H-bonds (Figure 7.) In the case of the PBP2a–linezolid antibiotic complex, the binding energy (-8.2 kcal/mol) was less than that of Theaflavin and Epicatechin gallate. In summary, Theaflavin was found to be the most effective lead candidate (phytochemical) for inhibition of PBP2a mediated MRSA infection.

CONCLUSION

Of the 25 phytochemicals tested, Theaflavin was found to be the most suitable ligand for 4dki receptor on the basis of binding affinity and hydrogen bonding of antibiotics with phytochemicals at the active site of protein. In vitro validation is necessary to propose it as a natural alternative to the use of antibiotics for the control of MRSA.

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Assessment of Fungal Secondary Metabolites as Quorum Sensing Inhibitors: A Computational Study on LasR of *Pseudomonas aeruginosa* and CviR of *Chromobacterium violaceum* Anti-QS Molecules Against LasR and CviR

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ABSTRACT

Current treatment for bacterial infections is largely dependent on antibiotics that inhibit microbial growth and viability. However, due to the adaptability and robustness of cellular processes in microbes, these approaches have resulted in evolution of multi drug resistant strains of bacteria. Therefore, it is quintessential to formulate different strategies against such drug resistant pathogens. A unique phenomenon of extracellular communication, Quorum Sensing (QS), is observed in bacteria for governing biofilm production and other virulence factors to express their pathogenicity. Thus, inhibiting QS can be an effective strategy against pathogens that remains to be exploited. In this study, we aim to find out potential LasR and CviR mediated quorum sensing inhibitors against *Pseudomonas aeruginosa* and *Chromobacterium violaceum* respectively. To find the potential inhibitors, fungal metabolites were used as fungi can be found in all type of habitat and has diverse secondary metabolites many properties. Further, the molecular interactions of receptor with different secondary metabolites was performed in UCSF Chimera and the results were analysed in Discovery Studio. Results suggest that, asnipyrone B, atromentin, rubrofusarin, TMC-256A1 and nigerazine B have potential dock score as compared to its original ligand. -11.3, -9.6, -10.5, -10.3, -10.0 kcal/mol are their score respectively for LasR and for CviR the scores are as -9.2, -9.2, -8.0, -7.3, -7.1 kcal/mol respectively. Generally, lower the energy produced higher is the binding affinity. Overall result suggest that, QSI can interact with LasR and CviR significantly (a LuxR homologue) leading to inhibition of QS. Since homologues of LuxR are inherently found in more than hundred species, such QSIs can pave a path towards the development of anti-infective agents with broad spectrum..

KEY WORDS: BIOFILM, *CHROMOBACTERIUM VIOLACEUM*, QUORUM SENSING, QUORUM SENSING INHIBITORS, *PSEUDOMONAS AERUGINOSA*, VIRULENCE.

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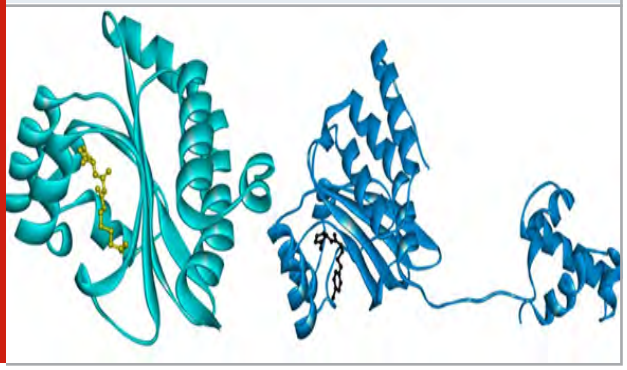
INTRODUCTION

Pathogenic microbes have evolved and developed mechanism for virulence. Different antimicrobial chemotherapy which is effective to various life-threatening diseases are mainly based on inhibition of growth of microorganism. Due to continuous usage of certain drugs for chemotherapeutic purpose it leads to the development of Multiple Antibiotic Drug Resistant (MDR) strains. Recent studies reveal that bacteria use cell to cell communication system known as quorum sensing. This communication system is mediated by the production of small signalling molecule which express different trait. Pathogenic microbes develop biofilms or different pigments which is responsible for the virulence development. This bacterial biofilm formation and virulence gene expression comes from QS. One novel way to get rid of these bacterial infections is by inhibiting or suppressing the cell to cell communications in pathogens (Tiwary et al., 2017).

Gram negative bacteria produces a signalling molecule to mediate QS. There are different classes of signalling molecules namely Acylated Homoserine Lactone (AHL), Furanosyl Borate Diester (FBD) and signalling peptides. From these classes AHL are most important. Their synthesis and detection promote many gram negative bacteria to engage in QS i.e. an intracellular signalling molecule that activates different virulence factor such as biofilm. AHL based signalling system is mediated by two proteins, an AHL synthase (member of LuxI family) and AHL receptor (members of LuxR family of transcriptional regulators). Low level of AHL is produced at less bacterial population density and the level increases at high bacterial population density (Harbottle A et al. 2008). When the value of AHL reaches a threshold concentration the signalling molecule binds to receptor followed by activation of targeted gene which regulates pathogenesis of pathogens.

Researchers all around world are engaged in finding the solutions to the problems emerging due to the usage of chemotherapy against *Pseudomonas aeruginosa* and *Acinetobacter sp*. in host having low immunity. These organisms show deadly threat to the hospitalized patients.

Figure 1: (a) LasR with 3-Oxo-N-[(3S)-tetrahydro-2-oxo-3-furanyl]-dodecanamide (OHN) (b)CviR with chlorolactone (HLC)



Mainly this infection is caught by the person who has recently gone through an organ transplant or undergoing cystic fibrosis treatment. Quorum sensing in pathogenic bacteria is highly responsible in biofilm formation and virulence gene expression (Fuqua et al., 2001; Whitehead et al., 2001). One way to fight against these pathogens is by interruption of QS. *P. aeruginosa* has two QS systems, the Las and Rhl; *C. violaceum* has QS system CviI/CviR; both possessing LuxI/LuxR homologue. QSIs involve three different strategies: (1) inhibiting the production of QS molecules, (2) degrading the signalling molecules, (3) blocking the receptor for signalling molecules. At present, the most promising strategy for successful inhibition of QS appears to be the blocking of signalling molecule. QS signal transduction can only be achieved by an antagonising binding of various inhibitors to its native protein (Mendes et al., 2009).

Figure 2: Superimposed ligands bound inside the hydrophobic pocket (LasR)

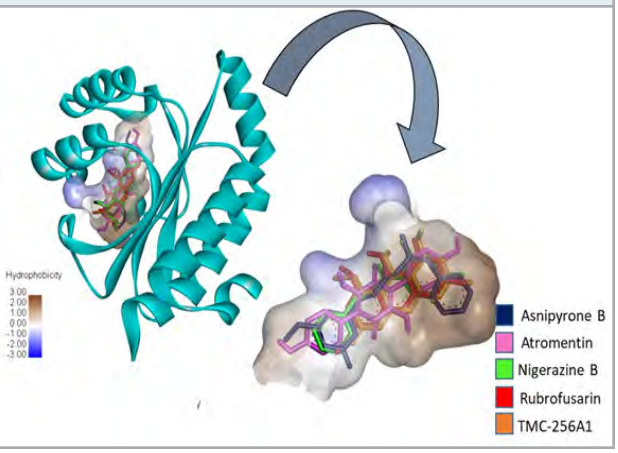
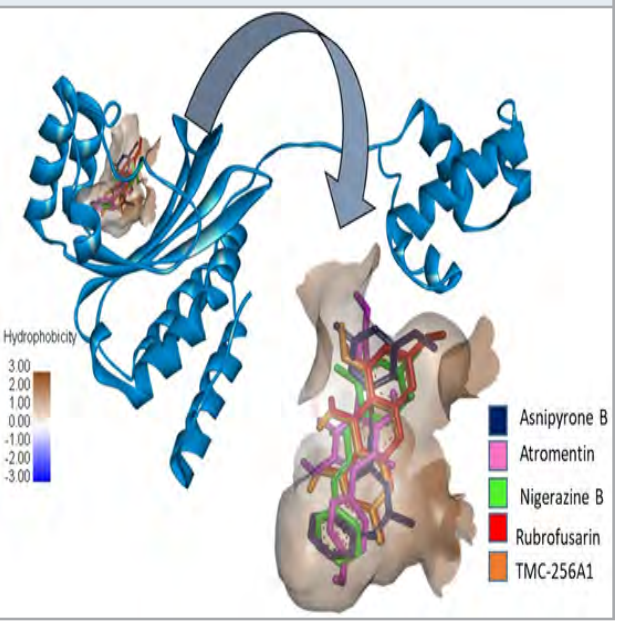


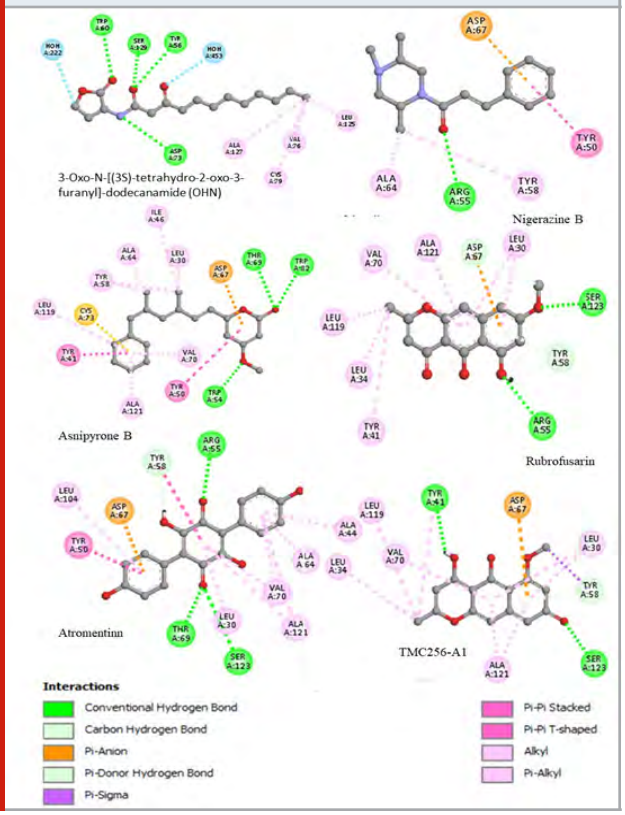
Figure 3: Superimposed ligands bound inside the hydrophobic pocket (CviR)



Various inhibitors of QS apart from the therapeutic antibiotics have been identified. The QSIs show many advantages as compared to the conventional antibiotics. Firstly, the pathogens may not develop resistance to QSIs. Secondly, QS is mainly responsible for the resistance of the bacteria. Thirdly, the LuxI/LuxR has been reported in most gram negative bacteria as using AHL as signalling molecule for QS. Thus, QSIs can show the great competence to the broad range of drugs. This mechanism will have great impact on bacterial resistance and its control. Many molecules have been reported as showing anti-QS properties which are derived from natural sources such as from herbal plants (Milhalik et al., 2008; Hussaini & Mahasneh, 2009). Metabolites derived from various fungi shows these properties. Fungi are source of many products as bioactive molecules, from antibacterial to antiviral, cytotoxic, anti-inflammatory, antioxidant, and many more.

In recent years, fungi associated with marine reveals the presence of Anti-QS metabolites. Many fungal metabolites derived shows medicinal property as they are bioactive compounds. Some of them can potentially be future medicines. Antibiotics are major contributions of fungi towards the human health and other death threats. Our study is focused on various fungal metabolites as a QS inhibitor. It comprises QS and screening of various secondary metabolites produced by fungi subsequent to their QSI activity. Various metabolite inhibitors were analysed for their activity against violacein (pigment) and biofilm that are produced by *Chromobacterium violaceum* and Pseudomonas aeruginosa respectively. Further analysis involves the interactions and binding positions of fungal metabolites within the active sites of CviR and LasR receptor proteins which were studied by molecular docking. To the best of our knowledge this is the first report on the inhibitory properties of various fungal metabolites against QS.

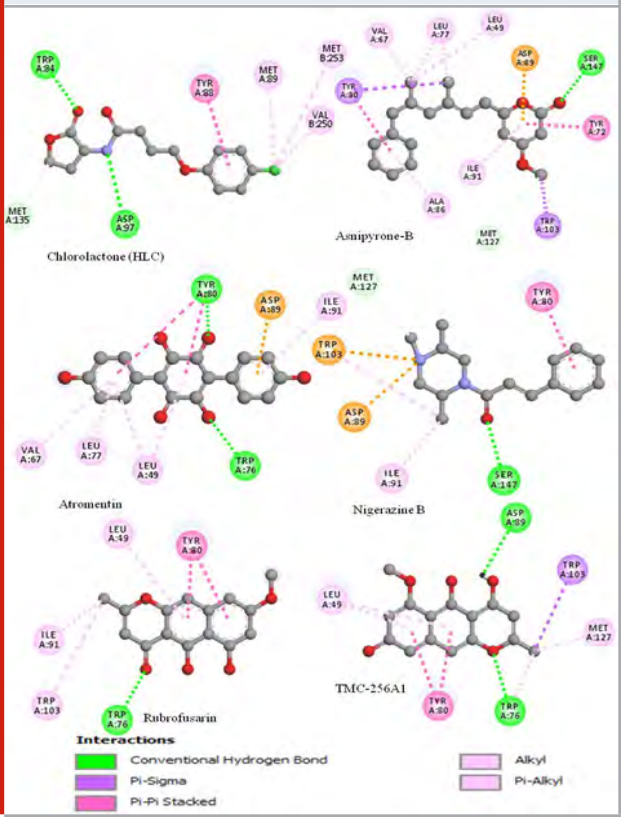
Figure 4: Poses of original and screened ligands with LasR



METHODOLOGY

Molecular Docking Studies: For the structure-based drug design strategy, molecular docking is widely used computational tool in which small molecules i.e. ligand docked into the active site of the tested receptor protein. These docked ligands are further ordered with reference to their binding affinity and positioning in active site. This technique is highly considered in studying protein-ligand interactions. For performing this analysis, the following steps were carried out (i) protein preparation (ii) ligand preparation (iii) in silico docking parameters and (iv) analysis of docking.

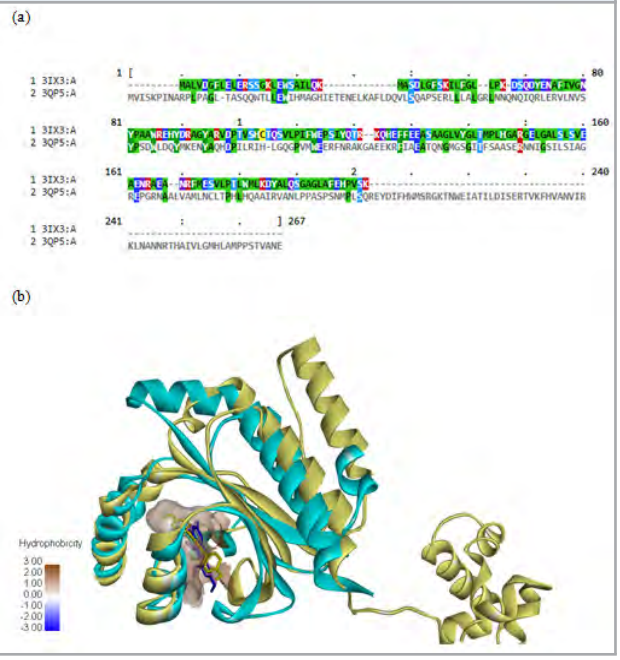
Figure 5: Poses of original and screened ligand with CviR



Protein preparation: For this study, the structures of two proteins, LasR (PDB ID: 3IX3) and CviR (PDB ID: 3QP5), along with 3-oxo-N-[(3S)-tetrahydro-2-oxo-3-furanyl]-dodecanamide and chlorolactone (HLC) were procured from PDB (protein data bank) respectively. Further, the protein structures were prepared in UCSF Chimera v1.13 (Pettersen et al., 2004). The DOCK PREP tool in Chimera performs several tasks to prepare structure for DOCK or for other calculations such as deleting water molecules,

adding hydrogen bonds, assigning partial charges, writing files in mol2 (Krivov et al., 2009). In DOCK PREP several operations are performed on chosen structure such as: delete solvent, delete non-complexes ions, change. Changes takes place as the modified residues are converted in the standard residues for which parameters are: Selenomethionine (MSE) to Methionine (MET) – changes MSE to MET by changing selenium to sulphur and adjust bond length of CG-SD and SD-CE to 1.81 and 1.78Å respectively. Then bromo-UMP (5BU) to UMP (U) in which 5BU changes to RNA residue UMP, methylselenyl-dUMP (UMS) to UMP in which UMS is converted into RNA residue UMP by replacing the methylselenyl moiety with oxygen atom and changes bond length to 1.430Å, methyl-dCMP (CSL) to CMP (C). Further add hydrogens, add charges, write Mol2 file.

Figure 6(a): Sequence alignment of CviR and LasR (b) Superimposed image of protein CviR and LasR with Ligands

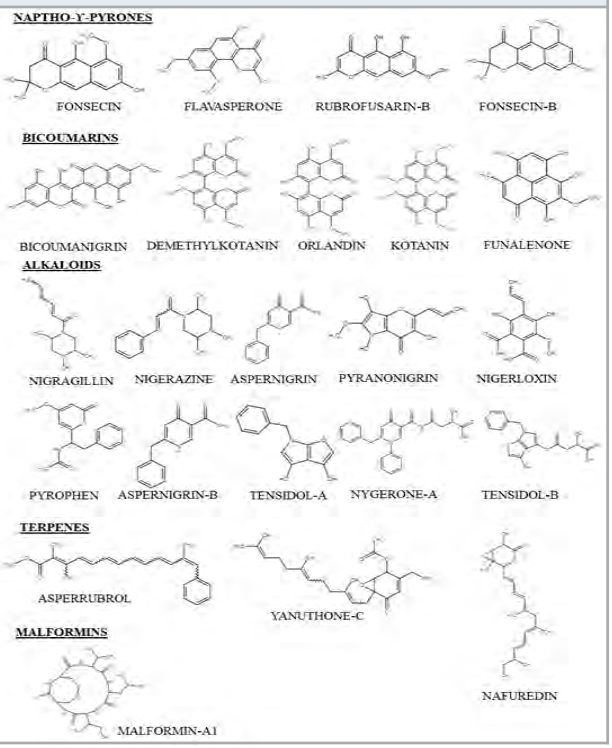


Many of these steps can be performed separately in Chimera but in dock prep option they are combined together for convenience. An extra molecule such as ligand or additional subunits are present but does not required in docking they are deleted before DOCK PREP is used. DOCK PREP does not delete molecules other than water or certain ions as it could be important for binding purpose.

Ligand preparation: Ligand preparation for the docking purpose was done in UCSF Chimera. The structures were built in Chimera under build structure option. Build structure can generate structures "from scratch" or modify existing molecule. There are several ways to start build structure, a tool in structure editing category. Different options such as start structure, modify structure, adjust bonds, adjust torsions, join model, invert and adjust bond angles are found under it. Smile or the PubChem CID were copied from the various sites. Next, those copied Smile were pasted in the build structure tool under structure editing in Chimera. The 3D structure of specific ligands appear and minimize option in the structure editing tool was selected. The electronegativity of ligand induces a change in bond angle and torsional force. The ligand is saved as Mol2.

In silico ligand-protein binding interactions: The experiments were performed for the study of interaction as one with the original ligand of the receptor and others with the molecules selected for QSI purpose. Both the proteins were prepared for docking purpose in Chimera under DOCK PREP tool. The charges were assigned as per AM1-BCC and computed using algorithm known as ANTECHAMBER (Wang et al. 2008). All the ligands used for in silico were retrieved from PubChem. Before performing docking, they were optimized using Gasteiger algorithm (Gasteiger & Jochum, 1979).

Figure 7: Structures of different Classes of Fungal Metabolites used under present study



The Chimera was used to perform receptor-ligand docking with the AutoDock Vina tool (Trot and Olson, 2010). The ligand binding site was chosen according to the crystallized ligand attached to the original protein and the coordinates were recorded. Further, the hydrophobic cavity of LasR and CviR were also determined in Discovery Studio. The AutoDock Vina algorithm was optimized with a set of parameters: (i) number of binding modes- 10; (ii) exhaustiveness of search- 8 and (iii) maximum energy difference- 3 kcal/mol.

Docking Analysis: Out of all the poses so generated, the one with maximum hydrogen bonds and minimum binding free energy change (kcal/mol) was selected.

This selected pose was further evaluated in Discovery Studio for examining the various bond formation by the functional group of ligands with the amino acids of protein. Discovery Studio also shows other hydrophobic interactions made by alkyl chains with the ligand.

RESULTS AND DISCUSSION

CviR structure bound with HLC (PDB ID: 3QP5) in a resolution 3.25Å was reported. CviR is homodiester protein possesses two binding domains, ligand binding domain [LBD] and DNA binding domain [DBD], which forms a cross-domain confirmation. Similarly, the structure of LasR protein directly synthesizes its autoinducer OHN. Its own ligand forms H-bonds with key amino acid residue, Ser129, Asp73, Trp60. Molecular docking was performed to investigate binding potential of the ligand chosen towards LasR and CviR protein. Docking protocol was standardized by the redocking of ligand OHN and HLC (Figure 1). They were able to dock in the active site of the protein. The ligand bound to the protein LasR and CviR is shown in figure A respectively. The co-ordinates were recorded and utilized to dock the other set of ligands.

The structures of asnipyrone B, rubrofusarin, TMC-256A1, atromentin and nigerazine B others were built using build structure tool in UCSF Chimera. Further their structures were optimized. The optimized structure was docked with the receptor using UCSF Chimera (AutoDock Vina tool). The docked result showed many different conformations.

Table 1. H-Bond, Hydrophobic Interactions, Energy in LasR

Ligand	H-Bond	Hydrophobic-Interactions	Energy (kcal/mol)
Asnipyrone B	Thr A:69,Trp A:82,Trp A:54	Ile A:46,Ala A:64,Leu A:30,Tyr A:58,Leu A:119,Cys A:73, Tyr A:41,Ala A:121, Tyr A:50,Val A:70	-11.3
Atromentin	Thr A:69,Ser A:123,Arg A:55	Leu A:104,Asp A:67, Tyr A:50,Ala A:69,Val A:70, Leu A:30,Ala A:121,Ala A:44	-9.6
Nigerazine B	Arg A:55	Ala A:64,Tyr A:58, Tyr A:50,Asp A:67	-10.0
Rubrofusarin	Asp A:67,Ser A:123,Tyr A:58,Tyr A:58,Arg A:55	Tyr A:41,Leu A:34, Leu A:119,Val A:70, Ala A:121,Leu A:30,Aspa :67	-10.5
Tmc-256a1	Tyr A:41,Ser A:123,Tyr A:58	Leu A:119,Leu A:34, Val A:70,Leu A:30,Asp A:67,Ala A:121,Ala A:121	-10.3

Table 2. H-Bond, Hydrophobic Interactions, Energy in CviR

Ligand	H-Bond	Hydrophobic-Interactions	Energy (kcal/mol)
Asnipyrone B	Met A:127, Ser A:147	Ile A:91,Ala A:86,Tyr A:72,Asp A:89,Leu	-9.2
Atromentin	Trp A:76,Tyr A:80	A:77,Leu A:49,Val A:67 Ile A:91,Asp A:89,Leu A:49,Leu A:77,Val A:67	-9.2
Nigerazine B	Met A:127, Ser A:147	Ile A:91,Asp A:89,Trp A:103,Tyr A:80	-7.3
Rubrofusarin	Trp A:76	Tyr A:80,Leu A:49,Ile A:91,Trp A:103	-8.0
TMC-256A1	Trp A:76,Asp A:89	Tyr A:80,Tyr A:80,Leu A:49,Trp A:103,Met A:127,Leu A:49	-7.3

Table 3. Energy (kcal/mol) of metabolites utilized for docking

Ligands	Energy (CviR) (kcal/mol)	Energy (LasR) (kcal/mol)
Asnipyrone-B	-9.2	-11.3
Asperic Acid	-7.4	-7.3
Aspernigrin-A	-8.6	-9.3
Asperubrrol	-6.2	-3.3
Atromentin	-9.2	-9.6
Bms	-7.2	-5.0
2-Carboxymethyl-3-Hexylbutanedioic AcidAnhydride	-7.0	-7.5
Demethylkotanin	-8.2	-4.6
Nigerazine-B	-7.7	-10.0
Fonsecin	-7.8	-9.1
Fonsecin-B	-7.8	9.2
Flavioin	-6.8	-8.3
Orlandin	-8.9	-4.7
Pyrophen	-8.0	-8.5
Kotanin	-7.7	-7.1
Hexylytaconic Acid	-6.2	-7.1
Nigerapyrone-E	-7.2	-7.5
Yanuthone-C	-7.8	-2.8
Fumonosin-B4	-5.2	-2.8
Malformin-A1	-5.0	-3.8
Maformin-C	-4.4	-3.4
Asnipyrone-B	-9.2	-11.3
Flavasperone	-7.1	-9.5
Nafuredin	-7.8	-4.8
Nigerasperone A	-7.8	-9.1
Farnesol	-7.1	-7.6
Funalenone	-7.2	-9.0
Nigerazine-A	-8.3	-9.0
Ochratoxin-B	-7.2	-7.0
Pestalamide-A	-9.2	-8.7
Pyranonigrin-A	-7.3	-8.6
Pyranonigrin-B	-6.7	-9.0
Pyranonigrin-C	-6.8	-8.3
Pyranonigrin-D	-7.1	-9.0
Rubrofusarin	-8.0	-10.5
Tensidol-A	-8.4	-8.4
Tensidol-B	-8.3	-9.5
Tensyuic Acid-A	-6.1	-6.7
Tensyuic Acid-B	-6.1	-7.0
Tensyuic Acid-C	-6.1	-7.3
Tensyuic Acid-D	-6.1	-6.9
Tensyuic Acid -F	-6.2	-6.8
Tmc-256A1	-8.1	-10.3
Nigeragillin	-6.6	-7.6
Nigerloxin	-6.7	-7.6
Nygerone-A	-9.9	-8.7
Yanuthone-C	-7.8	-4.6
3-Methyl-8-Hydroxy-4-Dec.	-7.3	-7.8

The best result of asnipyrone B showed the dock score -11.3 and -9.2kcal/mol in LasR and CviR respectively. Hydrogen bond formation with amino acid of ligand is Thr69, Trp82, and Trp54 for LasR and for CviR. Similarly, in the active site pocket of LasR and CviR receptor asnipyrone B showed H-bond interaction and hydrophobic interactions. Docked score for rubrofusarin for LasR and CviR are -10.5 and -8.0kcal/mol respectively. The superimposed ligand with LasR and in hydrophobic pocket is shown in Figure 2 and the superimposed ligand with CviR and its hydrophobic pocket is shown in Figure 3.

The presence of various ligands in the hydrophobic active sites of CviR and LasR receptors revealed the presence of interactions formed by hydrophobic amino acid residues and aromatic framework. Thus, the molecular docking studies demonstrated the capability of various ligands to hinder the mechanisms of QS. The H-bond and hydrophobic interaction of screened in molecule against LasR and CviR in terms of the docked scores are given in table 1. Table 2 shows the dock score of other molecules chosen for docking purpose. Figure 4 shows the hydrogen bond and hydrophobic interactions of ligand with the LasR receptor. Figure 5 shows the bonds and interactions with the CviR receptor. Figure 6 (a) shows the result of multiple sequence alignment (b) superimposed image of both the protein with ligand bound in hydrophobic pocket.

Due to continuous usage of the therapeutic drugs along with antibiotics, multi drug resistance has emerged as a threat (Soukareih et al., 2018). According WHO's Global Antimicrobial Surveillance System (GLASS) occurrence of MDR infection was found almost in half million people around world. Recent reports confirm serious situations of antibiotic resistance worldwide (Tournimbene et al., 2018). Many cases reported previously the on the deadly infections caused by *C. violaceum* and *P. aeruginosa* showing resistance against broad range of antibiotics. P. aeruginosa also infects patients causing cystic fibrosis and other liver, kidney, respiratory infections (Fantinatti et al., 2004; Justo & Duran, 2017). QS is known for the various virulence factor expression, biofilm formation sporulation. Therefore, it is necessary to develop different strategy for inhibiting it. In *P. aeruginosa* and *C. violaceum* this QS factor is basically LasR and CviR, a LuxR homologue which is responsible for QS. As it synthesizes its autoinducer, when this inducer reaches at high threshold, virulence factor is generated. Inhibition of this will stop the formation of biofilm and pigments (Kim et al. 2015). Several extracts from plants and halogenated furanones produced by marine algae Delisa Pulchra were reported as Anti-QS molecules (Rasmussen et al., 2008; Szabo et al., 2010; Koh et al., 2013).

Though fungi have been described as the producer of various secondary metabolites of therapeutic importance. There are no such reports of using those metabolites against QS. The metabolites of Aspergillus sp. selected for its various medicinal properties. Structures of metabolites respective to their classes are shown in Figure 7 (Pel HJ

et al. 2007; Ray AC and Eakin RE 1975; Henrikson JC et al. 2009). Fungal metabolites of selected strain were subjected to the initial screening for anti-QS activity against C. violaceum and P. aeruginosa. We have short listed some metabolites on the basis of its dock score obtained in molecular docking studies. *Aspergillus sp*. has been reported with possessing anti-microbial, anti-cancer, anti-inflammatory, anti-oxidant and other therapeutic properties. *Asnipyrone B* a metabolite of Aspergillus sp. is a polyketide derived polyene natural product isolated from *Aspergillus niger* (Liu et al., 2011). It is explained that, their molecular docking was conducted to explore different binding conformations of ligand with protein.

CONCLUSION

From all the studies on molecular docking it is deduced that asnipyrone *B, atromentin, rubrofusarin, nigerazine B* and TMC-256A1 show efficient binding to LasR and CviR as opposed to its native ligands OHN and HLC respectively. Its inhibitory effect has to be corroborated by experimental studies so that, it can be used as a natural antibiotic substitute against QS-driven virulence exhibited by Pseudomonas aeruginosa and *Chromobacterium violaceum*.

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Conflict of interest: The authors would like to declare that all the authors have equal contribution.

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Screening, Production and Characterization of Fungal Peroxidase

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ABSTRACT

The production of Manganese dependent peroxidase by white rot fungus *Aspergillus spp*. was studied in solid state and submerged cultures using agriculture and food wastes as substrate like wood chips, wheat straw, saw dust. White rot fungi secrete this enzyme to aid lignin degradation. The aim of this research is to obtain indigenous fungus producing manganese peroxidase from different sources, production of enzyme by Solid state fermentation and submerged fermentation and estimate enzyme activity. Screening was done by plate assay technique using Potato dextrose agar medium with 1.0 % methylene blue and microscopic morphology of fungi was studied by slide culture technique and *Aspergillus spp*. identified. Production of enzyme was done by using Manganese production medium. The enzyme activity was estimated by phenol red as substrate. The highest activities of manganese peroxidase were found in wood chips 1.72 U/mL by submerged fermentation. The best condition of manganese peroxidase activity was cultivation at low moisture (50-70%). Cultures with crude enzyme which contain high manganese peroxidase activity were more powerful to decolorization of dyes like; methylene blue, phenol red.

KEY WORDS: DYE DECOLORIZATION, LIGNIN, MANGANESE PEROXIDASE, PHENOL RED.

INTRODUCTION

White rot fungi are found in nature which have capability to degrade lignocellulolytic materials. Generically, white-rot fungi can be circulated into four groups, according to their ability to produce laccases and peroxidases (LiP, MnP, and VP) (Kuhar et al. 2007) (1) only peroxidases (*Phanerochaete chrysosporium*); (2) only laccase (*Schizophyllum commune*); (3) laccase and at least one of the peroxidases (*Lentinula edodes, Pleurotus eryngii, Ceriporiopsis subvermispora*);and (4) laccase and both peroxidases, MnP and LiP (*Trametes versicolor, Bjerkandera adusta*). The most frequently observed ligninolytic enzymes among the white-rot fungi species are laccases and MnP and the least are LiP and VP (Maciel et al. 2012). Manganese peroxidases belong to the family of oxidoreductases (Martinez et al, 2009; Hammel and Cullen, 2008). Activity of the enzyme was stimulated by simple organic acids, which stabilized the Mn^{3+}, thus producing diffusible oxidizing chelates (Glenn and Gold, 1985). Recently, physiological levels of oxalate in *P. chrysosporium* cultures have been shown to stimulate manganese peroxidase activity (Kuan and Tien, 1993).

The basic version of reaction mechanism of MnP is mentioned below and entails the oxidation of Mn (II)

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by compound I (MnP-I) and compound II (MnP-II) to profit Mn (III). In view of nonspecific landscape of its action, focus on biochemical properties of MnP has been made.

$MnP + H_2O_2 = MnP\text{-}I + H_2O$
$MnP\text{-}I + Mn^{2+} = MnP\text{-}II + Mn^{2+}$
$MnP\text{-}II + Mn^{2+} = MnP + Mn^{2+} + H_2O$

Mn (III) in turn facilitates the oxidation of organic substrates. The mandate for application of ligninolytic enzymes in industry and biotechnology is ever increasing due to their use in a variety of processes. Ligninolytic enzymes have potential applications in a large number of fields, including the chemical, fuel, paper, food, agricultural, textile, and cosmetic industrial sectors in addition to others (Karigar and Rao 2011). These enzymes are also directly involved in the degradation of various xenobiotic compounds. Due to the fact that ligninolytic enzymes are basically nonspecific, they are able to attack a series of molecules chemically similar to lignin including pesticides, polycyclic aromatic hydrocarbons, synthetic polymers, and synthetic dyes (Maciel et al. 2012).

Figure1: Fungal isolates from soil sample

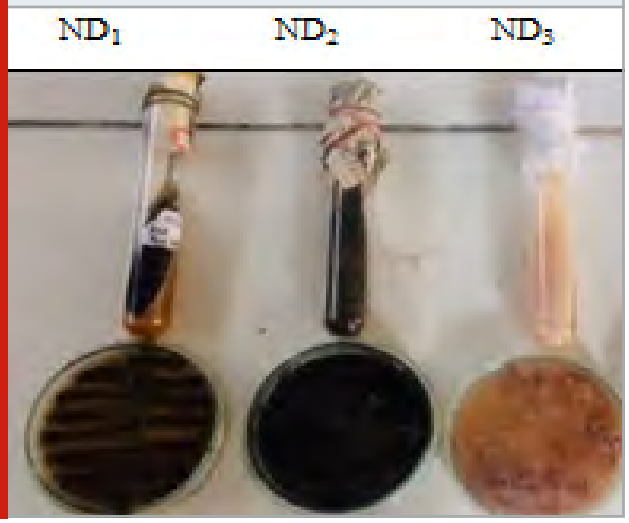
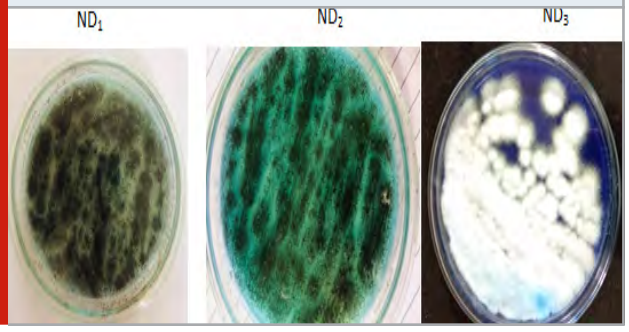


Figure 2: Methylene blue decolorization test



MATERIAL AND METHOD

Sample collection: Garden soil samples were collected from Swami Vivekanand Bridge, Lal Darwaja, Ahmedabad, Gujarat in sterile zip-lock bag and bring into the lab and preserved at 4 °C until further use.

Isolation and Screening: The samples were serially diluted up to $10^{-4}$and 100 µL of sample was spreaded on potato dextrose agar medium and incubated at 28 °C for 4 days. The fungal isolates were purified on new PDA plates and stored at 4 °C for further experiments. The pure isolates were screened by growth on PDA plates supplemented with 0.1% methylene blue (V. Sasikumar, 2014). Plates were incubated at 28 °C for 4 days and zone of decolorization of methylene blue was observed. Fungal colonies developed on the plates were purified and characterized by morphological and cultural characteristics.

Raw material: Several agriculture raw materials like wood chips, wheat straw and saw dust were used as carbon sources. All the raw materials were washed with tap water and dried in air-circulating oven at 70 oC until their moisture was removed and weight became constant. The dried material was then milled, and the resulting products were used as substrates.

MnP production:

(1) Submerged fermentation (SmF): The method of Steffen et al. (2000) was followed for SmF. The MnP production by submerged fermentation was carried out in 250 mL Erlenmeyer flask containing 100 mL medium supplemented with 5.0 gm % substrate like wood chips, saw dust and wheat straw. K_2HPO_4 0.005gm %; $MgSO_4 \bullet 7H_2O$ 0.001gm %; and $CaCl_2 \bullet 2H_2O$ 0.0005gm % were added in manganese peroxidase production medium. The pH of the medium was adjusted to 4.5 ± 0.1. Mixtures were sterilized by autoclaving at 121 °C for 15 min. Each flask was inoculated by mycelial plugs of 10 mm diameter and was incubated at 28 °C. The samples were taken at every 24 hours and analysed for manganese peroxidase production (Songulashvili G et al;2007)

Sample collection and processing: After every 24 hours of incubation, a volume of 5 mL broth was collected aseptically and centrifuged at 10,000 rpm for 10 min. The supernatants obtained were stored at 4 °C and used as crude enzyme and analysed for manganese peroxidase activity.

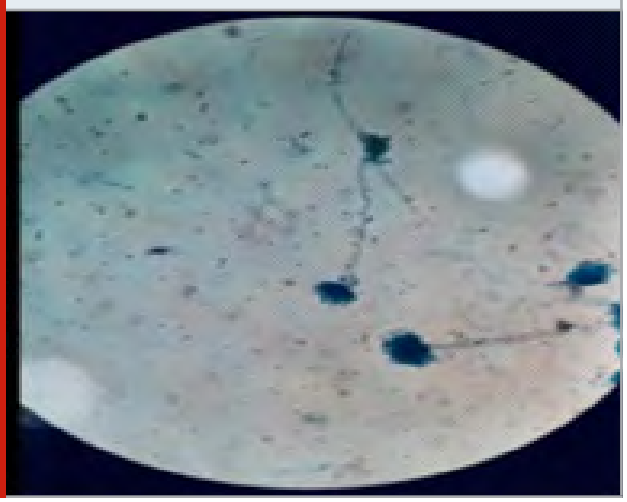
(2)Solid state fermentation (SSF): The MnP production by solid state fermentation was carried out in 1L Erlenmeyer flask containing basal medium where 50 g of wheat straw was taken as substrate and soaked in 100 mL salts solution. Wheat straw was used because it is

cheap and easily available in plenty amount, compared to wood chip. The following salts were added to give a final salt concentration of (in g/L) in basal medium: KH_2PO_{42}. 0; $MgSO_4 \bullet 7H_2O$ 0.5; $CaCl_2 \bullet 2H_2O$ 0.1 and KCL 0.5. The pH was adjusted to 5.0 (Vares, T., Kalsi et al; 1995). The mixture sterilized by autoclaving at 121 °C for 15 min. After sterilization and cooling the flasks were inoculated by mycelial plugs of 10 mm diameter and were incubated at 28 °Cor 15 days in static condition for production of MnP. After cultivation for 15 days the flasks were harvested by adding 50 mL sterilized distilled water and keeping on shaker for one hour. After one hour the content was filtered then centrifuged and the supernatant was analysed for manganese peroxidase activity.

Table 1. Cultural and morphological characters of ND1 fungal isolate

Fungal isolate	Cultural characters	Morphological characters
ND_1	Dry colony with black powdery growth.	Conidiophores are long arisen from foot cell , unbranched and ending in vesicles on which sterigmata are radially arranged bearing spherical spores in chain, Septate mycelia.

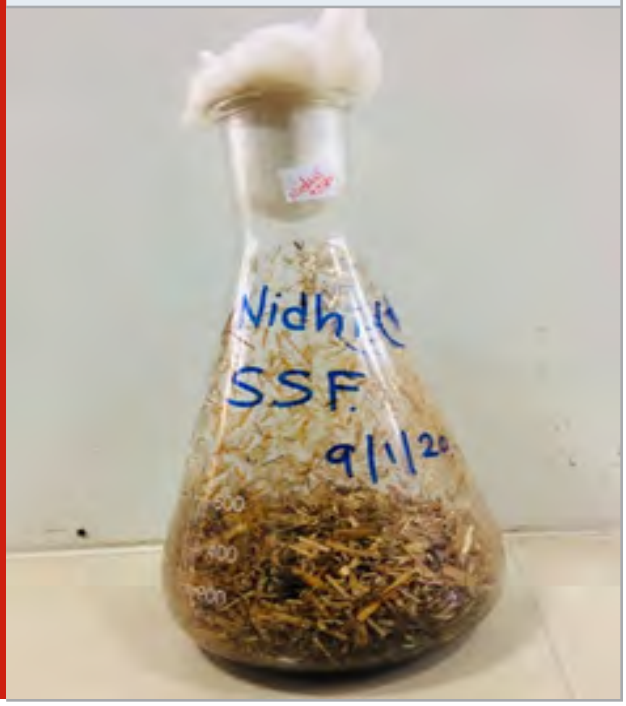
Figure 3: Microscopic observation of ND_1



Enzyme assay: Assay of MnP activity was based on oxidation of phenol red at 610 nm (Kuwahara et al, 1984). The assay mixture without enzyme source contained 0.1 mL 0.5% bovine albumin, 0.1mL 200 mM $MnSO_4$, 0.1mL 0.25M sodium lactate (pH 4.5), 0.1mL 2.0 mM H_2O_2 (prepared in 0.2 mM sodium succinate buffer pH 4.5) and 0.1 mL 0.1% phenol red as a substrate. Equal volumes of assay mixtures were provided into tubes and tubes were divided into two categories i.e. active and inactive. Tubes of both categories were pre-incubated at 30°C in a water bath for 10 minutes. An aliquot of 0.5 ml of culture supernatant as an enzyme source was added to each tube of the first category whereas, tubes of the second category received 0.5 ml of inactivated culture filtrate (boiled for 5 min). A tube of control was prepared by adding distilled water in place of enzyme with all above mentioned mixtures. All the tubes were further incubated after addition of enzyme for 5 minutes at 30°C. After incubation of 5 minutes, reaction in tubes of only the first category tubes was terminated by boiling the tubes for 5 minutes. The changes in absorbance of reaction mixture were monitored at 610 nm. MnP activity was expressed in IU where one unit of MnP was defined as the amount of enzyme that oxidize one micromole of phenol red per min.

In vivo dye decolorization experiment: To test the ability of isolates to decolorize synthetic dyes phenol red and methylene blue, each dye was membrane filtered through 0.45-µm cellulose nitrate filter (Boer, C.G.et al. 2004). 100 mg/mL of dye was added in 100 mL mineral salt medium and sterilized by autoclaving. The flasks were inoculated by 10 mm agar plug of isolates grown for 4 days. The flasks were incubated at 28°C for 3 days. Dye decolorization was determined spectrophotometrically at the wavelength of dye: 665 nm for methylene blue and 540 nm for phenol red (dos Santos Bazanella et al;2013) In control (abiotic control), the fungus was omitted. Percent decolorization of dye was calculated by considering the optical density of control flask as 100 % (0% decolourization).

Figure 4: Solid state fermentation



RESULT AND DISCUSSION

Isolation of organisms: Total three fungal isolates were obtained from garden soil collected from Swami Vivekanand Bridge, Lal Darwaja, Ahmedabad, Gujarat by serially diluted up to 10^{-4} and 100 µL of sample spreaded on potato dextrose agar medium and incubated at 28°C for 4 days.

Figure 5: Submerged fermentation

Screening: Screening was done using lignin model medium Potato dextrose agar with 0.1% methylene blue.

Dye decolorization plate assay: 0.1% Methylene blue is used for screening of manganese peroxidase enzyme producing fungi as it acts as lignin model. In the present study methylene blue dye decolorization is observed in ND_1 which is a positive result. From the dye decolorization plate assay, it can be stated that fungal isolate ND_1 gave potential decolorization zone and thus this isolate is processed further for production of enzyme and characterization. According to cultural and morphological characters, the isolated fungal culture is identified as *Aspergillus niger*.

Figure 6: MnP production using wood chips as carbon source (submerged fermentation)

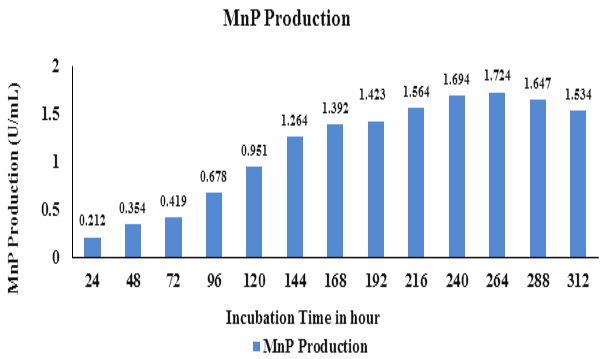
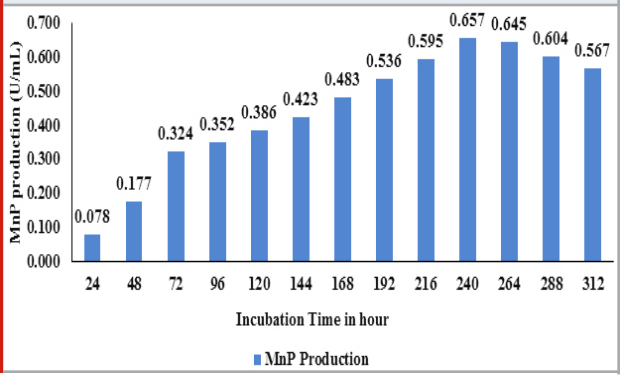
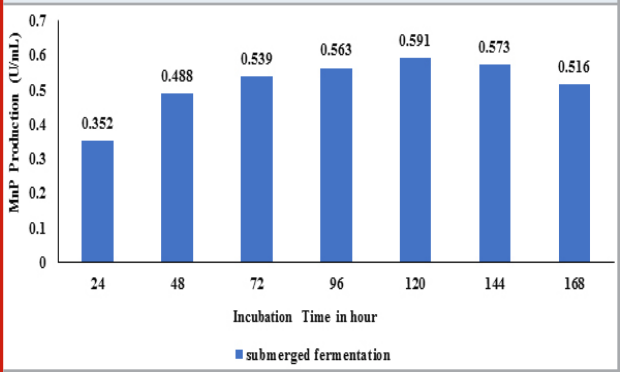


Figure 7: MnP production using saw dust as carbon source (submerged fermentation)



Manganese peroxidase production at SmF and SSF by Aspergillus niger. (ND_1): One fungal strain *Aspergillus niger* was selected after primary screening and was further used for production of manganese peroxidase using wood chips, saw dust and wheat straw as substrate. Submerged as well as solid state cultivation of cultures was carried out for production of manganese peroxidise. The same method was followed by Steffen et al. (2000) for production of MnP. The similar test system has already been successfully used for the selection of synthetic lignin, humic acid and coal-depolymerizing fungi (Steffen et al., 2000). Enzyme activity was monitored after every 24 hrs of incubation.

Figure 8: MnP production using wheat straw as carbon source (submerged fermentation)



The maximum enzyme activity was observed after 264 hours of manganese peroxidase production. Fungal isolate *Aspergillus niger* gave maximum production of 1.724 U/ml in submerged fermentation using wood chips as substrate. Fungal isolate *Aspergillus niger* gave maximum production of 0.657 U/ml in submerged fermentation using saw dust as substrate. The maximum

enzyme activity was observed after 240 hours. Fungal isolate *Aspergillus niger* gave maximum production of 0.591 U/ml after 120 hours in submerged fermentation using wheat straw as substrate. Fungal isolate *Aspergillus niger* gave maximum production of 0.597 U/ml after 144 hours in solid state fermentation using wheat straw as substrate.

Figure 9: MnP production using wheat straw as carbon source (solid state fermentation)

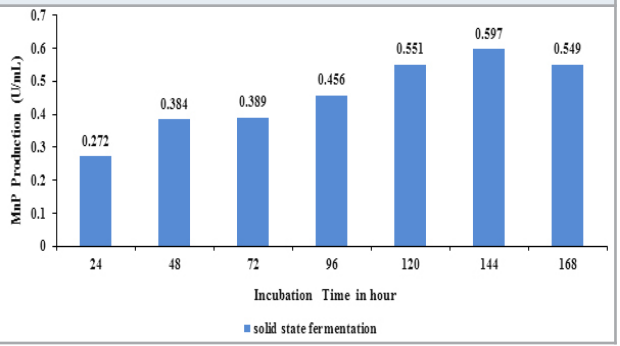


Figure 10: Comparison of solid state and submerged fermentation using wheat straw as carbon source for MnP production

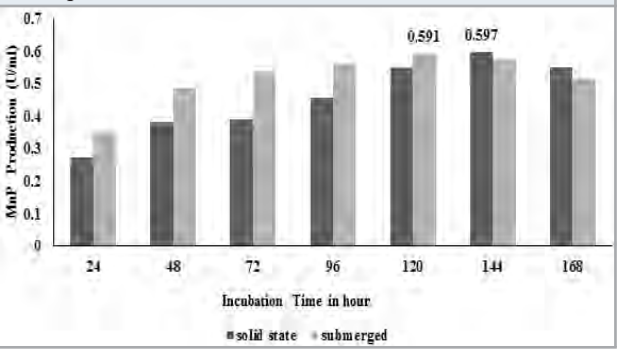


Table 2: Dye decolorization efficiency

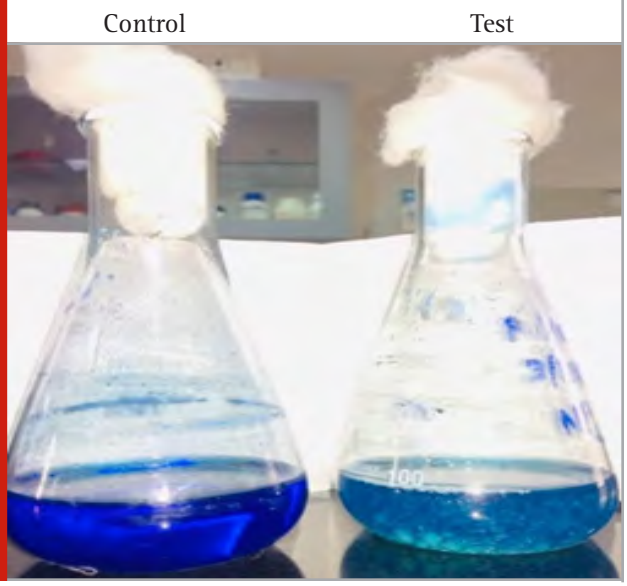
Dye	OD of Control	Decolorization of Control	OD of test	Decolourization of Test
Phenol red	1.251	0%	0.066	94.67%
Methy lene blue	1.560	0%	0.565	63.75%

With compare to submerged fermentation process, solid state fermentation gave slightly higher activity that is 0.597 U/ml after 144 hours. Thus, wheat straw can be used for SSF as it is cheap and easily available in plenty amount, compared to wood chip.

Dye decolorization: 100 mg/mL of dye was added in 100 mL mineral salt medium and sterilized by autoclaving. The flasks were inoculated by 10 mm agar plug of Aspergillus niger grown for 4 days and incubated at 28°C for 3 days. After incubation, observed the decolorization efficiency of dyes. Different dyes like phenol red and methylene blue are decolorized using manganese peroxidase producing fungus *Aspergillus niger*. Decolorization efficiency of *Aspergillus niger* was measured as 94.67% for Phenol red and 63.75% for Methylene blue after 3 days of incubation.

Figure 11: Phenol red decolorization

Figure 12: Methylene blue decolorization



CONCLUSION

Present research work was aimed to screen fungus producing manganese peroxidase from soil sample collected from Swami Vivekanand Bridge, Lal Darwaja, Ahmedabad, Gujarat. After screening on different lignin model stepwise, 1 fungal isolate Aspergillus niger was selected for further quantitative studies. Fungal isolate showed highest manganese peroxidase activities (1.72 U/ml) on 264 hours of incubation using wood chips as substrate in submerged cultivation. Isolated fungi *Aspergillus niger* shows 94.67 % decolorization of Phenol red and 63.75 % decolorization of Methylene blue after 3 days of incubation.

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Production, Characterization and Applications of Yellow Pigment Using Marine Bacteria Isolated from Coastal Region of South Gujarat

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ABSTRACT

Marine organisms are well-known for the natural pigment production viz carotenoids, flexirubin, xanthomonadine, prodigiosin, violacein and anthracene. Carotenoid pigments are known for their antioxidant, anticancer and antimicrobial activities. In the present study we have isolated 11 yellow pigment producing bacteria from the coastal region of South Gujarat. The morphological and cultural characteristics of the isolates were studied. It was observed that most of the isolates were Gram positive cocci and coco-bacilli. Out of 11 isolates 06 were found to synthesize dark pigments and rapid growth within 48-72 h on Zobell marine agar medium. Further these isolates were screened on the basis of their pigment production abilities in broth medium. The highest pigment production was 644.81mg/L by UKR-14. The pigments were extracted using methanol and further detected and confirmed by TLC. UV- visible spectrophotometric (λmax462nm) and FTIR analysis of the extracted pigment was performed and it was revealed that pigment belongs to the carotenoid family. The extracted pigment exhibits a significant antimicrobial activity against bacterial pathogens viz. *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*. The antioxidant activity of the pigment was assessed to be 45.68 %.

KEY WORDS: ANTIOXIDANT ACTIVITY, ANTIMICROBIAL ACTIVITY, BACTERIAL PIGMENTS, CAROTENOIDS, HALOPHILES.

INTRODUCTION

Human eyes can differentiate things based on the colors of the object; industries use synthetic colors to make products visually attractive. Majority of synthetic pigments are widely used owing to their low cost, higher stability and wide range of spectra (Fariq et al., 2019). Synthetic pigments made up of heavy metals and petroleum compounds which are toxic and unsafe for both environment and human health. Synthetic pigments are also reported to be carcinogenic, allergic, induce hyperactivity, toxic and organ damaging (Usman et al., 2018).

Synthetic yellow azo dyes were widely used in food, confectionary and beverage industries for bulk production of candy, soft drinks, chips, popcorn, cereals, sauces, baked goods. But few of the hazardous effects such as irritability, restlessness, depression, sleeping sickness and hyperactivity in children they are now banned in some countries (Sun et al., 2007). Due to these evil effects many

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of the synthetic dyes are now banned from use by the regulatory authorities. To counter the negative effects of synthetic colorants, globally researchers have focused on production of pigments from alternative natural resources including plants and microbes. Naturally occurring pigments and colored compounds from plants, animals, algae, fungi and bacteria are regarded as bio-pigments and are in use since ancestral days for coloring substances (Shatila et al., 2013).

Microbes are producers of a wide variety pigments such as carotenoids, prodiogiosin, flavin, indigoids, melamine, monascin, phenazine, prodiogiosin, quinone, bacteriochlorophyll and violacein (Ramesh et al., 2017). These pigments are also found to have pharmaceutical properties of antimicrobial, anticancer, antioxidant, antiviral, antitumor nature. Pigments are also found effective in healing and hence used in treating skin diseases, gastric ulcers, cardiovascular diseases, chemotherapy of tuberculosis and in protection against UV induced photo damage of cells (Tapia et al., 2019). Microorganisms are a potential source for bio-pigment with an advantage over plants in terms of availability; stability; cost efficiency; labor; yield and easy downstream processing (Tuli et al., 2015). Amongst bacteria, Halophiles are known for synthesis of various pigments, which are mostly carotenoid in nature and yellow to red in color. The carotenoids are a dominant class of pigment due to abundance and applicability with an estimated market share of $ 1.8 billion USD in 2020 (Villegas-Méndez et al., 2019).

Table 1. Colony characteristics of pigmented isolates

Sr. No.	Isolate code	Salt concn	Size	Shape	Margin	Pigment	Elevation	Opacity	Surface	Consistency	Grams nature
1	UKR-01	5%	1 mm	Round	Entire	Yellow	Raised	Opaque	Smooth	Moist	Grams positive coco-bacilli
2	UKR-02	5%	< 1 mm	Round	Entire	Yellow	Raised	Translucent	Smooth	Moist	Grams positive cocci
3	UKR-04	5%	2 mm	Round	Entire	Yellow	Convex	Opaque	Smooth	Moist	Grams positive cocci
4	UKR-05	5%	3 mm	Round	Uneven	Yellow	Flat	Opaque	Smooth	Moist	Grams positive cocci
5	UKR-06	5%	3 mm	Round	Uneven	Yellow	Flat	Translucent	Smooth	Moist	Grams positive cocci
6	UKR-07	3%	< 1 mm	Round	Entire	Yellow	Convex	Opaque	Smooth	Moist	Gram positive coco-bacilli
7	UKR-14	3%	2 mm	Round	Uneven	Yellow	Flat	Opaque	Smooth	Moist	Grams positive cocci
8	UKR-15	3%	2 mm	Round	Entire	Yellow	Convex	Translucent	Smooth	Moist	Gram positive
9	UKR-19	5%	2 mm	Round	Uneven	Yellow	Flat	Translucent	Smooth	Moist	Grams positive coco-bacilli
10	UKR-21	3%	2 mm	Round	Entire	Yellow	Umbonate	Opaque	Smooth	Moist	Grams positive cocci
11	UKR-22	5%	2 mm	Round	Entire	Yellow	Raised	Opaque	Smooth	Moist	Grams positive cocci

Figure 2: Growth of bacteria on ZMB a) Isolation of Yellow pigment producing bacteria b) UKR-4 c) UKR-5 d) UKR-14

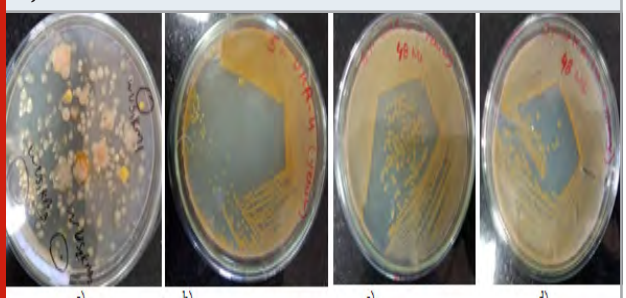


Figure 1: Sampling site (Umergaon and Nargol)

The anticancer property of yellow pigment from *Streptomyces griseoaurantiacus* is reported by Prashanthi and his coworkers, (2015). While Umamaheswari (2013), Sasidharan and his colleagues (2013) in separate research have found significant antioxidant activity of yellow pigment extracted from the YCD3b strain and *Exiguobacterium sp*. respectively. While similar reports of yellow pigment extracted from the Bacillus sp. with antifungal properties was reported recently by Dawoud and his labmates (2019). The present study focusses on isolating and production of such yellow pigment producing halophilic bacteria from the southern coast-lines of Gujarat. Further, the detection and characterization of the extracted pigment using TLC, UV-vis, FTIR spectroscopy techniques and determination of its biotechnological potential have been studied.

MATERIALS AND METHODS

Sample collection and isolation: Water samples were collected from different sites along coastal regions of Umergaon and Nargol villages of Valsad district, Gujarat as shown (Figure 1). Brine water were collected in sterile bottles from beaches and salterns and preserved 4 °C till further use. Each sample was serially diluted up to 10^{-5} and 0.1-ml sample was spreaded on Zobell marine agar (ZMB) medium with 3 %, 5%, and 10% (w/v) NaCl concentration, from last three dilutions. Plates were incubated at 30 °C for 48 -72hrs. Yellow pigment producing bacterial colonies were selected from incubated plates and transferred on new sterile ZMB plates and stored in 4 °C for further experiments.

Physiological and morphological features of isolates: The bacterial isolates were grown on 30 °C for 72hrs. The colony morphology and characters such as size, shape, margin, color, opacity, elevation, texture and Gram's reaction were studied.

Screening of isolates: Isolates were screened on the basis of dark pigment secretion in short time on ZMB agar medium. Further the pigment production was assessed by growing the cells in liquid broth.

Pigment Production: Zobell Marine broth (ZMB) (Hi-Media, India) supplemented with 5% (w/v) NaCl, pH 7.0 was used for the inoculum preparation. A loopful culture was inoculated in 20 ml in 100 ml Erlenmeyer flask containing sterile ZMB and incubated at 35 °C, 120 rpm on rotatory shaker for 48 h. Optical density of this activated culture was measured and adjusted to 1.0 abs at 600 nm with sterile distilled water. Pigment production was carried out in 100 ml ZMB (Hi- Media, India) supplemented with 5% w/v NaCl concentration and pH 7.0 in 250 ml flask. Production medium was inoculated with 2% (v/v) activated culture. and incubated at 35 °C, 120 rpm on a rotatory shaker for 48 h. After incubation log number of cells and pigment production was analyzed.

Table 2. Screening of isolates based on fast growth and dark pigment production on ZMB agar plate

Sr. No.	Isolate code	Incubation period				Remarks
		24	48	72	96	
1	UKR-01	+	++	++	++	Not selected
2	UKR-02	++	+++	++++	++++	Selected
3	UKR-03	+	+	+	++	Not selected
4	UKR-04	+++	++++	++++	++++	Selected
5	UKR-05	+++	++++	++++	++++	Selected
6	UKR-06	+	++	+++	++++	Selected
7	UKR-07	+	+	+	++	Not selected
8	UKR-14	+++	++++	++++	++++	Selected
9	UKR-15	+	+	+	++	Not selected
10	UKR-19	+	++	+++	++++	Selected
11	UKR-21	+	+	+	++	Not selected

Table 3. Pigment production in Broth

No.	Isolate code	Color	Pigment mg/L
1	UKR-02	Yellow	147.61
2	UKR-04	Yellow	281.48
3	UKR-05	Yellow	544.44
4	UKR-06	Yellow	117.4
5	UKR-14	Yellow	644.81
6	UKR-19	Yellow	39.58

Pigment extraction and quantification: The pigment solubility of collected cells from fermented broth by centrifugation 10000 rpm for 10 minutes were screened in various solvents (methanol, ethanol, acetone, distilled water, ethyl acetate hexane and chloroform). The mixture of cells and solvent was incubated at 60 °C for 20 min and visually observed for the colored supernatant. Methanol extraction procedure was used for pigment extraction (Shatila et al., 2013). Collected cell biomass was washed twice with distilled water and centrifuged as above. Cell pellet (harvested from 100 ml production broth) were re-suspended in 20 ml methanol and incubated for 30

min and centrifuged. Colored supernatant was collected in pre-weighted evaporating dish and dried at 40 °C. Pigment production was estimated by dry weight of pigment (Pathak and Sardar, 2012).

Characterization of extracted pigment: Extracted pigment was detected and confirmed using Thin Layer chromatography (TLC), while determination of λ_{max} was done by UV-Vis and Fourier-Transformed Infrared Spectroscopy (FT -IR) techniques.

Thin Layer chromatography (TLC): The dried pigment was dissolved in small amount of methanol and spotted on thin layer chromatography plate with silica gel G-60 F25 (Merck). After saturation for 20-30 minutes in solvent system consist of Methanol and Chloroform (6:4 v/v). The TLC plate was kept in chromatography chamber. The solvent system was allowed to run 75 % on TLC plate. TLC plate were carefully removed and allowed to air dry. The Rf value was calculated according to following formula.

$$Rf = \frac{\text{Distance traveled by compound}}{\text{Distance traveled by solvents}}$$

Determination of λ_{max}: Determination of λ_{max} helps find out maximum absorbance range of molecule, it was observed by UV-visible spectrophotometer. The extracted dried pigment was dissolved in absolute methanol and subjected to λ_{max} analysis in range of 300 - 800 nm in Jasco- 2000 UV- visible spectrophotometer.

Fourier-Transformed Infrared Spectroscopy (FT -IR): The presence of functional groups in extracted bioactive pigment were characterized by FT-IR spectra. The dried extracted pigment was applied on the surface area of crystal and measurements was carried out using range 400-4000, cm-1 transmission with 4 cm-1 resolution in FTIR (Bruker, Alpha).

Application of pigment: Antioxidant Activity DPPH (1, 1-Diphenyl-2-picryl-hydrazil): In the free radical scavenging activity of pigment, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was used as standard to assess antioxidant activity of pigments. 1 ml of extract (1000 µg/ml) were mixed with 2 ml 0.002% DPPH prepared in methanol. The mixture was incubated in dark for 30 min and disappearance of blue color of DDPH was measured at 517 nm by visible spectrophotometer (Electronics India). DPPH scavenging activity was calculated using the following formula

$$\text{Scavenging}_{\text{effect}}\ (\%) = [(A_{control} - A_{test}) / A_{control}] \times 100$$

Where,

$A_{control}$ = was the absorbance of the control reaction and

A_{test} = was the absorbance in the presence of test sample.

Antibacterial activity of the extracted pigment: Antimicrobial activity of extracted pigment was performed by well diffusion method. Four bacterial pathogens were selected and a 100 µl bacterial suspension was spread on to sterile Muller Hilton agar plate for each bacterium. In aseptic condition three wells were made using cup borer in each plate and labeled as negative control, positive control and test. Methanol (100µl) was treated as negative control, 100µl of streptomycin (50µg/ml) was taken as positive control. Test samples (100µl) each were prepared by mixing methanolic extract of the pigment (1000 µg/ml). Plates were incubated at 37 °C for 24h and the zone of inhibition was observed.

RESULTS AND DISCUSSION

Sample collection and isolation of pigment producing bacteria: Different pigment (pink, orange, brown and yellow) producing bacteria were isolated from the water samples on ZMB agar plates supplemented with 3% and 5% NaCl concentration. Total 11 yellow pigment producing bacteria were obtained and selected for the further studies.

Physiological and morphological features of isolates: Yellow pigment producing isolates (Figure 2) were purified on ZMB agar plates and subject to analysis of colony characterization as shown in Table 1. Isolates formed colonies, in round shape, with entire margin, convex or flat elevation, with opaque or translucent opacity and smooth surface. Gram staining of isolates showed that all isolates were Gram positive cocci except UKR-1, UKR-7 and UKR-19 which are the Gram-positive coco-bacilli.

Screening of isolates: Screening of the isolates on the basis of growth and pigment production by bacteria is shown in Table 2. Out of 11, 6 isolates produced dark pigment within the 48-72h which were selected for the pigment production in broth medium. As shown in Table 3 among these 6 isolates, UKR-14 produced highest pigment in broth medium which was selected for further investigations.

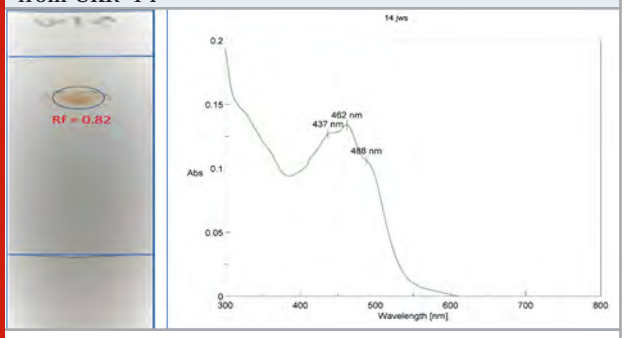
Pigment Extraction: Pigment solubility of the isolates were checked in different solvents where maximum pigment solubility was observed in methanol. Thus, methanol pigment extraction method was used for the further pigment extraction. Fariq et al., 2019 reported that *Halomonas aquamarina* MB598 producing lemon yellow color pigment is extracted by methanol.

Characterization of extracted pigment: The pigment extracted from the UKR-14 was characterized by TLC, UV- visible and FTIR spectroscopy.

Thin layer chromatography (TLC): Thin layer chromatography (TLC) analysis of extracted pigment from UKR-14 showed single yellow spot on TLC plate with 0.82 Rf value (Figure 3a). Rf values of Carotenoids pigments are reported in the range of 0.9 to 0.34. The standard β-carotene and pigment production from

Sarcophyton sp. is reported 0.78 Rf value which is near to our results (Kusmita et al., 2017).

Figure 3 a) TLC analysis and b) λ_{max} of yellow pigment from UKR-14

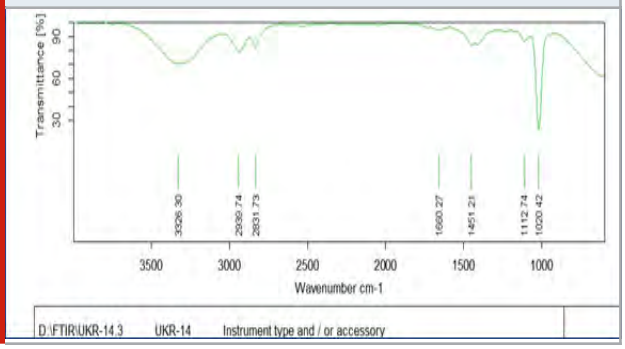


a) b)

Determination of λ_{max}: The UV- visible spectra of pigment from UKR-14 showed maximum absorbance at 462 nm along with two more absorbance peaks at 434 and 488 (Figure 3b). Maximum absorbance with three peaks in the range of 300-500 nm which is the characteristics of the carotenoid pigments. Garg and Aeron, (2016) reported similar results for yellow pigment from Bacillus sp. at 467, 493 and 527 nm.

Fourier-Transformed Infrared Spectroscopy (FT -IR): FTIR spectra stretches are the significant for confirming the functional groups present in the molecules. The crude extract of UKR-14 pigment spectra stretches were observed at 1020.42 is for the -C-O, 1451.21 represents for -CH2 alkenes, 1660.27 for C=C aromatic ring, 2831.13 C-H aromatic, 2939.74 –CH of alkenes, 3326.30 for –OH of alcohol (Figure 4).

Figure 4: FTIR spectra of extracted pigment from UKR-14



The same results were reported by (Neha et al., 2017) for β- carotene. Yellow pigments from the Bacillus sp., FTIR spectra also exhibited similar stretches (Dawoud et al., 2019). Broad stretch at 3326.30 for phenolic –OH and 2939.74 –CH of alkenes is the characteristic feature of carotene. From the results pigment was identified belonging to the carotenoid family.

Figure 5: Antimicrobial activity of pigment against *S. aureus*

Table 4. Antimicrobial activity of pigment extracted from UKR-14 against bacterial pathogens

Organism	Zone of inhibition
E. coli	6 mm
Pseudomonas Sp.	7 mm
Bacillus sp.	5 mm
Staphylococcus sp.	8 mm

Applications of extracted pigment Antioxidant activity DPPH (1, 1-Diphenyl-2-picryl-hydrazil): DPPH is a stable free radical and frequently used in the estimation of antioxidant activity of biomolecules and it is normally reduced by the antioxidant such as cysteine, glutathione, ascorbic acid and tocopherols (Sasidharan et al., 2013). Stable DPPH has blue color and after reduction it was converted in yellow, the reduction of blue color measured in spectrophotometer at 517 nm. The Pigment extracted from the UKR-14 showed 45.68 % DPPH scavenging activity. Umamaheswari S, 2013 reported isolates producing yellow pigment showed significant antioxidant activity in DPPH assay.

Antimicrobial activity: After incubation of plates at 37 °C zone of inhibition of pathogens were observed as shown in Figure 5. The UKR-14 pigment showed the antimicrobial activity against the bacterial pathogens as shown in Table 4. (Sibero et al., 2019). Yellow pigment extracted from the *Streptomyces sp*., *Micrococcus luteus* and *Flavobacterium sp*. showed significant antimicrobial activity against bacterial pathogens (Aruldass et al., 2018). Yellow pigment production from *Bacillus sp*. DBS4 showed antifungal activity (Dawoud et al., 2019).

CONCLUSIONS

In our study total 11 yellow pigment producing bacteria were isolated from marine water sample. Colony characterization of pigment producing isolates were

showed usually round, entire, opaque, moist and smooth colonies. Study of cell morphology conclude out of 11, 8 bacteria were Gram positive cocci and 3 were Gram positive coccobacilli. Screening of isolates showed that out of 18 isolates 6 isolates produced dark pigment after 48-72 h on ZMB agar medium and UKR-14 produced highest pigment 644.81 mg/L in broth medium. Solubility test showed that UKR-14 pigment was maximum soluble in methanol. TLC, UV- visible (λ_{max} 462 nm) and FTIR spectroscopy results showed that pigment belonged to the carotenoid family. DPPH assay of pigment showed 45.68 % antioxidant activity and significant antimicrobial activity against bacterial pathogens. Thus, UKR-14 pigment can be used as sources of yellow pigment in industries to replace the synthetic yellow pigments.

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A Walk into the EsaR Regulator of *Pantoea stewartii*: Inhibition of EPS Production By Interfering Quorum Sensing Pathway by Natural AHL Antagonist QS Inhibitors of EsaR

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ABSTRACT

The phenomenon of quorum sensing (QS) is the most widely studied in various gram positive and negative bacteria. Quorum sensing is a cell-to-cell communication system that regulates gene expression as a result of the production and response to signal molecules called autoinducers (AIs). The identification of QS inhibitors is considered a promising strategy for the development of anti-microbial agents from the natural bioactive compounds. *Pantoea stewartii* is a gram-negative enteric bacterium that primarily affects plants, particularly types of maize. In silico studies suggested the antagonistic binding of bioactive compounds to EsaR of *P. stewartii*, by hydrogen bonding and hydrophobic interactions, would reduce the virulence factor (Stewartan EPS production). Protein-ligand preparation is done prior to docking, making it accessible for ligand-receptor interaction using AutoDock Vina in Chimera. Analysis of the results was performed in BioVia Discovery Studio. Based on the binding energy and hydrogen bonds, various bioactive compounds were compared with AHL (N-acyl- homoserine lactone). *In silico* studies demonstrated the antagonist binding of eupatorin, catechin, aromadendrin, tricetin, rhamnazin and myricetin to EsaR would, in theory, reduce the EPS production and hence pathogenicity. Furthermore, the molecular docking analysis revealed that aromadendrin and rhamnazin bind more rigidly with EsaR receptor protein than the cognate signalling molecule AHL with the docking score of -10 kcal/mol and -10.2 kcal/mol respectively. From the results obtained, it is expected that the natural bioactive compounds can be used as an effective antimicrobial agents that acts by targeting the process of quorum sensing in pathogenic bacteria.

KEY WORDS: AUTOINDUCER, BIOACTIVE COMPOUNDS, IN SILICO, QUORUM SENSING.

ARTICLE INFORMATION

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INTRODUCTION

Bacteria have the ability to detect and respond to outside environmental stress by executing the pathway known as quorum sensing (QS). Environmental stress may include various factors such as nutrient deprivation, changes in physical parameters like pH, temperature, humidity and water availability. When the bacteria undergo such stress, they in regard respond to each other and become persistent in the situation. QS is a cell-to-cell communication process employed by a wide variety of gram-positive and gram-negative bacteria to harmonize cooperative behaviour for a particular function (Miller and Bassler, 2001). It is a process of regulating gene expression in response to the alteration in cell population density as well as environmental conditions (Miller and Bassler, 2001). Gram-positive and gram-negative bacteria abide by the quorum sensing communication pathways to control many physiological parameters like production of exopolysaccharide, biofilm, bioluminescence, virulence factors and toxin formation along with motility which are essential for a pathogenic or symbiotic relationship with the respective eukaryotic hosts (Juan and Neela, 2006).

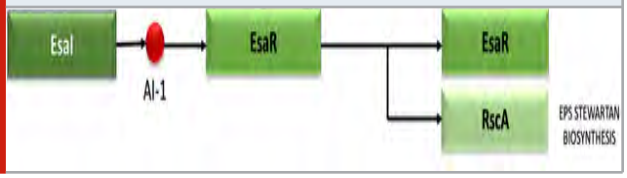
QS process despite being beneficial for the survival of bacteria, it is pathogenic to humans and plants due to their pathogenicity. Hence, it is of utmost importance to look for various strategies to successfully inhibit the production of signalling molecules causing the inactivation of QS pathway. One such strategy has been employed in the current study to hinder the normal QS mechanism. The quorum sensing pathway is generally divided into three categories: 1. LuxI/ LuxR type quorum sensing in gram-negative bacteria in which the acylated homoserine lactones (AHLs) are used as autoinducers; 2. Oligopeptide - two-component type quorum sensing in gram-positive bacteria that use the small auto-inducing peptide (AIPs) as a signal molecule and 3. LuxS-encoded autoinducer (AI-2) for quorum sensing in both gram-positive and negative bacteria (Li and Tian, 2012).

In different organisms, quorum sensing is activated by different types of autoinducers. N-acylated homoserine lactone (AHL) is the most common autoinducer molecule in the quorum sensing pathways of gram-negative bacteria. AHLs are synthesized by the homologues of LasI protein (Rasmussen and Givskov, 2006) which are then bind to the homologues of receptor LuxR protein (LuxR-AHL) and regulate the quorum sensing pathway. Hence, AHL mediated systems are a common target for inhibiting virulence in gram-negative bacterial families (Deryabin et al., 2019). Numerous strategies have been proposed for inhibiting AHL mediated QS pathways in various gram-negative bacteria. One such strategy for inhibiting quorum sensing is the use of bioactive compounds as QS inhibitors.

These bioactive compounds replace AHL in the quorum pathway and thus, decrease the virulence of the bacteria. Quercetin, a flavanol, successfully inhibits quorum sensing in *Chromobacterium violaceum* (Gopu et al., 2015). Here, instead of AHL, quercetin binds to CviR protein and inhibits the quorum sensing pathway. Similarly, gingerol, a compound found in ginger, can inhibit quorum sensing in *Psuedomonas aeruginosa* which is responsible for biofilm production due to binding of gingerol to LasR (Kim et al., 2015). This paper is focussed on the LuxR homologue, EsaR of phytopathogen *Pantoea stewartii* and inhibiting its QS pathway.

In the current study, various bioactive compounds (ligands) were evaluated by *in-silico* techniques for their ability to hamper the QS mechanism in *Pantoea stewartii*. This entire study is based on the relationship between the mechanism of quorum sensing and pathogenicity of organisms. *P. stewartii* is a bacterium that causes Stewart's wilt and leaf blight disease in maize plants. The bacterium produces EPS as a result of its quorum sensing pathway, and this EPS is the cause of virulence. It has been proved that EPS production is the factor for virulence in *Pantoea stewartii* (Roper, 2011). The production of this EPS is regulated by an autoinducer encoded by EsaI. As shown in Figure 1, EPS production is regulated by the activity of EsaR and EsaI where EsaR acts as a transcriptional repressor (Schu et al., 2014). When cell density is low, the EsaR protein binds to its recognition site, the EsaR box present on the DNA and obstructs the EPS production. But as the cell density increases, more AHL is produced.

Figure 1: Quorum sensing pathway of *P. stewartii*

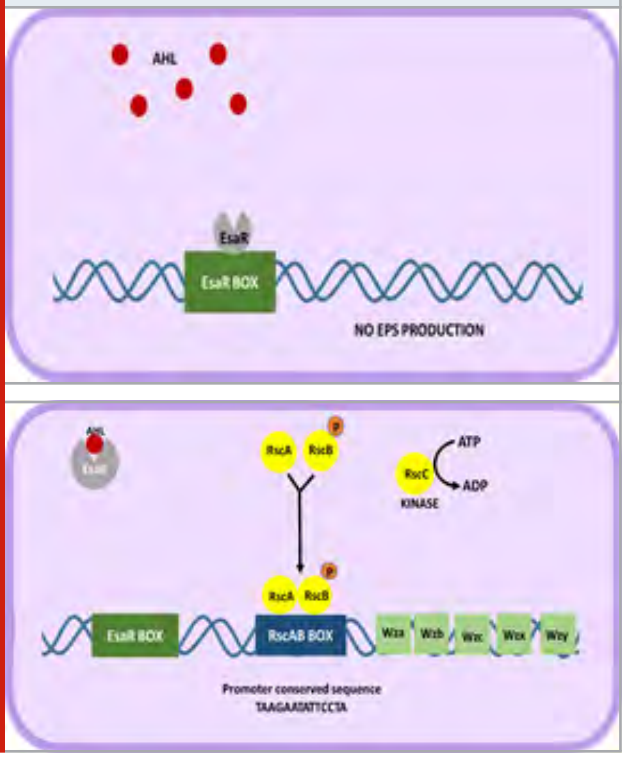


As shown in Figure 1., this AHL is produced by AHL synthase, EsaI. This AHL then binds to EsaR and changes its conformation which results in release of EsaR from the EsaR box as depicted in Figure 2. This activates the CPS operon responsible for the synthesis of carbomyl phosphate synthase in the cell. When the cell density is high then the RscC gets phosphorylated by ATP. This RscC further phosphorylates RscB. As shown in Figure 2., the phosphorylated RscB binds to RscA. RscA in combination with RscB binds to their recognition site on the DNA, the RscAB box. As they bind, the transcription is activated, which in turn causes activation of gene clusters; Wza, Wzb, Wzc, Wzx, Wzy. This results in the production of EPS (Burke et al., 2015). Thus, proving that EPS is produced only at high cell density. When this EPS is produced in abundance in the xylem of the infected plant, it becomes the cause of disease in maize plant (Schu et al., 2014). Other than EPS production, the swarming motility of *P. stewartii* is also QS regulated (Burke et al., 2015). A lack of AHL controls both motility and EPS production. So, AHL is vital for the pathogenicity of *P. stewartii*.

With encroachments in technology *in silico* studies have made major strides in the field of life sciences. In silico approaches play a vital role in analysing the interaction

between various biomolecules. In this study, the AHL molecule is replaced by bioactive compounds. These bioactive compounds (phytochemicals) bind to EsaR hence, inhibit the binding of AHL and EsaR. This causes changes in the regular quorum sensing mechanism of the bacterium. The bioactive compounds chosen mainly belonged to the class of alkaloids and flavonoids. This in silico study gives understanding about the interaction between various ligands (bioactive compounds) and the receptor (EsaR).

Figure 2: Quorum sensing mechanism in Pantoea stewartii. Inhibition of EPS synthesis in absence of AHL. Activation of EPS synthesis in presence of AHL



MATERIALS AND METHODS

Homology Modelling: The homology modelling of EsaR was performed based on the known crystal structure quorum sensing transcriptional activator (YenR) from *Yersinia enterocolitica* (SMTL ID: 5l07.1). The amino acid sequence of EsaR used to build the model was retrieved from Uniprot (ID: P54293). Out of all model AHL receptors known TraR from *Agrobacterium tumefaciens*, ExpR from *Erwinia chrysanthemi*, CarR from *Erwinia carotovora*, LasR, QscR and RhlR from *Pseudomonas aeruginosa*, TraR from *Agrobacterium tumefiecens*, AhyR from *Aeromonas salmonicid*, TraR is the closest to EsaR (Nasser and Reverchon, 2007) and its 3D structure was chosen to build the 3D model of EsaR using SWISS-MODEL (Benkert et al., 2010 and Waterhouse et al., 2018) QMEAN analysis of the modelled protein was performed using SWISS-MODEL server and Ramachandran plot analysis was performed using MolProbity v4.4 to check the psi and phi angles of the modelled EsaR to determine the overall torsion of the residues. ERRAT analysis was performed to reliably identify the regions of error in predicted protein crystal structure by examining the statistics of pairwise atomic interactions (Colovos and Yeates, 1993).

Protein Preparation: EsaR receptor protein was initially prepared for docking studies. The protein structure was prepared for docking by assigning the hydrogen atoms, charges, and energy minimization using DockPrep tool (Krivov et al., 2009). The charges were assigned as per AM1-BCC method that efficiently generate high quality atomic charges for protein and the charges were computed using ANTECHAMBER algorithm (Wang et al., 2008). The energy minimization was performed using 500 steepest descent steps with 0.02 A° step size and an update interval of 10. All the steps mentioned were performed in Chimera.

Ligand Preparation: Total 59 natural bioactive compounds were used for interaction assays that were retrieved from PubChem. Canonical SMILES of each ligand were taken, and their structures were developed. Before molecular docking, the ligands were recuperated by the addition of hydrogen and energy minimization using Gasteiger algorithm (Gasteiger and Jochum, 1979) in structure editing wizard of Chimera,that works on the chemoinformatic principle of electronegativity equilibration. The charges were assigned as per gasteiger method that efficiently generates high quality atomic charges for protein and the charges were computed using ANTECHAMBER algorithm and the files being saved in mol2 format.

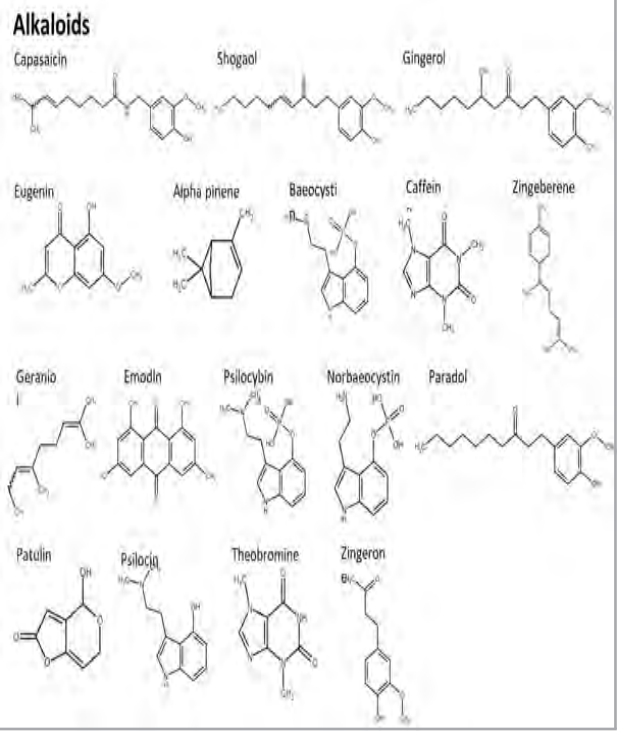
In-silico ligand binding Interactions: Receptor-ligand docking analysis was performed using AutoDock Vina (Trott and Olson, 2010) and the program was implemented as an add-on in Chimera. The ligand binding site was chosen based on the crystallized ligand attached in the original PDB file and the co-ordinates were recorded for docking of bioactive compounds. Superimposition of EsaR, CviR, QscR, SdiA, and RhlR also provided the information about the active site of EsaR and furthermore the hydrophobic cavity of EsaR was determined in BioVia Discovery Studio which represented the presence of active site similar to those four receptors. The coordinates of the hydrophobic cavity of the active site were used in the docking of 59 bioactive compounds.

Analysis of Docking: In the AutoDock Vina algorithm, the following parameters were set as (i) number of binding modes- 10; (ii) exhaustiveness of search- 8 and (iii) maximum energy difference- 3 kcal/mol. Following docking, the pose showing the maximum hydrogen bonds and the minimum binding free energy change (kcal/mol) as shown in the View Dock window were chosen. At last, they were further analysed in Discovery Studio for hydrogen bond and hydrophobic interaction formation by the functional groups of ligands with amino acids.

Comparison of Similar QS Receptor: 3-oxo-C6-HSL belongs to the AI-I class of autoinducers. Five other

receptors binding to the autoinducers of the AI-I class were selected. These receptors belong to the same or different gram-negative bacteria. RhlR (Uniprot ID: P54292) from Psuedomonas aeruginosa, CviR (PDB ID: 3QP6) from *Chromobacterium violaceum*, SdiA (PDB ID: 4Y17) from Escherichia coli, QscR (PDB ID: 6CC0) from *Psuedomonas aeruginosa* and SpnR from *Serratia marcescens* were selected for comparative studies. The original ligands of these receptors are C4-HSL (PubChem CID: 10130163), C6-HSL (PubChem CID: 10058590), 3-oxo-C8-HSL (PubChem ID: 127293), 3-oxo-C12-HSL (PubChem CID: 3246941) and 3-oxo-C6-HSL (Pubchem ID: 688505) respectively. A comparative study of the binding analysis of all these receptors with chosen ligands was performed. The binding analysis of these receptors with chosen ligands (bioactive compounds) was executed similarly using UCSF Chimera and Discovery Studio.

Figure 3: Structures of alkaloids retrieved from PubChem

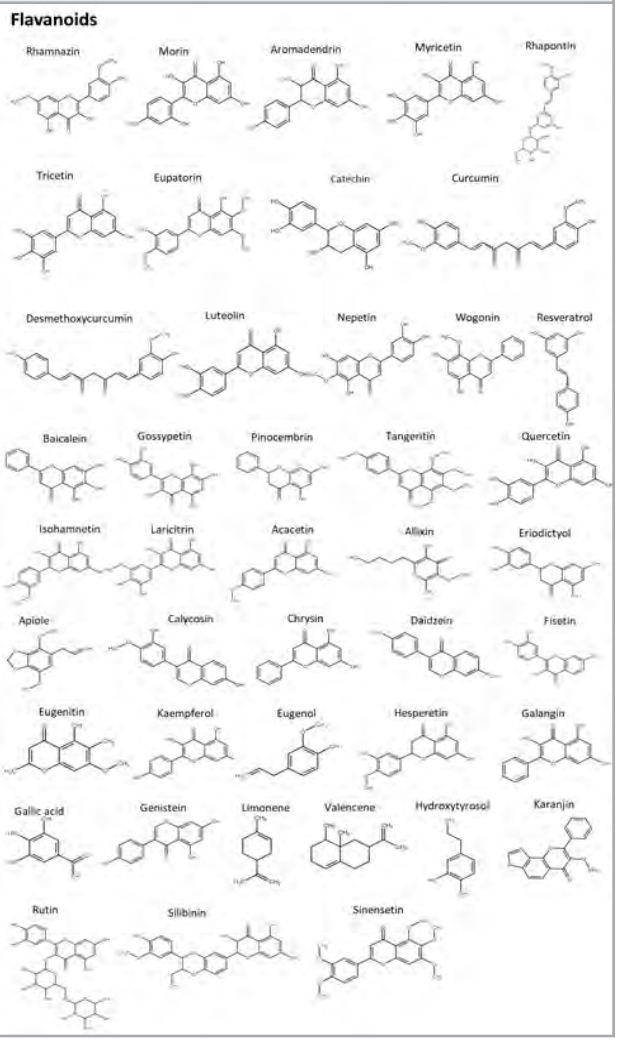


Further, multiple sequence alignment of all the six proteins (EsaR, QscR, CviR, SdiA, SpnR, and RhlR) was performed. The amino acid sequence of each protein in a FASTA format was retrieved and saved in notepad. The saved sequence was uploaded in a Clustal Omega, a multiple sequence alignment program. The output format chosen was ClustalW with character counts.

RESULTS AND DISCUSSION

Modelled EsaR: Structure and analysis of EsaR after homology modelling is shown in Figure 5. The model of EsaR was built using the protein sequence from the Uniprot entry with the ID:P54293. The YenR protein of Yersinia *enterocolitica* (PDB ID: 5107.1B) was used as the 3D template structure to build the model of EsaR. The QMEAN value of the model was -1.19 and Global Model Quality Estimation (GMQE) value was 0.59. Ramachandran plot analysis was done using MolProbity version 4.4, showing 94.7% of residues fall in the favoured region with only three amino acids 179THR, 88LEU and 134THR causing torsional strain by improper psi and phi angles, accounting for 0.66% outlier, were found in the built model. Eight amino acid, 241ILE, 108ASN, 152ILE, 152ILE, 167GLU, 191TRP, 219VAL, and 23LEU was identified in the rotamer outlier region.

Figure 4: Structures of flavonoids retrieved from PubChem

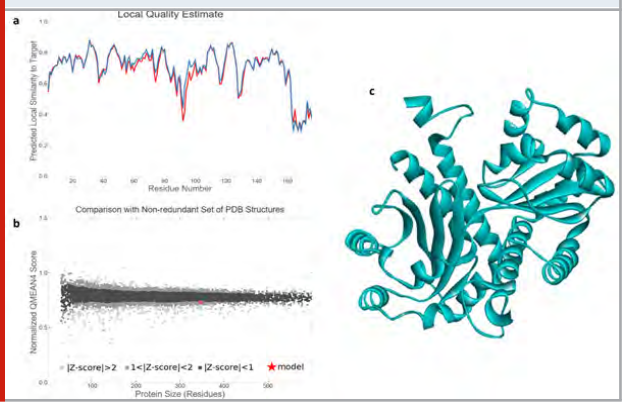


Molecular docking analysis: Not a lot is known about the natural QS inhibitors of *P. stewartii*. It is important to find natural inhibitors because they have more benefits than synthetic inhibitors. They are extracted from plants hence they are cost-effective. Since no antibiotics are used the bacteria will not acquire antibiotic resistance. There will be no side effects as these are natural compounds.

The docking analysis of EsaR with 59 bioactive compounds is depicted in Table 1. The original ligand AHL (3-oxo-C6-HSL) showed the interaction with

receptor ligand with a free energy change of binding -7.0 kcal/mol for the best pose which showed the formation of five hydrogen bonds with ASP63, SER97, SER97, SER97, and PHE94 as shown in Figure 6. However, zero hydrophobic interactions in the active site were observed. In order to replace the original ligand, the bioactive compounds should have free binding energy that is less than -7.0 kcal/mol. This is because, when the binding energy between the ligand and the receptor is less, the binding between them becomes stronger. Hence, a more stable ligand-receptor complex is formed. Other than the free binding energy, other aspects considered are the number of hydrogen bonds and hydrophobic interactions. If all the amalgamation of all three aspects is better than that of the original ligand and receptor binding only then, the natural ligand can replace the original ligand in the QS pathway.

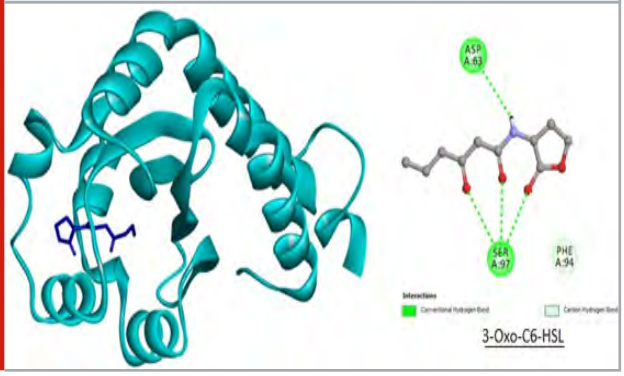
Figure 5: (a) Quality assessment of modelled EsaR, (b) QMEAN and (c) Modelled structure of EsaR



Overall, Flavonoids showed an effective binding capacity in comparison to the original ligand. Interaction of EsaR with rhamnazin (PubChem CID: 5320945) showed that the ligand effectively binds at the active site of the protein. The binding free energy change was found to be -10.2 kcal/mol as shown in Table 1. Four hydrogen bonds with PHE94, ASP63, SER121 and VAL103, the aromatic chain showed hydrophobic interaction with VAL65 and PRO64 in the form of Pi-alkyl bonds, Pi-Pi stacked with TRP78 and made Pi-anion bond with ASP63 were formed by rhamnazin, as shown in Figure 8. Rhamnazin is a flavonoid that can be extracted from *Rhamnus petiolaris* (Wagner et al., 1974). Its free binding energy is considerably less than that of AHL plus, it also harbours more hydrophobic interactions with EsaR. Thus, its binding capacity to EsaR is better than that of AHL. Rhamnazin is also reported to have anti-tumor activity (Yu et al., 2015). Hence, it has both medicinal properties as well as anti-QS properties.

Similarly, in the interaction of aromadendrin (Pubchem CID: 122850) and EsaR, the free binding energy was found to be -10.0 kcal/mol as shown in Table 1. The hydrogen bonds by aromadendrin were formed with SER121, ASP63, PHE94 and ASP87 and the aromatic moiety of aromadendrin made Pi-Pi stacked bond with TRP78 and Pi-alkyl bond with VAL65 as shown in Figure 8. Aromadendrin is a flavonoid that can be extracted from the cone of Pinus sibirica (Rogachev and Nariman, 2015). Its free bonding energy is significantly lower than that of AHL and more, it also has more hydrophobic interactions than AHL. Hence, its binding efficiency to EsaR will be more than that of AHL. Aromadendrin is reported to have anti-oxidant, anti-proliferative, and anti-inflammation activities (Cui et al., 2018). Aromadendrin is also a potential therapeutic medicine for type 2 diabetes and cardiovascular diseases (Zhang et al., 2011, Cui et al., 2018). From this, we can conclude that on top of having a great medicinal value aromadendrin is also an anti-QS molecule.

Figure 6: *In silico* assessment of AHL (3-Oxo-C6-HSL) binding with modelled EsaR



Effective binding of eupatorin (Pubchem CID: 97214) and tricetin (Pubchem CID: 5281701) was observed, with EsaR with the binding energies of -9.8 kcal/mol and -9.3 kcal/mol respectively as shown in Table 1. They show four hydrogen bonding with SER121, ASP87, PHE94, VAL103 and SER121, TRP78, ASP87, and PHE94 as shown in Figure 8. The aromatic chain showed hydrophobic interaction with VAL65, TRP,7 and ASP63 in the form of Pi-alkyl, Pi-Pi stacked, Pi-anion bonds and VAL65, PRO64, and ASP63 in the form of Pi-alkyl and Pi-anion bonds as shown in Figure 8. Eupatorin and tricetin are flavonoids extracted from the leaves of Orthosiphon stamineus and pollen of Myrtaceae family (Laavola et al., 2012, Zhou et al., 2006). The free binding energy of both compounds is less than that of AHL but more than aromadendrin and rhamnazin. Thus, eupatorin and tricetin show enhanced binding with EsaR than AHL but not better than aromadendrin and rhamnazin. Eupatropin has anti-proliferative, anti-angiogenic and anti-inflammatory properties (Dolecková et al., 2012, Laavola et al., 2012). Tricetin has anti-inflammatory and antitumor activity (Hsu et al., 2010). Therefore, it can be said that both these compounds have anti-QS properties on top of their medicinal properties.

The interaction of EsaR with catechin (Pubchem CID: 9064) indicated that the ligand effectively binds at the active site with the binding free energy change of -8.9 kcal/mol with five hydrogen bonds and three hydrophobic bonds as shown in Table 1. The hydrogen bonds by ligand were formed with ASP87, ASP63, ASP63, TRP78, and SER121. The aromatic moiety of catechin made three

unique hydrophobic interactions in the form of Pi-anion bond with ASP63; Pi-Pi stacked bond with TRP 78 and Pi-alkyl bond with VAL65 as shown in Figure 8. Catechin is also a flavonoid obtained from cocao fruits, wine and green tea (Donovan et al., 2006, Singh et al., 2011). Its binding energy is less than that of AHL. Since the binding energy is lesser and hydrophobic interactions are more than that of AHL, it binds better to EsaR. Catechins are antioxidants and have anti-tumor activity too (Singh et al., 2011). Hence, catechin possesses both medicinal and anti-QS properties.

Figure 7: Interaction of various best docked bioactive compounds in the binding cleft of modelled EsaR

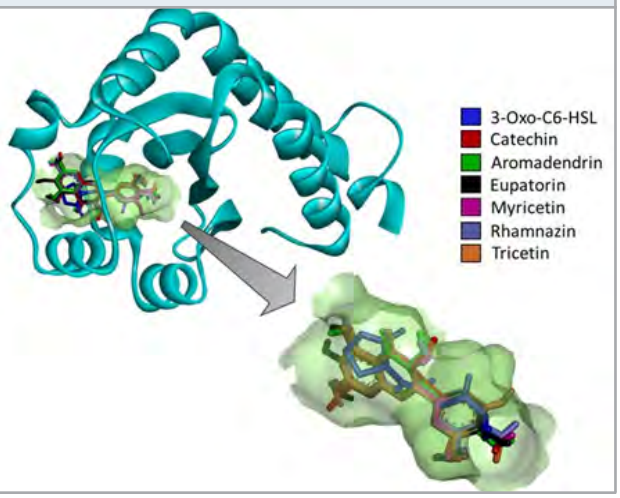
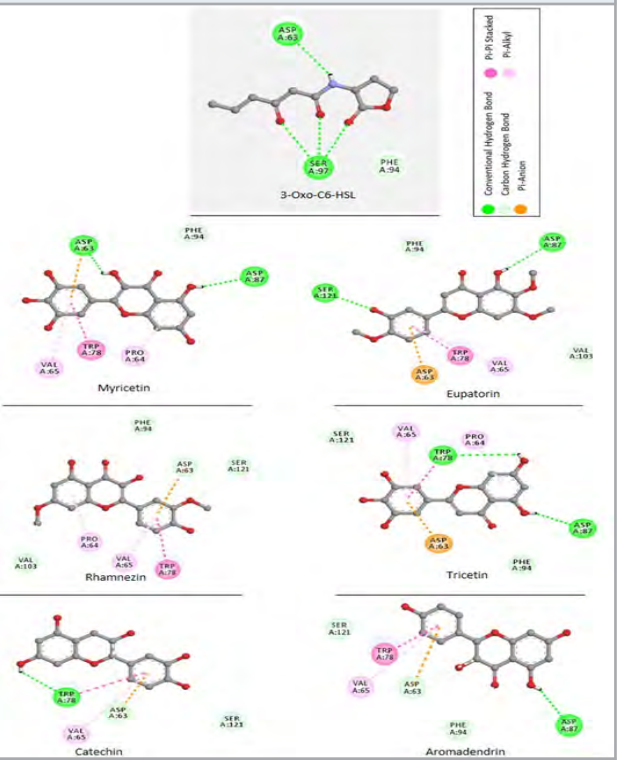


Figure 8: Interaction of screened bioactive compound with EsaR by hydrogen bonding along with other hydrophobic interactions



The interaction between EsaR and myricetin (Pubchem CID: 5281672) depicted the binding free energy to be -9.5 kcal/mol as shown in Table 1. It also displayed five hydrogen bonds with SER121, ASP63, ASP87, PHE94 and SER121. Also, the aromatic moiety of myricetin only made three hydrophobic interactions; Pi-Pi stacked bond with TRP78, Pi-alkyl bond with VAL65 and Pi- Anion bond with ASP63 as shown in Figure 8. Myricetin is a flavonoid that can be extracted from foods like berries, tea and red wine (Ong and Khoo, 1997). Its free binding energy is less than that of AHL and all the three aspects are better than AHL therefore, it will bind more rigidly to EsaR than AHL. Myricetin has anti-oxidant, anti-carcinogenic and anti-viral properties (Ong and Khoo, 1997). This proves that myricetin has various medical properties on top of being an anti-QS molecule.

All these bioactive compounds showing good results bind in the hydrophobic cavity of EsaR, as shown in Figure 7. They act as an antagonist to AHL because none of the compounds bind to the amino acids on EsaR with which the AHL molecule binds. Thus, instead of being agonist they act as antagonist. So, even though the bind with different amino acids on EsaR, their binding is more efficient than the binding of AHL and EsaR. Furthermore, all these ligands tightly bind to EsaR and can successfully prevent the binding of AHL to EsaR. Hence, they can act as successful QS inhibitors.

Figure 9: (a) Multiple sequence alignment of protein sequences of CviR, EsaR, RhlR, QscR, SdiA, SpnR (b) Superimposition of EsaR with RhlR, QscR, CviR, SdiA and SpnR. Ligands showing good results are present in the hydrophobic cavity

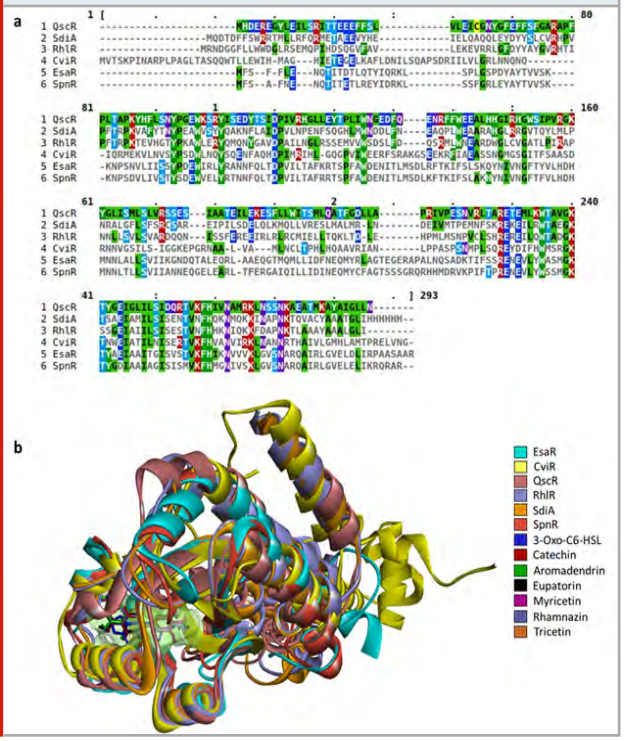


Table 1. Computational interactions predicted interactions made by various ligands with EsaR.

Ligands	Hydrogen Bonds	Hydrophobic Bonds	Energy
AI-1	ASP:63, SER:97, SER:97, SER:97, PHE:94	-	-7 kcal/mol
ALKALOIDS			
Capasaicin	ASP:63, SER:121, SER:97, THR:30	PHE:94, PHE:94, LYS:98, VAL:103, VAL:65, TRP:78, ASP:63	-8.3 kcal/mol
Psilocybin	SER:97, VAL:103, TRP:78, ASN:104, ASP:63, SER:97	ILE:93, PRO:64, PRO:64, ASP:63, TRP:78	-7.2 kcal/mol
Shogaol	SER:97, SER:121, ASP:63, PHE:94	VAL:65, ILE:102, TRP:78, ASP:63, PHE:94	-7.4 kcal/mol
Alpha pinene		PRO:63, PRO:64, ILE:93, VAL:65, VAL:65, TRP:78	-6 kcal/mol
Baeocystin	ASN:104, ILE:102, TRP:78, VAL:103	ASP:79, PHE:94, ASP: 87, TRP:78, VAL:103	-6.2 kcal/mol
Caffeine	ILE:102, PHE:94, ASP:87, VAL:103, SER:97	PHE:94, PHE:94, PRO:64, ASP:87	-5.5 kcal/mol
Emodin	ARG:55, SER:44, SER:44, THR:8, GLN:5	ARG:55	-6.5 kcal/mol
Geraniol		ILE:93, VAL:65, TRP:78, TRP:78, TRP:50, TYR:46	-6.2 kcal/mol
Gingerol	ASP:63, SER:97, VAL:103	ALA:28, TRP:50, ASP:87	-7.4 kcal/mol
Norbaeocystin	PHE:94	VAL:103, PHE:94, ASP:63, ASP:63, ASP:87	-6.4 kcal/mol
Paradol	SER:97, PHE:94	ASP:63, TRP:78, VAL:65, ILE:102, PHE:94	-7.1 kcal/mol
Patulin	SER:97, SER:121	TRP:78, VAL:65	-6.1 kcal/mol
Psilocin	SER:97	ILE:93, PRO:64, PRO:64, VAL:65, TRP:78, ASP:63, ASP:63	-6.8 kcal/mol
Thiobromine	TRP:78	TRP:78, VAL:65, VAL:65, ASP:63, ILE:93, PRO:64	-5.9 kcal/mol
Zingerone	SER:121, SER:97, TRP:78	VAL:65, PRO:64, ILE:93, ASP:63	-6.7 kcal/mol
Zingiberene		ILR:93, PRO:64, PRO:64, LEU:84,	-7.7 kcal/mol
		TRP:50, TRP:78, TRP:78, TYR:46, ILE:102	
FLAVANOIDS			
Rhamnazin	PHE:94, ASP:63, SER:121, VAL:103	PRO:64, VAL:65, ASP:63, TRP:78	-10.2 kcal/mol
Morin	TRP:78, ASP:63, PHE:94, PRO:64	ILE:102, VAL:65, TRP:78, PRO:64, ASP:63	-10.1 kcal/mol
Aromadendrin	SER:121, ASP:63, PHE:94, ASP:87	TRP:78, VAL:65	-10 kcal/mol
Myricetin	SER:121, ASP:63, ASP:87, PHE:94, SER:121	TRP:78, VAL:65, ASP:63	-9.5 kcal/mol
Eupatropin	SER:121, ASP:87, PHE:94, VAL:103	ASP:63, TRP:78, VAL:65	-9.8 kcal/mol
tricetin	SER:121, TRP:78, ASP:87, PHE:94	VAL:65, TRP:78, PRO:64, ASP:63	-9.3 kcal/mol
Rhapontin	SER:121, SER:121, SER:97, TRP:78	TRP:78, VAL:65, ASP:63, PRO:64, ASP:87	-8.9 kcal/mol
Catechin	ASP:87, ASP,63, TRP:78, SER:121	VAL:65, TRP:78, ASP:63	-8.9 kcal/mol
Resveratrol	VAL:103, ILE:102, SER:97, SER:121	VAL:65, VAL:65, ALA:28	-8.5 kcal/mol
Curcumin	SER:94, PHE:94, ASN:104, THR:30	ALA:28	-8.4 kcal/mol
Desmethoxy Curcumin	PHE:94, SER:97, SER:97, THR:30	ALA:28, TRP:78	-8.1 kcal/mol
Luteolin	TRP:78, ASP:87, SER:121	VAL:65, PRO:64,ASP:63, TRP:78	-10.1 kcal/mol
Nepetin	TRP:78, SER:121, PHE:94	VAL:65, ASP:63	-9.9 kcal/mol
Wogonin	PHE:94, TRP:78, VAL:103	ASP:87(UF), PRO:64, TRP:78, VAL:65, ASP:63	-8.5 kcal/mol
Baicalein	ASP:87, PRO:64, PHE:94	TRP:78, VAL:65, ASP:63	-9.6 kcal/mol
Gossypetin	PRO:64, PHE:94, SER:121	VAL:65, TRP:78, ASP:63	-9.1 kcal/mol
Pinocembrin	SER:97, PHE:94, ASP:87	ASP:63, TRP:78, VAL:65, ILE:93, PRO:64	-8.5 kcal/mol
Tangeritin	VAL:65, TRP:78, PRO:64, THR:83, VAL:103	ASP:63	-7.1 kcal/mol
Quercetin	SER:121, ILE:102, SER:97	PRO:64, VAL:65, TRP:78, ASP:63	-8.4 kcal/mol
Isohamnetin	ASP:63, PHE:94, SER:121	TRP:78, PRO:64, VAL:65	-10.1 kcal/mol

Laricitrin	SER:121, ASP:63, SER:97, PRO:64	TRP:78, VAL:65, ASP:63	-7.1 kcal/mol
Acacetin	ASP:87, PHE:94, TRP:78	VAL:65, Asp:63, PRO:64	-10.2 kcal/mol
Allixin	SER:121	PRO:64, TRP:78, VAL:65, ILE:66	-5.6 kcal/mol
Apiole	THR:83	ASP:87, PRO:64, TRP:78, VAL:65	-6.4 kcal/mol
Calycosin	ASP:87,ASP:63	ASP:63, TRP:78, VAL:65, SER:121	-8.3 kcal/mol
Chrysin	PHE:94, PRO:64	ASP:63, TRP:78, VAL:65	-9.4 kcal/mol
Daidzein	ILE:102	PRO:64, TRP:78, VAL:65, ASP:63	-7.8 kcal/mol
Eriodictyol	PHE:94, PRO:64, VAL:103	TRP:78, VAL:65	-7.2 kcal/mol
Eugenitin	PHE:94, PRO:64, VAL:103, ASP:87	TRP:78, VAL:65	-7.2 kcal/mol
Kaempferol	ASP:63, PHE:94	TRP:78, PRO:64, VAL:65	-10.2 kcal/mol
Eugenol	VAL:103, SER:97	TRP:78, VAL:65, PRO:64, -6.1 kcal/mol	
		VAL:65, ILE:93, ASP:63	
Fisetin	ASP:87, SER:121, SER:97, SER:97	PRO:64, VAL:65, VAL:65,	-8.2 kcal/mol
		ASP:63, TRP:78, ILE:102	
Galangin	ASP:87, PRO:64, PHE:94	ASP:63, TRP:78, VAL:65	-9.9 kcal/mol
Gallic Acid	TRP:78, SER:97	VAL:65, PRO:64, ASP:63, ASP:63	-5.9 kcal/mol
Genistein	ILE:102, VAL:103, ASP:87, ASP:63	VAL:65, PHE:94, ASP:63	-8.3 kcal/mol
Hesperetin	ASP:87, PHE:94, SER:121	VAL:65, TRP:78, ASP:63	-10.1 kcal/mol
Hydroxytyrosol		ASP:63, TRP:78, VAL:65	-6.2 kcal/mol
Karanjin	ASP:79, PRO:64	VAL:65, TRP:78, TRP:78,	-7.9 kcal/mol
		TRP:78, ASP:63	
Limonene	-	ALA:28, TYR:54, ILE:66,	-6.7 kcal/mol
		TYR:46, TRP:50,	
		TRP:78, TRP:78, VAL:65, VAL:65	
Rutin	ASN:115, LYS:34	LEU:117, PHE:70, PHE:40 (UF)	-5.4 kcal/mol
Silibinin	VAL:109, ASN:115	ILE:148	-5.1 kcal/mol
Sinensetin	ARG:55, THR:8, GLN:5, GLN:12, LEU:2	ILE:51	-6.9 kcal/mol
Valencene	-	TRP:50, TRP:78, VAL:65, ILE:102, PRO:64	-8.1 kcal/mol

Comparison of Similar QS Receptor: The hydrophobic active sites of EsaR, QscR, SdiA, RhlR, CviR, and SpnR in a superimposed state are represented in Figure 8. All these receptors belong to the LuxR family of protein but their structure is not identical. But it was observed that the active sites of all the AHL receptors had similar structures that included a four stranded antiparallel β-sheet and three α-helices on each side. The cavity was found to be formed by cluster of hydrophobic and aromatic residues. Superimposition of all six protein receptors also showed the ligand binding cleft were identical and therefore may have similar ligand binding properties. Thus, if such bioactive compounds bind efficiently binds with EsaR then it may also bind to QscR, SdiA, RhlR, CviR, and SpnR, with similar affinity, contributing to its anti-quorum sensing property. As shown in Figure 9(a), the sequence of these proteins is not very similar except for some conserved amino acids i.e. less than 10 % similarity was observed. Albeit their ligand binding site or active site remains the same as shown in Figure 9(b).

CONCLUSION

From the above results we can conclude that bioactive compounds: rhamnazin, aromadendrin, eupatorin, tricetin, catechin, and myricetin can efficiently replace autoinducer (3-oxo-C6-HSL) in the QS pathway thereby, effectively interfering with the quorum sensing mechanism of *Pantoea stewartii*. The outcome of the present study could pave a path for the control of EPS production in *P. stewartii* and simultaneously reducing its pathogenicity.

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Conflict of interest: All the authors have contributed equally in this paper.

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Screening and Optimization for Cellulase Production by Soil Bacterial Isolates JRC1 and JRC2

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ABSTRACT

Cellulase production by soil bacteria can be beneficial as the cellulase production rate is comparatively advanced due to the hasty bacterial rate and bacteria are competitively less or nil pathogenic as compared to fungi. Screening of bacteria and optimization of fermentation conditions such as substrate concentration, pH, temperature, incubation period are imperative for successful production of cellulase. The present study is conducted to produce cellulase by local isolates JRC1 and JRC2 using Carboxymethylcellulose (CMC) as substrate. Following that, cellulase produced from isolates were partially purified using ammonium sulphate precipitation method for the characterization of cellulase. Cellulase was successfully produced and optimized with substrate concentration 3%, pH 7, and temperature 35 ^{0}C and inoculum size 10%. It provides current data for cellulase production by soil bacteria as well as its optimization procedures and an acumen into the various mechanisms bacteria uses to tolerate, survive and carryout processes that could potentially lead to the eco-friendly tactic for bio-conversion of cellulose. It also provides biotechnological aspects, application of cellulase research.

KEY WORDS: BIOCONVERSION, CELLULASE, CELLULYTIC BACTERIA, ENZYME PRODUCTION, OPTIMIZATION.

INTRODUCTION

Cellulase play significant role in natural biodegradation processes in which plant lignocellulosic materials are efficiently degraded by cellulolytic bacteria. The bioconversion of cellulose into glucose is nowadays recognized to comprise of two steps in the enzyme system. In the initially stage , β-1, 4 glucanase breakdowns the glucosidic linkage to cellobiose, which is a glucose dimer with a β-1, 4 linkage as contrasting to maltose, a complement with a α-1, and 4 linkage. Subsequently in the second stage, this β-1, 4 glycosidic linkage is fragmented by β-glycosidase.

Cellulase is one of the most convenient enzyme found on the earth. Which is used in several industries now a days for various purposes. Cellulase is mainly used to break such a complex polysaccharide substance that is known as cellulose. Which is the most lavish and most leading biomass on earth.it is the primary product of photosynthesis in terrestrial environments and most copious renewable bio resource produced in biosphere. Cellulose accounts for 50% of the dehydrated mass of herbal biomass and approximately 50% of the dehydrated weight of subordinate sources of biomass

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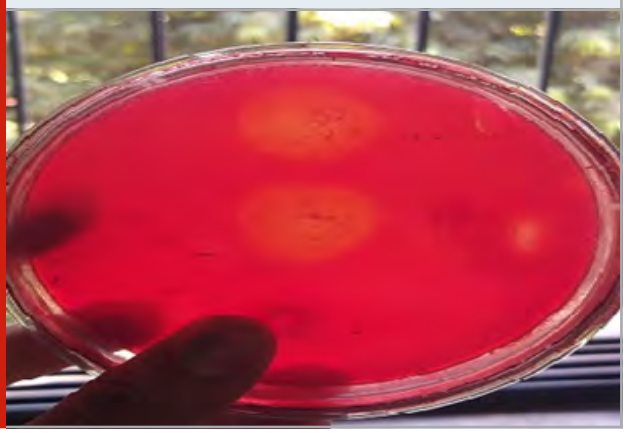
such as agricultural trashes(Patagundi, Shivasharan, & Kaliwal, 2014).

Cellulase is mainly produced by the several organisms such as bacteria, fungi, protozoa, and actinomycetes. However the growth rate, genetic engineering is so concerned bacteria are more preferential as their growth rate is normally higher and it is to manipulate bacterial gene as compared to other organisms also it has the nil or least pathogenicity as compared to fungus. The cellulolytic property of some bacterial genera such as Cellulomonas (Saratale, Saratale, Lo, & Chang, 2010), Cellvibrio, (Berg, von Hofsten, & Pettersson, 1972) Pseudomonas (Kasana, Salwan, Dhar, Dutt, & Gulati, 2008) Bacillus (Shankar & Isaiarasu, 2011) and Micrococcus (Mmango-Kaseke, Okaiyeto, Nwodo, Mabinya, & Okoh, 2016) has been reported.

Now a days cellulase is tremendously used in various industries including paper and pulp, detergent, fermentation, food, (Bhat & Bhat, 1997; Phitsuwan, Laohakunjit, Kerdchoechuen, Kyu, & Ratanakhanokchai, 2013; Ryu & Mandels, 1980) and many more as described in this research. So, cellulase enzyme will be the furthermost thrilling technology of future. Continuous research for developments in marked features for cellulase production (such as rate, substrate specificity, and specific activity) is looked for to accomplish improved techno economic feasibility. The present work was performed to optimize the nutritive and environmental constraints for cultivating cellulase production by bacterial isolates JRC1 and JRC2.

Figure 1: Zone of clearance on CMC Agar plate



MATERIALS AND METHODS

Screening and Isolation of Cellulase Producing Bacteria: Cellulolytic bacteria were isolated from soil by the dilution pour plate or spread plate method using CMC (Carboxymethyl cellulose) agar media. Tenfold serial dilutions of each sample was taken. The modified medium composition of CMC agar (g/L) E: CMC 10; KH_2PO_4 1.0; $MgSO_4.7H_20$ 0.5; NaCl 0.5; $FeSO_4.7\ H_2O$ 0.01; NH_4NO_3 0.3; agar 12 (Kuhad, Gupta, & Singh, 2011) and the pH is adjusted to 7.0; the plates were then incubated at 30 0C, 35 ^{0}C, 40 ^{0}C, and 45 ^{0}C for 24-48 hours. To envisage the clear zone of cellulose hydrolysis, the plates were flooded with an aqueous solution of 0.1% Congo red for 15 min and washed with 1 % NaCl (Sethi, Datta, Gupta, & Gupta, 2013). To specify the cellulolytic activity of the organisms, diameter of the clear zone around colonies on CMC agar was measured. Beside, a more quantitative assay method was used to determine the cellulose activity of the selected bacterial isolate in liquid medium. The cellulase activity of each culture was measured by determining the amount of reducing sugars liberated by using a 3,5-Dinitrosalicylic acid (DNSA) method (Miller, 1959). Bacterial isolates showing highest activity were selected for optimization of cellulase production.

Bacterial Identification on the Basis of Morphological and Biochemical Characteristics: The bacterial isolates JRC1 and JRC2 were preliminarily identified by means of morphological, cultural and biochemical tests. The examination includes colony morphology, gram's reaction and various biochemical tests such as methyl red, VP, Sugar fermentation, citrate utilization, starch utilization, lipid utilization, urea utilization, gelatine hydrolysis, H2S production, indole production, casein hydrolysis, catalase test and dehydrogenase test. The results were compared to the bergey's manual of determinative bacteria for partial identification (Sobur et al., 2019).

Table 1. Morphological and cultural characterization of cellulase producing isolates JRC1 and JRC2

Isolates	Colony characteristics On Nutrient Agar medium	On CMC Agar plate	Morphological characteristics
JRC1	Small, circular, entire, raised, smooth, Opaque with no pigment	Growth observed with small , circular, smooth colonies	Gram positive Big rods arranged single and in chains Actively motile
JRC2	Medium, circular, entire, raised, smooth, moist, Translucent with no pigment	Growth observed with medium, circular, moist colonies	Gram positive Big rods arranged in long chains. Actively motile

Cellulase enzyme production by submerged fermentation: Cellulase enzyme produced was checked by the submerged fermentation (Tolan & Foody, 1999) process with the modified medium composition that is (g/100mL) CMC 2g; Peptone 0.2g; $FeSO_4$ 0.1; KH_2PO_4 0.1; $MgSO_4$ 0.1 and pH adjusted to 7.0; 50ml of the medium is taken in the 250 mL Erlenmeyer's flask. The flasks were sterilized in autoclave at 121 ^{0}C at 15 lbs pressure for 20 minutes. The inoculum medium was incubated at 37 ^{0}C in rotatory shaker for 24 hour. Then it is transferred to fermentation medium, the composition of the fermentation medium is same, as the inoculum medium except the concentration of CMC is 1% instead of 0.5%; after the successful transformation of inoculum medium into fermentation medium, fermentation medium was kept at 37 ^{0}C in rotatory shaker for 72 hours with 150 RPM.

Table 2. result of biochemicaltests

NO	Name of the test	JRC1	JRC2
1	Methyl red test	-	
	-		
2	VP test	-	-
3	Sugar fermentation		
	1.glucose	⊥	⊥
	2.manitol	⊥	⊥
	3.xylose	⊥	⊥
	4.lactose	⊥	⊥
	5.maltose	-	-
	6.sucrose	-	-
4	Citrate utilization test	-	-
5	Starch utilization	+	+
6	Lipid utilization		
	+	-	
7	Urea utilization	-	-
8	Indole production	-	-
9	H2s production	-	-
10	Gelatine hydrolysis	-	-
11	Casein hydrolysis	+	+
12	Catalase test	+	+
13	Dehydrogenase test	+	+
NOTE:	(-)Negative, (+)Positive, (⊥) presence of only acid		

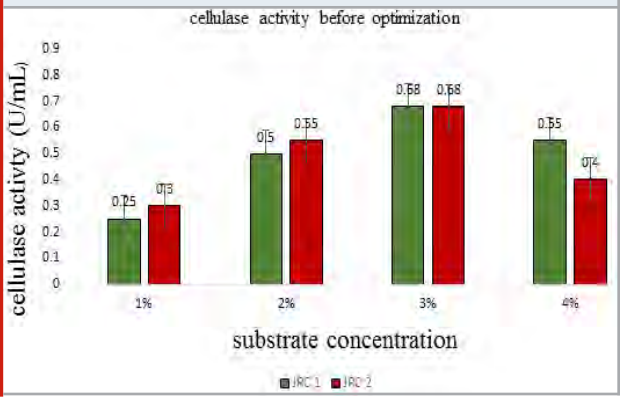
Production of Crude Enzyme: After the end of fermentation period the culture medium was centrifuged at 1500 rpm for 15 minutes to obtain crude enzyme(Shanmugapriya, Saravana, Krishnapriya, Mythili, & Joseph, 2012).

Cellulase Enzyme Assay: Cellulase enzyme activity was measured by using DNSA method (Miller, 1959). CMCase activity, which uses Carboxymethylcellulose as substrate in brief reaction mixture composed of 1mL of crude enzyme plus 1mL of 1% of CMC solution made up in the phosphate buffer pH 7 as substrate. The reaction was carried out at 55 ^{0}C for 20 minutes. The reaction was determined by adding 3 mL of DNSA reagent. The colour was then developed by boiling mixture for 5 minutes. OD of samples was measured at 575 nm against a blank having all other reagents except the crude enzyme. The quantity of reducing sugar unconfined by the hydrolysis was measured. The amount of CMCase required to release 1µmole of reducing sugar per mL per minute under given assay condition is determined as enzyme unit (EU) (Lokhande & Pethe, 2017).

Optimization for cellulase production: Furthermore optimization methods were carried out for different parameters cited following to improvise the cellulase activity, different modified methods have been adapted from the references cited following as well: pH Fermentation broth containing the optimum concentration of substrate and carbon source are taken and the pH of the broth is adjusted to 5.0, 7.0, 9.0 and 11.0 in different flasks with the help of 1 N HCl and 1 N NaOH (Acharya, Acharya, & Modi, 2008). The cultures are inoculated and incubated at optimum temperature. At the end of incubation period, the cell-free culture filtrate is taken and used as crude enzyme.

Temperature: Fermentation medium with pH 7 was inoculated with 24 hour grown selected bacterial strain. The broth was incubated at different temperatures ranging from 30 ^{0}C, 35 ^{0}C, 40 ^{0}C, 45 ^{0}C and 50 ^{0}C for 24 hour (Liang, Zhang, Wu, Wu, & Feng, 2014). After sufficient incubation period, the cell-free culture extract is gained and used as crude enzyme.

Figure 2: cellulase activity before optimization



Substrate concentration: Fermentation medium with pH 7 was inoculated with 24 hour grown selected bacterial strain with different concentration of CMC as substrate such as 1%, 2%, 3% and 4% which is incubated at optimum temperature for 24-72 hours. After 72 hours of successful incubation period, the cell-free culture extract is obtained and used as enzyme source.

Inoculum size: Inoculum size to be transferred in to production medium is checked for the optimization by taking different inoculum size of 6%, 8%, 10% and 12 % which is further transferred to an ideal production medium with overnight grown culture and incubated at optimum temperature for 24-72 hours.

RESULTS AND DISCUSSION

Bacteria with cellulase production potential were isolated from soil and on the Basis of their morphological, cultural and biochemical characteristics, the efficient two isolates were designated as JRC1 & JRC2. As shown in the figure 1 cellulose degrading bacteria were isolated on CMC agar which shows the clear zone of hydrolysis with diameter of 8.1 mm and 8.7 mm of JRC1 and JRC2 respectively. Screening of bacteria was accompanied by using the Congo red test as a initial study for identifying cellulase producers. After 3 days of incubation, both the two isolates JRC1 and JRC2 confirmed positive results in the Congo red test. Since the sole carbon source in CMC agar was cellulose, therefore the result of the test were a strong manifest that cellulase was produced in order to degrade cellulose. Partial Bacterial Identification was done on the basis of morphological, cultural and biochemical characteristics as described below in the table. As mentioned in figure 2 preliminary cellulase enzyme assay for cellulase activity shows maximum cellulase activity about 0.68 U/mL with the substrate concentration of 3 % and the standard pH of 7.0, and temperature of 37^{0}C. this result was taken before the optimization. As compared to 0.07 U/mL (Ariffin, Abdullah, Umi Kalsom, Shirai, & Hassan, 2006) isolates JRC1 and JRC2 gives comparatively higher activity.

Figure 3: Effect of pH on cellulase activity

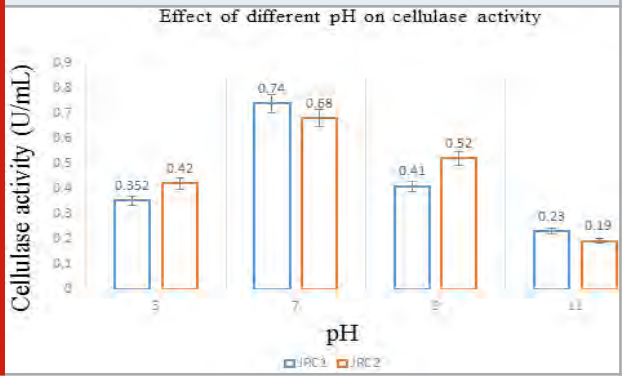


Figure 4: Effect of Temperature on cellulase activity

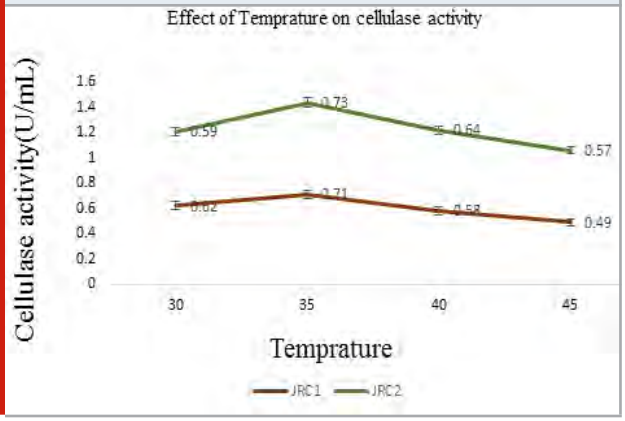


Figure 5: Effect of Substrate concentration on cellulase activity

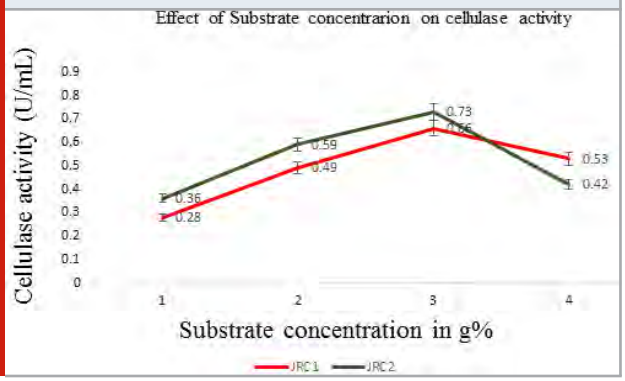
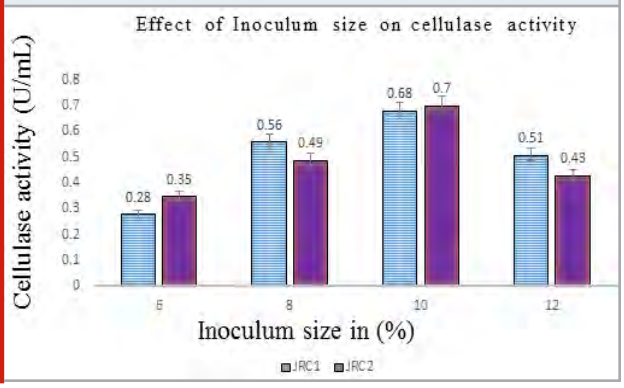


Figure 6: Effect of Inoculum size on cellulase activity



Optimization for cellulase production: Effect of pH: Both the isolates were indorsed to grow in medium of various pH ranging from 5.0 to 11.0 in odd digits. Maximum enzyme activity up to 0.74 U/mL and 0.68 U/mL was observed at pH of 7.0 in case of JRC1 and JRC2 respectively (Figure: 3). which is found to be fairly higher than (Ariffin et al., 2006) which was 0.6 U/mL in case of *B. pumilus*.

Effect of Incubation Temperature: cellulase activity substantiated at various temperatures exposed that both the isolates JRC1 and JRC2 generated determined cellulase production up to 0.71 U/mL and 0.73 U/mL respectively at 35 0C (Figure: 4). Where in case of ASN 2 (Irfan, Safdar, Syed, & Nadeem, 2012) it was nearly 0.15 U/mL .

Effect of Substrate concentration on cellulase activity: to check the maximum enzyme activity by means of substrate concentration, various amount of CMC (in g %) was taken ranging from 1 to 4 %. it is proved that isolates JRC1 and JRC2 shows maximum enzyme activity 0.66 U/mL and 0.73 U/mL respectively at substrate concentration of 3 % (Figure: 5). Which is comparatively higher than the *micrococcus spp*. Which is around 0.02 U/mL with substrate concentration of 2 % (Immanuel, Dhanusha, Prema, & Palavesam, 2006).

Effect of Inoculum size on cellulase activity: cellulase enzyme activity was also been influenced by the inoculum size ranging from 6 to 12 (in g %). Where

results obtained showed that 10% inoculum size bought the highest cellulase production compared to other inoculum size (in g %) (Figure: 6). In this existing work we are trying to determine the effect of inoculum size on cellulase activity. First time we are reporting the optimum inoculum size for cellulase production which is 10%.

CONCLUSION

The objective of existing work was to isolate cellulose degrading bacteria with potential of more cellulase production capability from soil. This study clearly demonstrate the production of cellulase from isolates JRC1 and JRC2 which produced maximum yield of cellulase. Optimum temperature and pH were determined as 35 ^{0}C and pH 7.0, substrate concentration and inoculum size were found to be 3 % and 10% respectively. Cellulase production is carefully dignified in bacteria and for improving its production capacity these controls can be amended. Cellulase yields appear to relay on complex association having a range of factors like inoculum size, pH, temperature, substrate concentration, presence of inducers and so forth.

Conflict of interest: There is no conflict of interest regarding the data published and authorship.

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Screening of Marine Bacteria for Lipase Production Using Waste Cooking Oil

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ABSTRACT

During the process of cooking, fresh cooking oil goes through many adverse alterations that confine its further consumption. Disposal of this type of polluting trashes has become progressively upsetting matter. Thus, appropriate waste management of waste cooking oil is needed. The present study focuses on screening and characterization of marine isolates and assessment of their capability to utilize waste cooking oil as a substrate for lipase production. Total of 23 bacteria isolates were screened for lipase production. Out of which 14 isolates resulted in orange fluorescence under UV light on Rhodamine olive oil agar plates detecting the lipase production. Out of 14 isolates, 2 potential strains were selected to study lipase activity. The lipase present in the broth was analyzed by titrimetric method. Results reveal that the bacterial strain BD-15 exhibited the highest lipase production in the broth after 48 h, which was 7.83 U/mL. Various parameters such as oil concentration, carbon source, dextrose, inoculum size, nitrogen source were studied to determine the optimum conditions for lipase production.

KEY WORDS: LIPASE PRODUCTION, MARINE BACTERIA, OLIVE OIL, RHODAMINE, WASTE COOKING OIL.

INTRODUCTION

Improvements in the arena of science and technology have led to abundant advances in various fields, which have also made serious problems of pollution such as liberations of unsafe gases, disposal problems of the risky industrial wastes as well as domestic waste in gaseous, solid or liquid forms. Production of useful products or energy production is the modern attitude for effective waste management and exploitation of waste products across the globe (Atlin et al., 2001). All over the globe, cooking oil is one of the most important constituents in food preparation. Cooking oil is used in extensive quantity for food frying either in-home, restaurants or in the food industry. Various physical and chemical changes that oil undergoes during the frying include viscosity, change in colour, calories count, change in odour etc. These alterations after continued cooking make the oil unhealthy for human intake. Generally, these types of oils are used in domestic animal feeds, consequently, such harmful kinds of stuff may passage down to food chain towards human, this can result in food safety complications (Lam et al., 2010).

Waste cooking oil (WCO) can solidify and blocks pipelines if not properly discarded. Degradation of WCO in pipelines can cause corrosion of concrete or metal elements. Apart from these, it may cause various health problems which

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include gastrointestinal problems and even mutagenesis (Pandare., 2015). Therefore, the use of WCO for the production of lipase can reduce and recycle wastes as well as it acts as an alternative to other substrates for lipase production (Bankovic-Ilic et al., 2012).

Lipases are triacylglycerol acyl hydrolases (E.C. 3.1.1.3) that catalyze the hydrolysis of triacylglycerol into fatty acid and glycerol (Navvabi et al., 2018). Based on the market values, lipases comes after proteases and carbohydrates, and are the 3rd largest group of the enzymes (Basheer et al., 2012). lipases are considered very significant from an industrial point of view, because of their greater production potential on a large scale (Bharathi et al., 2019). Lipases have many potential applications in various fields such as in food and dairy industry for enhancement of flavours of various foods and to speed up various processes such as cheese ripening, In detergent industry for removal of fatty or oily stains, in pharmaceutical and medical industry for various purposes (Navvabi et al., 2018). Major lipases which are commercially useful are of microbial origin (Veerapagu et al., 2013). Lipases from microorganisms have various advantages such as stability, broad substrate specificity and selectivity due to which they are widely used in industries (Dutra et al., 2007; Griebeler et al., 2011).

The environment of marine ecosystem varies from nutrient-rich conditions to limited-nutrient conditions. So, only a few microorganisms can survive there. The marine environment includes low temperature, high salt concentration, high pressure, and low temperatures as well as different lighting conditions. Therefore, there is a significant difference between enzymes produced by marine microorganisms and terrestrial microorganisms (Ramani et al., 2013). Lipases produced by marine microorganisms are generally extracellular and are influenced by medium composition such as carbon source, nitrogen source etc. (Veerapagu et al., 2013). The present study deals with the use of waste cooking oil as a substrate for lipase production using marine bacteria and optimization of various parameters such as substrate concentration, carbon source, inoculum age, nitrogen source, etc. to increase the lipase production.

MATERIAL AND METHODS

Materials: Waste cooking oil samples were collected from different local food stalls located in Ahmedabad, Gujarat, India. All chemicals and media used were of analytical grade.

Screening of marine bacteria for lipase production: Laboratory preserved marine bacterial isolates were screened for their lipase producing capabilities on Rhodamine agar medium. Nutrient agar was incorporated with 1% v/v rhodamine solution (containing 0.001% w/v rhodamine) and 1% olive oil to prepare Rhodamine olive oil agar medium. Organisms showing orange fluorescence under UV light (260 nm) on Rhodamine agar plate were considered positive (Lanka et al., 2015)

Optimization of various parameters for lipase production: From selected pure culture, a single colony was inoculated in 100 mL Erlenmeyer flask having 30 mL sterile yeast extract peptone dextrose broth (YEPD). The medium consist (g/L): yeast extract, 10; peptone, 10; dextrose, 10; supplemented with 1% v/v waste cooking oil and pH 7.0 adjusted with 1 N NaOH. After inoculation, the flask was incubated under shaking conditions at 150 rpm for 24 h at 30±2 °C for inoculum preparation. The same medium was also used for the production of lipase (Ginalska et al., 2004; Dandavate et al., 2009). If otherwise mentioned, all the experiments for optimization of lipase were carried out in triplicates in 250 mL Erlenmeyer flasks with 100 mL working volume. One factor at a time approach was used to optimize various parameters such as waste cooking oil concentration 0.5, 1, 2, 4% v/v; carbon sources (1% w/v) namely dextrose, maltose, fructose, rice flour, water chestnut flour, cornflour, dextrose concentration of 1, 2, 3, 4% w/v; age of inoculum 5, 17, 24, 48 h, nitrogen sources (1% w/v) namely yeast extract, peptone, beef extract, ammonium chloride, ammonium nitrate, ammonium sulfate, were studied.

Enzyme activity assay: Lipase activity was measured after a regular time interval using titrimetric assay (Mahmood et al., 2017). In short, the culture broth was centrifuged at 10,000*g* for 10 min at 4 °C, the cell-free extract was used as the crude enzyme. A one-millilitre cell-free extract was mixed with 5 mL of olive oil emulsion and 2 mL of phosphate buffer in a 100 mL Erlenmeyer flask. Flasks were kept on a shaker at 150 rpm for 30 min. After 30 min, the reaction was stopped by adding 1 mL of chilled acetone and 1-2 drops of phenolphthalein indicator were added. The free fatty acid liberated was titrated with 0.05 N NaOH until the faint pink colour appeared. One unit of lipase unit was defined as the amount of enzyme required to release 1 µmol of fatty acid per minute under the assay conditions. The enzyme activity was expressed as units (U) per millilitre of enzyme extract and was calculated using the below Equation.

$$\text{Lipase activity (U/mL/min)} = \frac{\text{Normality of NaOH} \times \text{volume of NaOH titrated} \times 1000}{\text{Incubation time (in min)} \times \text{volume of crude enzyme}}$$

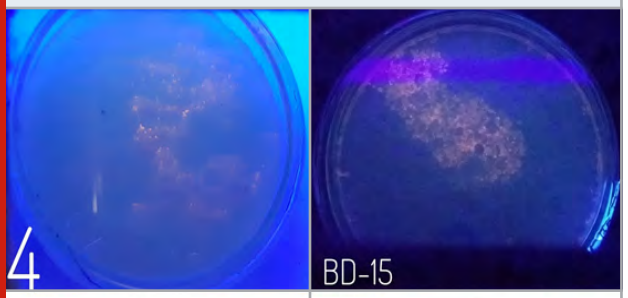
RESULT AND DISCUSSION

Screening of marine bacteria for lipase production: Total 23 marine bacterial isolates were screened for their ability to produce lipase using the Rhodamine olive oil agar plate method (Data not showed). Isolate BD-15 and 4 produced a bright orange fluorescence on Rhodamine olive oil agar plates (Figure 1) and hence, were selected for further studies. The principle behind the method is that the orange fluorescence is produced around the colonies due to interaction between hydrolyzed substrates and Rhodamine B that can be seen under the UV light (Olusesan et al., 2009). This method is advantageous because Rhodamine B does not have any adverse effect on the growth of microorganisms and it does not alter the physiological properties of the microorganisms (Kouker and Jaeger, 1987; Thompson et al., 1999)

Optimization of various parameters for lipase production:

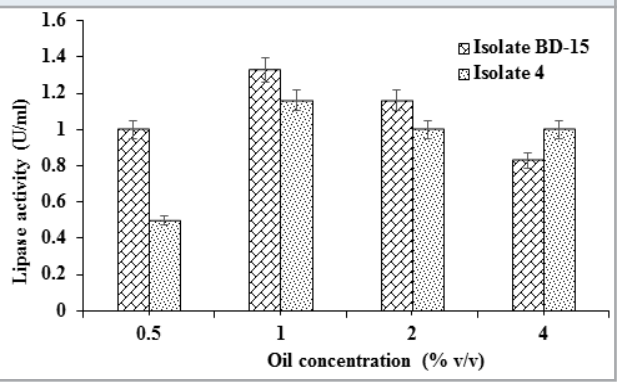
Effect of oil concentration on lipase production: As can be seen from results depicted in Figure 2, WCO concentration of 1% v/v waste cooking oil was best suited for lipase production because, on both sides of this, there was a decrease in enzyme production. Isolate BD-15 and isolate 4 showed 1.33 U/mL and 1.16 U/mL lipase production respectively after 24 h of incubation (Figure 2). Thus, it can be said that above 1% v/v of WCO concentration, has an inhibitory effect on cell activity which in turn showed less lipase production.

Figure 1: Orange fluorescence under the UV light indicates the production of lipase by (a) isolate 4 and (b) isolate BD-15



Effect of carbon source on lipase production: To check the effect of various carbon sources on lipase production, different carbon sources were supplemented in the medium. The results as shown in Figure 3, the lipase production was higher when dextrose was supplied as a carbon source in the production medium. Isolate BD-15 and isolate 4 showed 2.25 U/mL and 1.66 U/mL lipase activity respectively after 24 h of incubation (Figure 3), which shows 1.69 and 1.43 fold increase in lipase production respectively.

Figure 2: Effect of oil concentration (% v/v) on lipase production (24 h)



Effect of dextrose concentration on lipase production: Different concentrations of the dextrose were supplemented into the medium to check the effect of dextrose concentration on the lipase production (Figure 4). Among different concentrations of dextrose studied, 2% w/v was found to have the best impact on lipase production. Isolate BD-15 and isolate 4 showed 3.46 U/mL and 2.05 U/mL lipase production respectively after 24 h of incubation, which shows 2.60 and 1.76 fold increase in lipase production respectively.

Figure 3: Effect of carbon source (1% w/v) on lipase production (24 h)

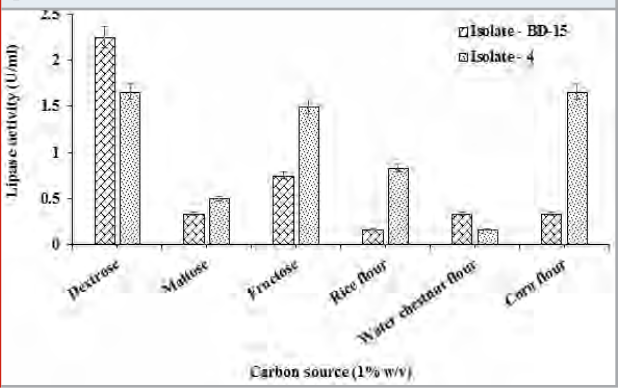
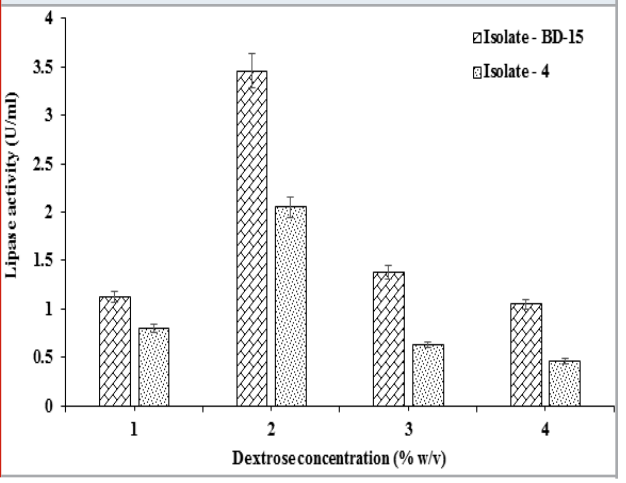


Figure 4: Influence of dextrose concentration (% w/v) on lipase production (24 h)



Effect of inoculum age on lipase production: To observe the effect of inoculum age, inocula of different age were inoculated in the production medium. As seen from Figure 5, the lipase production increased with inoculum age till the age of 24 h, after that, the production decreased. Hence, 24 h old inoculum was considered best suited for lipase production by the selected bacterial cultures. Isolate BD-15 and isolate 4 showed 4 U/mL and 3.33 U/mL lipase production respectively after 24 h of incubation (Figure 5), which shows 3 and 2.87 fold increase in lipase production respectively.

Effect of nitrogen source on lipase production: To check the effect of nitrogen source on lipase production, various organic as well as inorganic nitrogen sources were incorporated into the medium. It was observed that the lipase production was higher when two nitrogen sources added together, namely yeast extract and peptone in the broth at the concentration of 1% w/v of each. Whereas,

when these two nitrogen sources were supplemented individually in the medium, the lipase production was comparatively lower. Isolate BD-15 and isolate 4 showed 7.83 U/mL and 3.5 U/mL enzyme production respectively after 48 h of incubation (Figure 6), which shows 5.86 and 3.01 fold increase in lipase production respectively.

Figure 5: Effect of inoculum age (h) on lipase production (24 h)

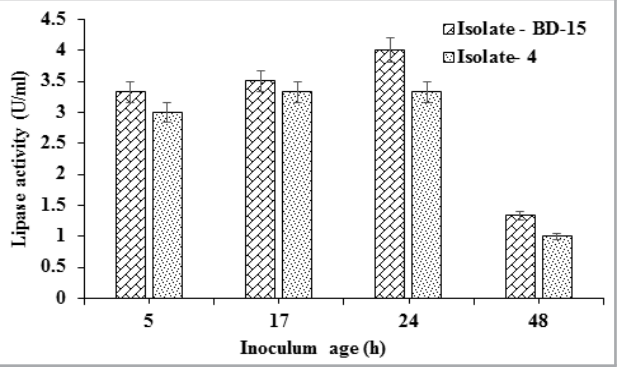
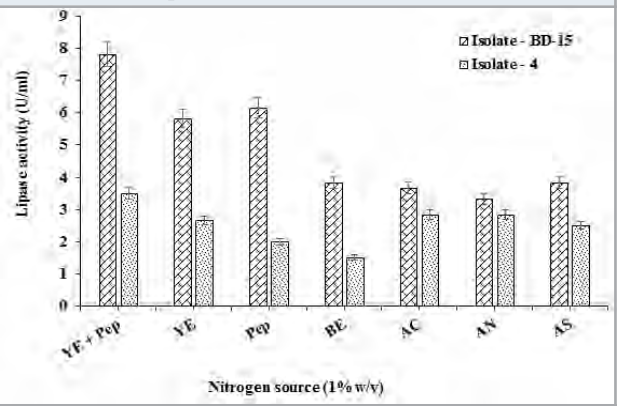


Figure 6: Effect of nitrogen source (% w/v) on lipase production (48 h)
(Note: YE= yeast extract; Pep= peptone; BE= beef extract; AC= ammonium chloride; AN= ammonium nitrate; AS= ammonium sulphate)



CONCLUSION

The main objective of the study was to produce the high yield of lipase from marine bacterial strain utilizing waste cooking oil as the substrate. The bacterial isolate BD-15 produced 7.8 U/mL of lipase enzyme after optimization which was 5.86 fold higher than the unoptimized condition. Further studies on other parameters such as salt concentration, pH, temperature need to be investigated.

Conflict of interest: There is no conflict of interest.

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Screening of Biosurfactant Producing Bacteria and Optimization of Production Conditions for *Pseudomonas guguanensis* D30

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ABSTRACT

Screening of potential biosurfactant producing bacteria from oil contaminated sites was carried out. Total thirty bacterial isolates were obtained using mineral salt medium (MSM) supplemented with and without 2% (v/v) engine oil. Among the thirty isolates obtained, three isolates D20, D27 and D30 showed positive result in biosurfactant screening tests like, drop collapsed test, oil displacement activity, hemolytic assay and emulsification index ($E_{24\%}$). Isolate D30 was selected as the potent biosurfactant producer from the three isolates as showed the highest emulsification index ($E_{24\%}$) of 36.66%. It was identified as *Pseudomonas guguanensis* D30 by 16S rDNA sequencing. To get the highest biosurfactant production by *Pseudomonas guguanensis* D30, culture conditions optimized were Bushnell Haas medium, 8% (v/v) yellow mineral oil, 6% (v/v) inoculum size and 5 days incubation period at 35 ± 2 °C, 150 rpm on a rotary shaker. It has resulted the rise to 70.00% emulsification index.

KEY WORDS: BIOSURFACTANT, BUSHNELL HAAS MEDIUM, EMULSIFICATION INDEX ($E_{24\%}$), *PSEUDOMONAS GUGUANENSIS* D30, SCREENING.

INTRODUCTION

Biosurfactants are the biologically synthesized extracellular amphiphilic surface active secondary metabolites (Janek et al., 2013). They consists of hydrophilic as well as hydrophobic domains, which ultimately provide the organism an ability to reduce the surface and interfacial tensions between two liquid phases and hence increases the solubility and biodegradability of hydrophobic and/or insoluble organic compounds (Van-Hamme et al., 2006). The carbohydrate, amino acid, phosphate group or some other compounds can be present as the hydrophilic domain whereas usually a long chain fatty acid can be present as the hydrophobic domain (Lang, 2002). Based on molecular weight biosurfactants are classified into two groups as higher molecular weight and lower molecular weight biosurfactants; while based on the chemical structure they are classified as glycolypids, lypopeptides, phospholipids, fatty acids, polymeric microbial surfactants and particulate biosurfactants (Vijayakumar and Saravanan, 2015). The structure of biosurfactants depends on the carbon:nitrogen ratio and influences the total production (Janek et al., 2013).

Environmental problems are increased due to the use of synthetic surfactants which are toxic and mostly resistant to the biodegradation in ecosystem. Hence, to

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replace the synthetic surfactants researchers are currently taking more interest in the biosurfactant production as they are less toxic, environment friendly, biodegradable products with extensive applicability in different fields; like petroleum, agricultural, bioremediation, pharmaceutical, cosmetics and food (Kaskatepe, 2015; Shah et al., 2016).

An extensive range of bacteria are reported for biosurfactant production but maximum reports are accounted with *Pseudomonas* and *Bacillus* genera like, *Pseudomonas aeruginosa, Ps. putida, Ps. stutzeri and Bacillus subtilis, B. licheniformis, B. pumilus* (Janek et al., 2013; Pereira et al., 2013). These microbes are widely studied for the production of rhamnolipids, lipopeptides and surfactins. The characteristics of biosurfactant vary from strain to strain and microbial origin; therefore, it is essential to assess different available strains for their biosurfactant potential and their characterization (Panesar et al., 2011). In the present study, biosurfactant producing *Pseudomonas guguanensis* D30 was isolated from oil contaminated soil. Furthermore, optimization of culture conditions, for biosurfactant production were carried out.

METHODOLOGY

Isolation of biosurfactant producing bacteria: Seven samples (6 from soil and 1 from water) were collected from three different oil contaminated sites near Ahmedabad and Vadodara of Gujarat, India. Mineral salt medium (MSM) containing $NaNO_3$ (15 g/L), KCl (1.1 g/L), NaCl (1.1 g/L), $FeSO_4.7H_2O$ (0.00028 g/L), K_2HPO_4 (4.4 g/L), KH_2PO_4 (3.4 g/L), $MgSO_4.7H_2O$ (0.5 g/L), $MnSO_4$ (0.05 g/L), Na_2HPO_4 (0.44 g/L), 2% (v/v) engine oil (EO) and pH 7 ± 2 was used for enrichment. Approximately 10 g samples were inoculated sterile MSM broths prepared in 250 mL Erlenmeyer flasks supplemented with and without 2% EO and incubated at 35 ± 2 °C on a rotary shaker (150 rpm) for 7 days. After incubation period, the samples were approximately diluted and spread on the same MSM agar plates and incubated at 35 ± 2 °C for 7 days. During the incubation period plates were regularly observed for growth of colonies. The cultural and morphological characteristics of the different isolated colonies were recorded. The isolates obtained were stored at 4 ± 2 °C in a refrigerator on respective MSM agar slants and maintained by sub-culturing after every 30 days.

Screening and determination of biosurfactant efficiency: For detection of biosurfactant, culture supernatant was used directly. It was obtained by centrifugation of the culture broth at 12,000 rpm (4 °C) for 15 min.

Drop collapsed test: It is a qualitative test. An oil (EO) coated glass slide was observed after 1 minute of placing a drop of the culture supernatant (approximately 10 µL). The presence of biosurfactant indicated by collapsed drop of the supernatant and spreaded on the oil coated surface (positive test). But if the drop leftovers as such after 1 minute, the test is recognized as negative. This test was carried out with 1% sodium dodecyl sulfate (SDS) as positive control and distilled water as negative control (Barakat et al., 2017).

Oil displacement test: In a clean Petri plate, 20 mL distilled water was taken, then 1 mL oil (EO) was added onto the surface to form a thin layer on the water surface. 100 µL fresh cell free supernatant was carefully placed on the center of the oil layer. If the oil displaced and zone of clearance formed within 1 min of the supernatant added it indicates presence of the biosurfactant. Distilled water and 1% SDS were used as negative control and positive control, respectively (Thavasi et al., 2011).

Emulsification index ($E_{24\%}$) activity: Emulsion activity of the culture supernatant was detected by addition of 2 mL of oil (engine/paraffin oil) to the equal volume of cell-free supernatant, mixed with a vortex for 2 min and allowed to stand for 24 hours at 35 ± 2 °C. Distilled water was used as a negative control and 1% SDS was used as the positive control. The percent $E_{24\%}$ was calculated by measuring the height of emulsified layer (mm) and total height of the liquid (mm) using formula as shown below (Khan et al., 2017):

$$E24\% = \frac{\text{Total Height of the emulsified layer}}{\text{Total height of the liquid}} \times 100$$

Hemolytic activity: Hemolytic activity is used to determine biosurfactant production ability. Isolated bacterial cultures were spotted on 5% blood agar medium and plates were incubated at 35 ± 2 °C for 48 h. After incubation period zone of clearance was observed around the colonies for hemolysis. It was correlated with the production of biosurfactant. The observation was recorded as α-greenish halo (incomplete hemolysis), β-clear zone (complete hemolysis) and γ-no change around the spotted colony (no hemolysis) (Barakat et al., 2017; Carrillo et al., 1996).

Characterization and identification of potent biosurfactant producing bacteria: The selected potent biosurfactant producing bacterial isolate D30 was studied for its morphological and cultural characteristics on MSM agar plate supplemented with oil. The identification of isolate D30 was carried out using 16S rDNA sequencing and a phylogenetic tree was prepared by BLAST with the database of NCBI Genebank.

Optimization of biosurfactant production by *Pseudomonas guguanensis* D30:

Inoculum preparation: First of all, different media were screened for inoculum preparation viz. bushnell haas (BH), mineral salt medium (MSM), nutrient broth (NB) and luria burtani (LB). To prepare an inoculum, a small growth from the agar slope culture was transferred into 50 mL of each sterile medium in 250 mL Erlenmeyer flask and incubated at 35 ± 2 °C, 150 rpm for 24 h on an orbital shaker. Absorbance of each culture was measured as optical density (OD) at 600 nm after 24 h. The medium showing the best growth was used for further study as an inoculum preparation medium. For inoculation, cells were separated by centrifugation at 10,000 rpm for 5

minutes, supernatant was removed and pellet of cells was resuspended in sterile distilled water to get an OD of 1.0 at 600 nm. A 2% (v/v) was used as an inoculum.

Emulsification index ($E_{24\%}$) with different oils: To check the effect of biosurfactant produced, emulsifying efficiency was carried out with different oils like engine oil, cotton seed edible oil, paraffin oil, mineral oil and yellow mineral oil. Sterile 50 mL broth of MSM medium supplemented with 2% engine oil (pH 7.0) was prepared in 250 mL Erlenmeyer flask, inoculated with 2% (v/v) inoculum and incubated at 35 ± 2 °C, 150 rpm on an orbital shaker for 5 days and estimated for biosurfactant production. The cell free supernatant was checked for $E_{24\%}$ activity same as mentioned in above section.

One-factor-at-a-time optimization: Media, inoculum size, incubation period, carbon (oil) source and different concentrations of oil were tested for optimizing the biosurfactant production. $E_{24\%}$ activity was measured as the response. Samples were withdrawn periodically and the cell free supernatant was obtained upon centrifugation at 12,000 rpm (4 °C) for 15 minute and was mixed with paraffin oil to measure $E_{24\%}$ activity.

Effect of different media: Three media viz. BH, NB and MSM were tested for biosurfactant production. Sterile 50 mL of each medium supplemented with 2% engine oil (pH 7.0) were prepared in 250 mL Erlenmeyer flask, inoculated with 2% (v/v) inoculum and incubated at 35 ± 2 °C, 150 rpm on an orbital shaker for 5 days.

Table 1. Colony characteristics of isolates obtained on MSM agar (without oil)

Isolate No.	Size	Shape	Margin	Elevation	Consistency	Opacity	Surface	Pigmentation
D1	Small	Round	Entire	Convex	Moist	Opaque	Smooth	Dark Yellow
D2	Small	Round	Entire	Low Convex	Moist	Opaque	Smooth	Creamy White
D3	Small	Round	Entire	Flat	Moist	Opaque	Smooth	Light Yellow
D4	Medium	Round	Entire	Convex	Moist	Opaque	Smooth	Light Pink
D5	Small	Round	Entire	Low Convex	Moist	Opaque	Smooth	Dirty Yellow
D6	Medium	Round	Entire	Convex	Moist	Opaque	Smooth	Creamy White
D7	Medium	Round	Entire	Convex	Moist	Opaque	Smooth	Creamy White
D8	Small	Round	Entire	Low Convex	Moist	Opaque	Smooth	Yellow
D9	Large	Round	Entire	Low Convex	Moist	Opaque	Smooth	Creamy White
D10	Small	Round	Entire	Low Convex	Moist	Opaque	Smooth	Creamy White
D11	Large	Elliptical	Irregular	Pulvinate	Moist	Translucent	Smooth	Creamy White
D12	Medium	Elliptical	Irregular	Pulvinate	Moist	Translucent	Smooth	Creamy White
D13	Large	Round	Entire	Low Convex	Moist	Opaque	Smooth	Yellow
D14	Large	Round	Entire	Convex	Moist	Opaque	Smooth	White
D15	Pinpoint	Round	Entire	Low Convex	Moist	Opaque	Smooth	White
D16	Small	Round	Entire	Low Convex	Moist	Opaque	Smooth	Yellow

Table 2. Colony characteristics of isolates obtained on MSM agar (with 2% engine oil)

Isolate No.	Size	Shape	Margin	Elevation	Consistency	Opacity	Surface	Pigmentation
D17	Pinpoint	Round	Entire	Low Convex	Moist	Opaque	Smooth	Orange
D18	Small	Round	Entire	Low Convex	Moist	Opaque	Smooth	Yellow
D19	Large	Round	Entire	Low Convex	Dew drop	Opaque	Translucent	-
D20	Small	Round	Entire	Convex	Moist	Opaque	Smooth	Light Orange
D21	Intermediate	Round	Entire	Low Convex	Moist	Opaque	Smooth	Creamy White
D22	Small	Round	Entire	Low convex	Moist	Opaque	Smooth	Yellow
D23	Small	Round	Entire	Low convex	Moist	Opaque	Smooth	Orange
D24	Small	Round	Entire	Low convex	Moist	Opaque	Smooth	Yellow
D25	Large	Round	Entire	Raised	Moist	Opaque	Smooth	Yellow
D26	Medium	Round	Entire	Raised	Dew drop	Vetreous	Smooth	-
D27	Small	Round	Entire	Convex	Moist	Opaque	Smooth	Dark Orange
D28	Pinpoint	Round	Entire	Convex	Moist	Opaque	Smooth	White
D29	Medium	Round	Entire	Low Convex	Moist	Opaque	Smooth	Yellow
D30	Small	Round	Regular	Low Convex	Moist	Opaque	Smooth	Light Orange

Table 3. Results of screening tests for biosurfactant detection

Isolate	Drop collapsed test	Oil displa cement test	Emulsifi cation ($E_{24\%}$)	Hemolytic activity
D1	-	-	1.78	-
D2	-	-	0.35	-
D3	-	-	0.35	-
D4	-	-	3.80	-
D5	-	-	0.35	-
D6	-	-	3.80	-
D7	-	-	0.35	-
D8	-	-	0.35	-
D9	-	-	1.78	-
D10	-	-	0.35	-
D11	-	-	0.35	-
D12	-	-	1.78	-
D13	-	-	1.78	-
D14	-	-	0.35	-
D15	-	-	0.35	-
D16	-	-	0.35	-
D17	-	-	1.78	-
D18	-	-	1.78	-
D19	-	-	12.50	-
D20	+	+ +	34.78	-
D21	-	-	14.28	-
D22	-	-	0.35	-
D23	-	-	11.66	-
D24	-	+	18.75	-
D25	-	-	14.28	-
D26	-	-	0.35	-
D27	+	+ +	33.33	+
D28	-	-	0.35	-
D29	-	-	1.78	-
D30 Distilled water	+ +	+ + +	36.66	+ +
(Negative control) 1% SDS	-	-	-	-
(Positive control)	+ + + +	+ + + +	82.60	-

*Where "+ sign" showing the degree of positive response.

Effect of inoculum size: To evaluate optimum inoculum size for biosurfactant production 2%, 4%, 6%, 8%, 10% and 12% (v/v) of inoculum was inoculated in sterile 50 mL BH broth (pH 7.0) supplemented with 2% (v/v) engine oil in 250 mL Erlenmeyer flask and incubated at 35 ± 2 °C, 150 rpm on an orbital shaker for 5 days.

Effect of carbon (oil) source: To evaluate the effect of different carbon/oil sources; yellow mineral oil (YO), engine oil (EO), cotton seed edible oil (FO) and glucose were supplemented at final concentration of 2% (v/v or w/v) in the 50 mL sterile BH broth (pH 7.0) in 250 ml flasks. They were inoculated with 6% (v/v) inoculum and incubated at 35 ± 2 °C, 150 rpm on an orbital shaker for 5 days.

Effect of incubation period: In order to optimize the harvesting time of biosurfactant produced, sterile 50

mL BH broths (pH 7.0) supplemented with 2% (v/v) yellow mineral oil (YO) were prepared in 250 mL flasks. The media were inoculated with 6% (v/v) inoculum and incubated at 35 ± 2 °C, 150 rpm on an orbital shaker up to 11 days.

Figure 1: Drop collapsed test

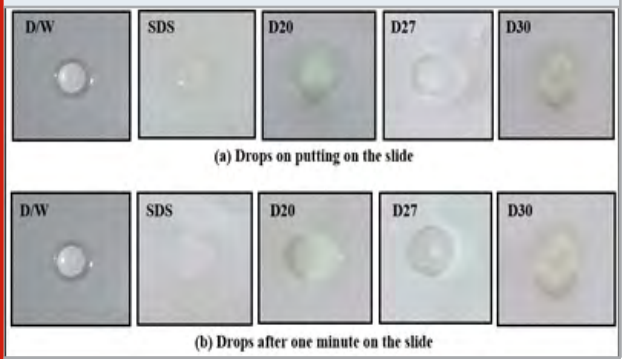


Figure 2: Oil displacement test with engine oil (a) Emulsification activity and (b) hemolytic activity

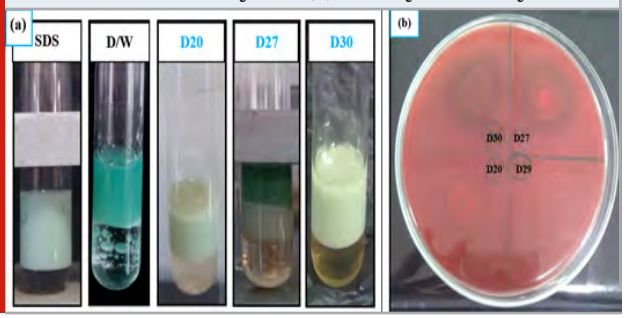
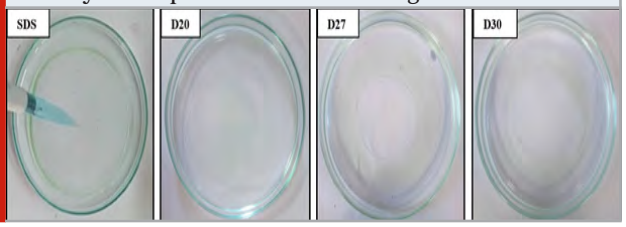


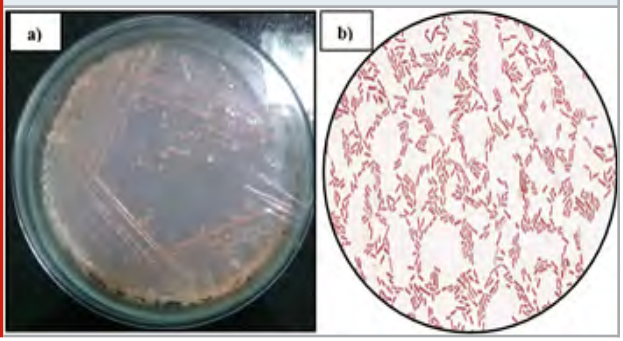
Figure 3: (a) Emulsification activity and (b) hemolytic activity Oil displacement test with engine oil



Effect of oil concentration: Since yellow mineral was found the best, it was selected as major carbon source and different concentrations were tested for biosurfactant production. Sterile 50 mL BH broths (pH 7.0) in 250 mL Erlenmeyer flasks were supplemented with different concentrations of YO (2%, 4%, 6%, 8% and 10%; v/v), inoculated with 6% (v/v) inoculum and incubated at 35 ± 2 °C, 150 rpm on an orbital shaker for 5 days.

Biosurfactant production using optimized medium: The optimized culture conditions for Pseudomonas guguanensis D30 found were BH medium, 8% yellow mineral oil, 6% (v/v) inoculum size and 5 days incubation period at 35 ± 2 °C, 150 rpm on a rotary shaker. An experimental run was kept using these optimized conditions and emulsification activity was measured.

Figure 4: Colony characteristics and gram's staining of isolate D30. (a) Growth of D30 on MSM agar supplemented with 2% EO and (b) Gram's staining of D30



RESULTS AND DISCUSSION

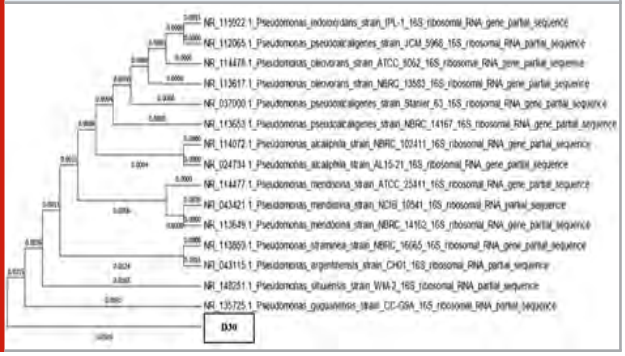
Isolation of biosurfactant producing bacteria: Total thirty bacterial strains were isolated from the enriched samples using MSM agar plates supplemented with and/or without oil. Majority of the isolates have shown growth after 5 days of incubation and the mature colonies were observed after 7 days at 35 ± 2 °C. Among thirty, fourteen isolates were obtained using MSM supplemented with oil and sixteen isolates on MSM plates without oil. The colonial characteristics of the purified isolates were recorded from the same medium and as shown in the above Tables 1, 2. The pure culture of selected isolates was transferred on MSM agar slants supplemented with and without 2% EO and stored at 4 °C in refrigerator. The cultures were maintained by sub-culturing after every 30 days on the respective medium.

Nwaguma et al. (2016) isolated 29 bacterial strains using MSM and 4 isolates were selected based on their responses in screening tests. Bodour et al. (2003) reported 45 positive isolates for biosurfactant production using MSM containing 2% (w/v) glucose. 105 oil degrading bacterial strains were isolated using Bushnell Haas agar with 2% (v/v) crude oil for screening of the biosurfactant producing organisms (Thavasi et al., 2011). Total 185 bacterial strains were isolated using BH, Reasoner's 2A (R2A) and GP2 medium with glucose 1% (w/v) or petroleum 1% (w/v) as a carbon source for biosurfactant production by Batista et al. (2006) and highest number of positive isolates were found with glucose.

Screening and determination of biosurfactant efficiency: Screening tests were done for all the 30 bacterial isolate and results were recorded in Table 3. Isolates D20, D27 and D30 showed positive response in different screening tests used to determine the biosurfactant produced. All the three isolates showing positive tests were isolated from oil supplemented media, none of the isolate from plates without oil was positive. So, presence of oil may be working as an inducer for production of biosurfactant. The culture supernatant showing positive results were shown for their drop collapsed test in Figure 1, oil displacement test in Figure 2, emulsification index ($E_{24\%}$) and hemolytic activity in Figure 3.

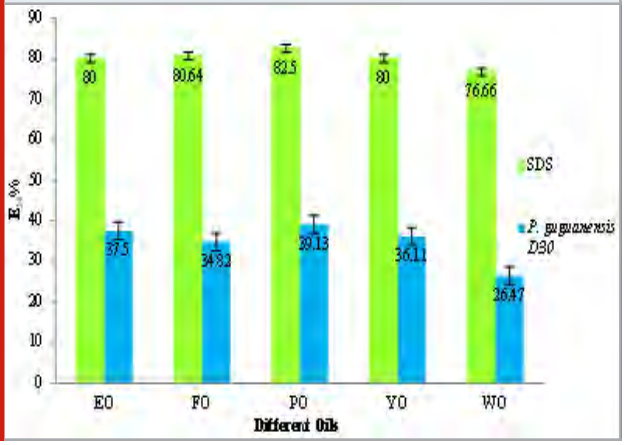
Thavasi et al. (2011) reported positive biosurfactant producing bacteria using drop collapsed and oil displacement test, among which most of the isolates are belongs to the *Pseudomonas sp*. and *Bacillus sp*. *Pseudomonas aeruginosa* ATCC-10145 a highly positive strain showed 8.0 cm of oil displacement (El-Sheshtawy and Doheim, 2014). Mouafi et al. (2016) reported positive hemolytic activity, oil displacement test with 28.2 cm2 zone and 46.6% $E_{24\%}$ activity for *Bacillus brevis* to confirm the biosurfactant production.

Figure 5: Phylogenetic tree of D30 (Evolutionary relationships of taxa)



The biosurfactant producing *Bacillus amyloliquefaciens* SH20 and *Bacillus thuringiensis* SH24 isolates the emulsification index of 57% and 56%, respectively (Barakat et al., 2017). *Bacillus* subtilis SPB1 strain was reported as the highest biosurfactant producer based on the size of hemolysis zone using blood agar medium (Kamoun et al., 2005). *Klebsiella pneumoniae* IVN51 a hydrocarbon degrading isolate showed 47.8% emulsification and 78.5 mm2 of oil displacement with 6 mm zone of blood hemolysis (Nwaguma et al., 2016). Among the positive isolates (D20, D27, D30), isolate D30 showed the highest activity in drop collapsed, oil displacement, hemolytic activity as well as $E_{24\%}$ (36.66%). Therefore, isolate D30 was selected for further study of biosurfactant production.

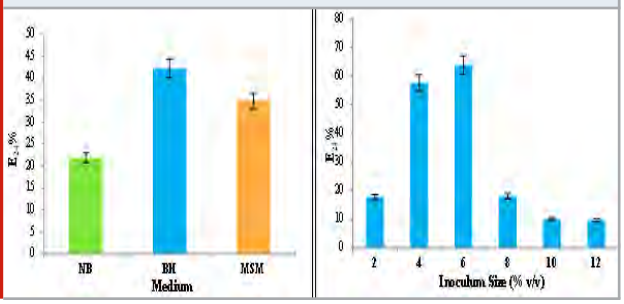
Figure 6: Emulsification index ($E_{24\%}$) of biosurfactant with different oils



Characterization and identification of potent biosurfactant producing bacteria: The cultural characteristics of isolate D30 showed its appearance as small, round, slightly convex, regular margin, smooth, opaque, and light orange pigmented colonies on MSM agar supplemented with 2% EO (Figure 4a). The Gram staining observation revealed that isolate D30 is a Gram negative, long rod shaped bacteria arranged as singly and in chains (Figure 4b). Identification of the isolate D30 was carried out using 16S rDNA sequencing. The sequence obtained from 16S rDNA were compared with those available in NCBI Genbank database using BLAST program and the similarity percentage of this isolate was 87% with strain *Pseudomonas guguanensis* CC-G9A. Hence, the strain was named as *Pseudomonas guguanensis* D30. The phylogenetic tree of the isolate *P. guguanensis* D30 is shown in Figure 5 which reveals its evolutionary relationship with other strains.

As like ours, many *Pseudomonas sp*. strains are reported for biosurfactant production. A glycolipid producer, *Pseudomonas sp*. BS1 was isolated (Liu et al., 2011). Ozdal et al. (2017) reported a rhamnolipid producing strain *Pseudomonas aeruginosa* OG1. A strain of *Pseudomonas aeruginosa* ATCC-10145 was studied for biosurfactant production (El-Sheshtawy and Doheim, 2014). A rhamnolipid producing bacterium *Pseudomonas putida* BD2 was isolated from Arctic soil (Janek et al., 2013). Sousa et al. (2011) isolated a high yield rhamnolipid producing and various hydrocarbons emulsifying bacterium, *Pseudomonas aeruginosa* MSIC02. Meliani and Bensoltane (2014) studied the capacity of fluorescent *Pseudomonas aeruginosa* to produce biosurfactant. The rhamnolipid producing strain of *Pseudomonas* was isolated (Nitschke et al., 2011).

Figure 7: Effect of different media and inoculum size on *P. guguanensis* D30



Optimization of culture conditions for biosurfactant production by *Pseudomonas guguanensis* D30:

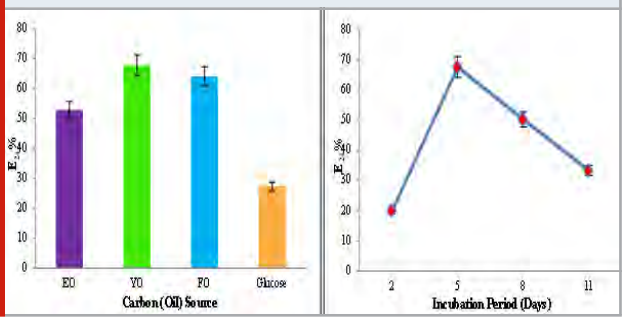
Inoculum preparation: Among the four tested broths for inoculum preparation luria burtani (LB) showed maximum growth in 24 h according to the optical cell density (OD) measured at 600 nm. Hence, LB broth was selected for inoculum preparation in further studies.

Emulsification index ($E_{24\%}$) with different oils: The $E_{24\%}$ activity of the crude biosurfactant produced by *Pseudomonas guguanensis* D30 was tested with different oils like engine oil, cotton seed edible oil, paraffin oil,

mineral oil and yellow mineral oil. Results showed that crude biosurfactant and SDS (1%) have maximum emulsification efficiency of 39.13% and 82.5% with paraffin oil, respectively (Figure 6).

A biosurfactant producing strain *Pseudomonas aeruginosa* showed good emulsification of 27.94% with gasoil (Meliani and Bensoltane, 2014). The highest emulsification activity of 46.6% was found with olive oil for the biosurfactant produced by Bacillus brevis (Mouafi et al., 2016). *Bacillus subtilis* CN2 was reported for lipopeptide production which showed $E_{24\%}$ with different oils viz., hexane, cyclohexane and motor oil as 81%, 84% and 76%, respectively (Bezza and Chirwa, 2015). The highest emulsification index of biosurfactant produced by *Bacillus cereus* DRDU1 was reported with kerosene 55.33% (Adamu et al., 2015). Ramani et al. (2012) reported maximum emulsification index i.e., 60% of biosurfactant produced by *Pseudomonas gessardii*

Figure 8: Effect of carbon (oil) source and incubation period on *P. guguanensis* D30



with olive oil amongst the tested other oils like, palm, kerosene, diesel and petrol.

One-factor-at-a-time optimization: One-factor-at-a-time approach was used to optimize various physical and chemical culture conditions for biosurfactant production by *Pseudomonas guguanensis* D30 and the response was measured on terms of their $E_{24\%}$ activity performed with paraffin oil.

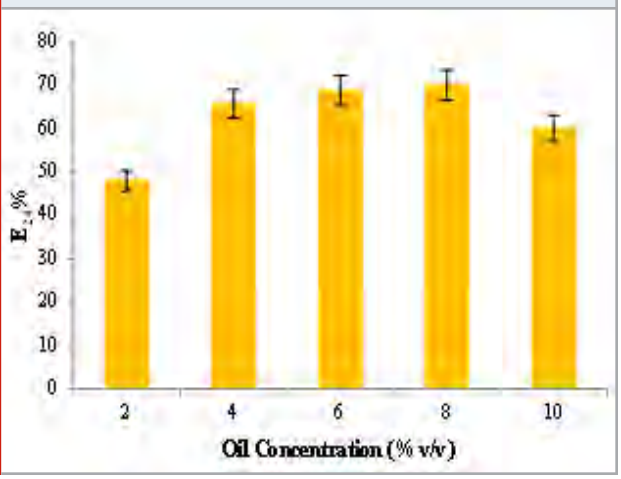
Effect of different media: Among the three different media evaluated for biosurfactant production, BH medium supported maximum $E_{24\%}$ (42.22%) activity followed by MSM (34.84%) and NB (21.88%) when supplemented with 2% engine oil (Figure 7). Hence, BH medium was selected for further study. Biosurfactant production by *Pseudomonas aeruginosa* ATCC-10145 and *Bacillus subtilis* NCTC-1040 using bushnell haas mineral salt (BHMS) medium was reported (El-Sheshtawy and Doheim, 2014). *Bacillus sphaericus* EN3 and *Bacillus azotoformans* EN16 have shown better biosurfactant production with BH medium (Adamu et al., 2015). Biosurfactant production from *Bacillus cereus* DRDU1 was also carried out with modified BH medium (Borah and Yadav, 2017).

Effect of inoculum size: Biosurfactant production at different inoculum size (2%, 4%, 6%, 8%, 10% and 12%; v/v) is shown in Figure 7. Result showed that $E_{24\%}$ index was increasing as the inoculum increased from 2% (17.74%) to 6% (64%) but decreased from 8% onwards. Hence, 6% (v/v) inoculum size was selected for subsequent experiments with *Pseudomonas guguanensis* D30.

Pseudomonas aeruginosa OG1 showed optimum biosurfactant production with 4% (v/v) inoculum size (Ozdal et al., 2017). Two novel isolates *Ochrobactrum anthropi* HM-1 and *Citrobacter freundii* HM-2 were isolated and reported for rhamnolipid production with 2% (v/v) inoculum size (Ibrahim, 2017). *Pseudomonas gessardii* strain was studied for biosurfactant production and 2% (v/v) inoculum size was used for optimum production (Ramani et al., 2012). Makkar and Cameotra (1998) reported 2% (v/v) inoculum size of the biosurfactant producing *Bacillus subtilis* strain for optimum production using modified MSM.

Effect of carbon (oil) source: Among the four carbon/oil sources tested yellow mineral oil has shown the highest $E_{24\%}$ activity of 67.64% followed by cotton seed edible oil (63.89%), engine oil (52.81%) and glucose (27.28%) (Figure 8). Hence, yellow mineral oil was selected as a carbon source for biosurfactant production by *Pseudomonas guguanensis* D30.

Figure 9: Effect of oil concentration (% v/v) on *P. guguanensis* D30



Optimization of rhamnolipid production by *Pseudomonas aeroginosa* OG1 using waste frying HM-2 have been reported with waste fried oil as a carbon source to produce optimum rhamnolipid (Ibrahim, 2017). Meliani and Bensoltane (2014) used 2% (v/v) of gas oil as carbon and energy source for biosurfactant production by *Pseudomons* strains in mineral medium. Hexadecane and soybean oil were used as carbon sources for production of rhamnolipid by *Pseudomonas aeruginosa* (Perfumo et al., 2013). Chebbi et al. (2017) reported the 1% olive oil (v/v) as the best carbon source for *Pseudomonas aeruginosa* W10.

Effect of incubation period: As shown in Figure 8, maximum $E_{24\%}$ activity was observed on the 5th day

(67.64%) of incubation as compared to other days. Thus, it was selected as the optimum incubation period for the biosurfactant harvesting produced by *Pseudomonas guguanensis* D30. Similar results were reported with *Pseudomonas sp*. for biosurfactant production on the 5th day in shaking condition at 30 °C (Liu et al., 2011). *Bacillus sphaericus* EN3 and *B. azotoformans* EN16 were reported to produce maximum biosurfactant on the 8th day (Bezza and Chirwa, 2015). Mouafi et al. (2016) reported incubation at 33 °C for 10 days as the optimum incubation period for biosurfactant production by *Bacillus brevis*.

Effect of oil concentration: The highest $E_{24\%}$ activity (70%) was measured with 8% yellow mineral oil and the lowest of 47.95% with 1% YO. The higher concentration of 10% YO showed decline in $E_{24\%}$ (60.11 %) as shown in Figure 9. Therefore, 8% of YO was selected as optimum concentration for biosurfactant production. Liu et al. (2011) used 5% (v/v) crude oil for biosurfactant production from *Pseudomonas sp*. BS1. Among different oils 2% (v/v) crude oil was reported as the best carbon source for biosurfactant production from *Stenotrophomonas sp*. B-2 (Gargouri et al., 2017). A biosurfactant production from *Pseudomonas aeruginosa* with 1% (v/v) tapis gas oil was investigated (Meliani and Bensoltane, 2014).

Biosurfactant production using optimized medium: Crude biosurfactant was extracted from the optimized medium and culture conditions i.e. 50 mL BH medium (250 ml flask), 8% yellow mineral oil, 6% (v/v) inoculum size and 5 days incubation period at 35 ± 2 °C, 150 rpm on a rotary shaker. The $E_{24\%}$ index of the supernatant of *Pseudomonas guguanensis* D30 was found 70% using optimized medium. Biosurfactant producing *Pseudomonas aeruginosa* MTCC 2297 showed maximum emulsification activity of 70% with optimum parameters (Panesar et al., 2011). The two alkaliphilic biosurfactant producing strains of *Bacillus* sp., SH24 and SH20 showed optimum emulsification activity of 60% and 69% at pH 11, 30 °C (Barakat et al., 2017). *Klebseilla sp*. FKOD36, the biosurfactant producing strain showed higher emulsification index of 66% with mineral salt media, 2.5% (v/v) inoculum size and 1% (v/v) crude oil as a carbon source (Ahmad et al., 2016).

CONCLUSION

The biosurfactants are biologically synthesized surface active agents, they are proved as good replacement of synthetic surfactants because of their low toxicity and environmental friendly nature. In the present study, biosurfactant producing bacteria *Pseudomonas guguanensis* D30 isolated from local oil well was a potent producer which is confirmed by all major screening tests. The optimization studies maximizes the biosurfactant production with conditions BH medium, 8% YO, 6% inoculum size and 5 days incubation period giving the highest $E_{24\%}$ index of 70%, which makes this organism competent enough. This can be further standardized for enhancement of production and may be applied for some commercial use.

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Bacillus pumilus – a Marine Bacteria: Unexplored Source for Potential Biosurfactant Production

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ABSTRACT

Biosurfactants are secondary metabolites produced by microorganisms. They exhibit the properties of the surface and interfacial tension reduction, emulsion stabilization, promote foaming, specific selectivity and specific activity at extreme temperatures, pH, and salinity. Marine bacteria have emerged as a potential source for these natural products since the last decade. Their surface-active properties make them useful for wide applications such as detergents, wetting and foaming agents, solubilizers, emulsifiers and dispersants. This study aimed to produce biosurfactant from marine bacteria isolated from the coastal region of Bhavnagar, Gujarat, India. Out of 120 bacterial isolates, 22 halophiles were screened and one giving the highest reduction in surface tension was selected for the study. Screening showed that Isolate PK2WM3, a Gram-positive isolate that gave the highest oil displacement area, emulsification index and positive drop collapse test with the high reduction in surface tension of 55 mN/m, after 7 days of culturing in mineral salt medium and 2% (v/v) hexadecane at 30 °C and agitation of 200 rpm. An extraction method, using chloroform: methanol (2:1) as the solvents, gave the highest biosurfactant yield, which was 8 g/L. TLC and FTIR analysis confirmed that the biosurfactant produced by the selected marine bacterial isolate PK2WM3 is composed of the lipopeptide. The potential isolate was identified by 16S rRNA gene sequencing analysis and Biolog® Microbial Identification Systems which identifies microorganism from its phenotypic pattern in the Biolog® microplate which identified the isolate as *Bacillus pumilus*.

KEY WORDS: *BACILLUS PUMILUS*, BIOLOG®, FTIR, MARINE BACTERIA, SURFACE TENSION.

INTRODUCTION

Marine microorganisms are predominant in the marine environment as well as extreme environments. The oceans have a comparatively narrow range of pH, salinity and temperature. These microorganisms are potential metabolically and physiologically to be adapted and survive under extreme pH, temperature, pressure, and saline environment (Das et al. 2010; Thavasi et al. 2014). Biosurfactants (BS) are classified according to their molecular structure e.g., glycolipids, lipopeptides, phospholipids, lipoprotein, fatty acids and polymeric biosurfactant. They also have a range of different properties such as surface tension reduction, emulsification, foaming and wetting (Banat et al. 2014; Marchant and Banat 2012).

Marine biosurfactant can enable bioavailability, dispersion, emulsification and degradation of hydrocarbon

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(Das et al. 2010; Mapelli et al. 2017). Their low-toxicity and biodegradability make them potential users for large-scale industrial production and subsequent environmental disposal where they can be readily biodegraded (Irorere et al. 2017; Uzoigwe et al. 2015).

In recent years, more attention is focused on the application of the BIOLOG® system to identify and characterize microorganisms (bacteria, yeasts and fungi). *Bacillus* species constitute a diverse group of bacteria widely distributed in soil and the aquatic environment (Parvathi et al., 2009). Taxonomically they are from the genus of Bacillaceae family belonging to Bacillales order of class, members of genus *Bacillus* are usually spore-forming Gram-positive bacteria that have rod shape and aerobic. Few publications are devoted to the study of the *Bacillus* species isolated from the marine environment. Due to their ubiquity and capability to survive under adverse conditions, heterotrophic *Bacillus* strains are hardly considered to be species of certain habitats (Claus 1986).

Bacillus species are known to produce biosurfactants such as lichenysin, surfactin, fengycin, pumilacidin, iturin and bacillomycin (Vater et al., 2002). BS production was seen during anthracene degradation by a marine alkaliphile *Bacillus licheniformis* (MTCC 5514). A novel *Bacillus stratosphericus* FLU5 strain produced biosurfactant of lipopeptide family which revealed hydrocarbon remobilization on contaminated soil (Hentati et al., 2019). Recent advances in phenotypic and chemotaxonomic methods have improved the ability of systems to resolve bacterial identities at the species level. In this study, we used the Biolog® GEN III identification system and 16S rRNA gene sequencing method. As compared to biosurfactant produced from mesophilic microorganisms, less work has been done on marine biosurfactant. Therefore this study is carried out to explore marine isolates for novel biosurfactant.

MATERIALS AND METHODS

Screening of biosurfactant producing bacteria: Total 120 marine bacterial isolates procured from the departmental preserved culture collection were screened for their biosurfactant production in Minimal Salt Medium (g/L): K_2HPO_4 1.8, NH_4Cl 4.0, $MgSO_4 \bullet 7H_2O$ 0.2, $FeSO_4 \bullet 7H_2O$ 0.01, NaCl 30.0, pH 7.0 with 0.1% crude oil (as an inducer) by the following methods.

Oil spreading technique: In oil spreading assay (Safary et al., 2010), 40 µL of crude oil was placed to the surface of 40 mL of distilled water in a petri dish forming thin oil layer on it. After that, 10 µL of culture supernatant was gently placed on the centre of the oil layer. Clear zone formation by displacing oil indicates the presence of biosurfactant. The diameter of the clear zone on the oil surface was visualized under visible light and measured after 30 seconds, which was correlated to the surfactant activity, also known as an oil displacement activity.

Haemolysis test: For the preparation of blood agar, a basal layer of sterile nutrient agar (HiMedia, India) amended with 3% glucose was overlaid with sterile agar containing 5% blood. Actively grown isolated cultures were streaked on blood agar plates. These plates were incubated for 48 to 72 h at 35±2 °C and then observed for the presence of clear haemolytic zone around the colonies, which indicated the presence of biosurfactant producing organisms (Anandaraj and Thivakaran, 2010).

Drop-collapse test: The bacterial cultures were inoculated in minimal salts medium (MSM) with 0.1% crude oil and incubated for 48 h. Drop collapse test, as described by Jain et al., (1991) was performed to screen the biosurfactant production. Crude oil was applied to the solid glass surface of a microscope glass slide and 250 µL of the supernatant was placed on the oil-coated surface and drop size was observed after 1 min with the help of a magnifying glass. The result was considered to be positive when the diameter of the drop was increased by 1 mm from that which was produced by distilled water that was taken as the negative control (Youssef et al., 2004).

Emulsification index (%EI): Emulsification assay was carried out using liquid hydrocarbon (Cooper and Goldenberg, 1987). In a test tube, 2 mL of hydrocarbon was taken, to which 2 mL of cell-free supernatant obtained after centrifugation (10,000*g* for 5 min) of the culture (after incubation) was added and vortexed for 2 min to ensure homogenous mixing of both the liquids. The emulsification activity was observed after 24 h and it was calculated using the formula:

$$EI_{24}(\%) = \frac{Total\ height\ of\ the\ emulsified\ layer}{Total\ height\ of\ the\ liquid\ layer} \times 100$$

Surface tension reduction: The selected isolates were grown in MSM broth at 32±2 °C on incubator shaker rotating at 180 rpm for 48 h. The broth was then centrifuged at 10,000*g* for 10 min, 35 mL of the cell-free supernatant was then checked for the reduction in the surface tension using tensiometer based on DuNuoy ring detachment method, with water as the standard (giving surface tension of 70±2 mN/m). Surface tension reduction was calculated by taking uninoculated sterile broth as 100% and was considered as indirect observation of surfactant production by the isolates.

Production and extraction of biosurfactant: The cultures were inoculated in 500 mL MSM broth to which 2% (v/v) of crude oil was added. The cultures were incubated at 25±2 °C for 7 days with shaking conditions. After incubation, the cultures were centrifuged at 5000*g*, 4 °C for 10 min to remove the bacterial cells. To the supernatant thus obtained, 1 M H_2SO_4 was added to adjust the pH 2. Equal volumes of chloroform:methanol was added in the ratio of 2:1 (v/v). These mixtures were shaken well to ensure proper mixing and were left overnight at 4 °C. Grey white coloured precipitate seen at the interface between the two liquids proved the presence

of biosurfactant. They were collected by centrifugation at 10000*g* for 20 min at 4 °C. For further extraction of biosurfactant, 10 mL of chloroform:methanol was added in the ratio of 2:1 (v/v) to the precipitated pellet and incubated in a rotary shaker at 30 °C for 15 min with 250 rpm agitation. Then content was centrifuged at 10000*g* for 20 min at 4 °C and supernatant was evaporated by drying at room temperature. The dried content was dissolved by dispensing in sodium phosphate buffer (pH 7) and stored at 4 °C (Chander et al., 2012).

Identification of biosurfactant by TLC and FTIR: A small quantity (~10 mg) of crude biosurfactant was dissolved in chloroform and from the obtained solution 10 µL was applied onto TLC Silica gel $60F_{254}$ plate (Merck, India). Once dried, the plate was allowed to run in a solvent system of chloroform:methanol:water (65:25:4 v/v). When the solvent reached the top, the plate was removed and allowed to air dry. Then the plate was evenly sprayed with ninhydrin reagent (0.5% w/v solution prepared in anhydrous acetone) for the presence of amino acids and development of red spot (Dusane et al., 2011) and also kept in iodine vapour chamber for development of yellow spot to check the presence of lipids in biosurfactant. FTIR was used to understand the overall chemical nature of the extracted biosurfactant. This technique helps to explore the functional groups and the chemical bonds present in the crude extract. The sample was dissolved in chloroform and analysed (Brucker, USA). Infrared (IR) absorption spectra were obtained using a built-in plotter. IR spectra were collected over the range of 400–3800 cm^{-1}. The spectrum was studied to interpret the chemical nature of the biosurfactant.

Identification of bacterial isolate by 16S rRNA gene sequencing: Genomic DNA of the selected isolate PK2WM3 was extracted using Genei Pure Bacterial DNA purification kit-117290. The 16S rDNA fragment was amplified using high-fidelity PCR polymerase. Universal 16S rDNA primers and instant ligation kit 105611 were used for the process. PCR product was cloned in pGEMT vector and 1.5 kb insert were sequenced in ABI3100 (16 capillaries) sequencer. The obtained sequence was aligned with similar sequences of GenBank using NCBI BLASTn algorithm for its molecular identification (Altschul et al., 1990). Identification was done based on the maximum percentage similarity with an already existing similar sequence in the database. For phylogenetic analysis, the 16S rRNA gene sequences of the type strains of *Bacillus pumilus* were taken along with the sequence of isolate PK2WM3. The alignment was carried out using the ClustalW algorithm in the software MEGA 6.

Table 1. Screening of halophilic marine bacterial isolates for traits of biosurfactant production

Isolates	Screening tests				
	Heam olysis	Drop collapse	Oil displacement (cm)	Emulsification index (%)	Surface tension (Nm/m)
9	+	+	3.0	45.45	61.3
10	+	+	4.0	30.46	59.7
11	+	+	1.2	-	-
15	+	+	2.8	20.0	-
16	+	+	3.0	23.07	-
17	+	+	1.5	-	-
20	+	+	1.0	-	-
27	+	+	3.0	42.85	67.5
28	+	+	2.5	38.0	70.1
BD1	+	+	2.0	40.0	65.9
BD11	+	+	-	-	-
BD16	+	+	5.0	65.21	60.5
BD17	+	+	1.0	-	-
BD22	+	+	3.0	46.6	65.5
PK2WM3	+	+	7.5	70.40	51.53
PK2WM16	+	+	3.0	23.07	67.50
K3W5	+	+	1.0	10.3	-
K3W6	+	+	3.0	20.0	68.25
K3W7	+	+	0.5	7.5	-
K3S5	+	+	1.0	14.20	-
NSM2	+	+	6.0	50.50	58.03
NST6	+	+	5.5	45.25	51.66

Key, + positive test; - Negative test

Identification of the isolate PK2WM3 using Biolog® GEN III™ microplate: The identification of isolated bacteria was performed by GEN III MicroPlate™ test panel of the Biolog® system. The test panel facilitates a "Phenotypic Fingerprint" of the microorganism which can then be used to identify them to a species level. The GEN III MicroPlates™ enables testing of Gram-negative and Gram-positive bacteria in the same test panel. The test panel contains 71 carbon sources and 23 chemical sensitivity assays. This assay dissects and analyzes the ability of the microbial cell to metabolize all major classes of compounds. It also involves the determination of other essential physiological properties such as pH, salt and lactic acid tolerance, reducing power, and chemical sensitivity. All the reagents used were from Biolog, Inc., USA. Fresh overnight grown culture of the bacterial isolate was tested as recommended by the kit manual. The bacterial suspension was prepared by removing bacterial colonies from the plate surface with a sterile cotton swab and agitating it in 5 mL of 0.85% saline solution. The bacterial suspension was adjusted in inoculating fluid IF-A to achieve a 90–98% transmittance (%T=90) using a Biolog® turbidimeter. A 150 µL of the suspension was dispensed into each well of a Biolog® GEN III microplate. The plates were incubated at 30±3 °C. After incubation, the phenotypic fingerprint of purple wells was compared to the Biolog's database.

RESULTS AND DISCUSSION

From 120 marine bacterial isolates, 22 halophiles those were able to grow from 15-30% w/v salt were selected for biosurfactant screening. Isolates 9, 10, 27, 28, BD-1, BD-16, BD-22, PK2WM3, PK2WM16, K3W6, NSM2 and NST6 showed a positive result for all five tests (Table 1). Among them isolate PK2WM3 was found to be potential showing positive test for both haemolysis and drop collapse, 7.5 cm clear zone in oil displacement test, 70.40% emulsification index value and 51.53 Nm/m reduction in surface tension. It was observed that strain with higher emulsification activity showed high oil displacement. The haemolysis test is generally carried out as a primary (qualitative) screening of bacteria to confirm its ability to produce biosurfactant (Walter et al., 2010; Youssef et al., 2004). The drop collapse test relies upon destabilization of liquid droplets by surfactants (Plaza et al., 2006). Biosurfactant production decreases the surface tension of the supernatant. As a result, the shape of the supernatant droplets was large as compared to control (distilled water/sterile broth). The oil spreading test results are considered positive when a clear zone is formed on the oil layer of the supernatant as shown in Figure 1. Oil displacement test was developed where the clearing zone was formed as oil being displaced by the presence of biosurfactant (Morikawa et al., 2000; Youssef et al., 2004).

Production of biosurfactant by isolate PK2WM3: Maximum yield of 8 g/L biosurfactant was produced after 7 days of incubation. Similar work was reported by (El- Sersy et al., 2012) who reported 76.3 mg/L of lipopeptide biosurfactant production from *B. subtilis* SDNS strain. Figure 2 depicts that surface tension of broth that decreased with cell proliferation indicating an increase in biosurfactant production.

Characterization of produced biosurfactant: Results of TLC showed red and yellow spots, indicating the presence of peptides and lipids in the structure of produced biosurfactant (Figure 3a). This preliminary analysis revealed lipopeptide nature of the biosurfactant. The result of FTIR spectrum showed (Figure 3b) a broad absorbance peak (centred around 3296.65 cm-1), which is a typical characteristic of carbon-containing compounds with amino groups. Sharp absorbance peaks at 1412.25 cm^{-1}, 1449.57 cm^{-1}, 2833.23 cm^{-1} and 2944.36 cm^{-1} are indicative peaks of aliphatic chains (-CH^{3-} and -CH^{2-}).

Figure 1: Screening of biosurfactant (a) Haemolysis test, (b) Oil displacement test, (c) Emulsification index %EI_{24}

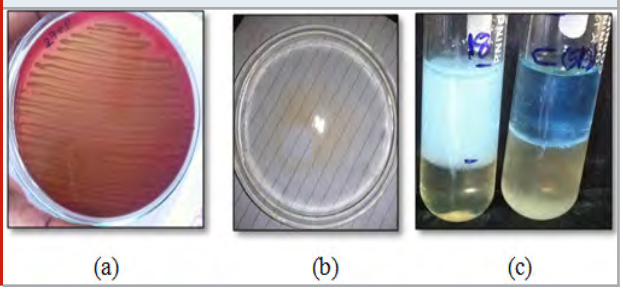


Figure 2: Growth kinetics and surface tension reduction by isolate PK2WM3

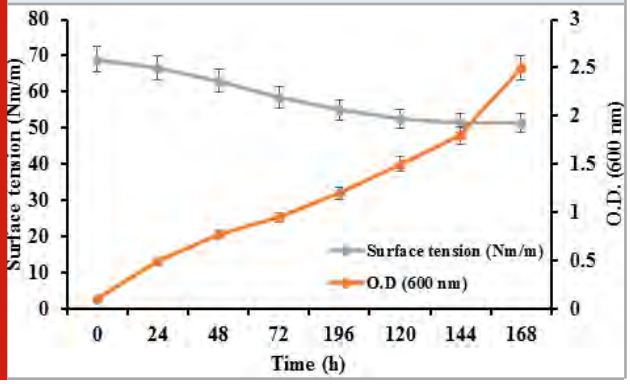


Figure 3: Characterization of biosurfactant (a) TLC (b) FTIR

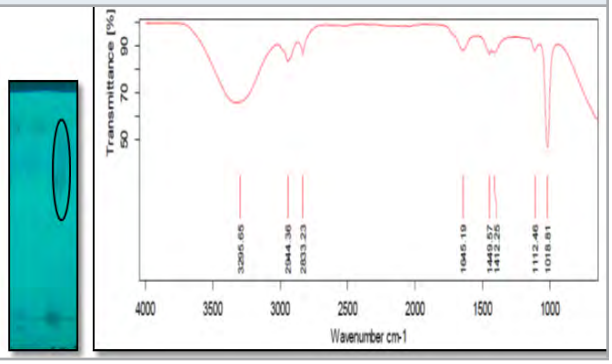
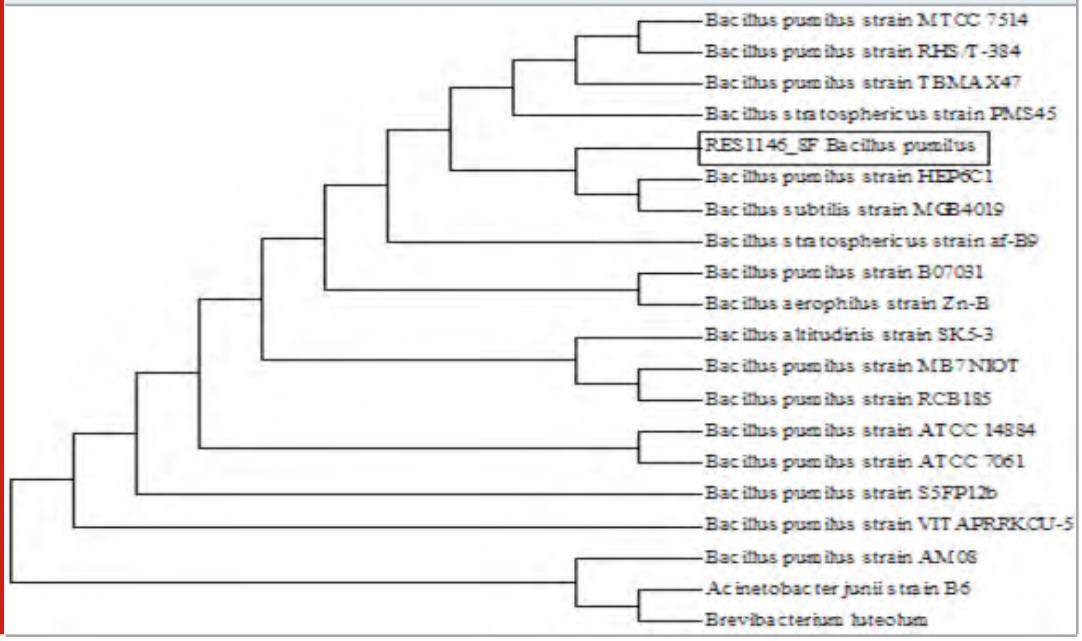


Table 2. Metabolic profile of marine bacterial isolate PK2WM3 using the Biolog® GEN III microplate

Substrate		Result
Sugars	Dextrin, D-trehalose, D-cellibiose, Gentibios, Sucrose, D- turanose, Stachyose, D-raffinose, α-D-lactose, D-melibiose, β- Methyl-D-glucoside, D-salicin, N-acetyl-D-glucosamine, N- acetyl-β–D-mannosamine, N-acetyl-D-galactosamine, N-acetyl neuraminic acid, α-D-glucose, D-mannose, D-fructose, D-galactose, 3-Methyl glucose, D-fructose, L- fucose, Inosine D-maltose, L-rhamnose	+ - -
Lactic acid	1% Sodium lactate Fusidic acid, D- serine	+ -
High pH	pH6, pH5	+
NaCl (%)	1, 4, 8	+
Hexose- PO_4	D-glucose-6-PO_4, D-fructose-6-PO_4	+
Hexose acids	Pectin, Quinic acid	+
Amino acids	D- galacturonic acid, L- galactonic acid lactone, D- gluconic acid, D-glucuronic acid, glucuronamide, Mucic acid, D- saccharic acid Gelatin, Glycyl-L-proline, L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, L-histidine, L-pyroglutamic acid	- +
Carboxylic acids, esters and fatty acids	D-lactic acid methyl ester, L-lactic acid, Citric acid, α-keto- glutaric acid, D-malic acid, L-malic acid, Bromo-succinic acid, Tween 40, γ-Amino-butyric acid, α-Hydroxy- D,L-butyric acid, Acetoacetic acid, Acetic acid, Propionic acid, Acetic acid, Formic acid, Lithium chloride, Potassium tellurite, Azotreonam, Sodium butyrate p- Hydroxy-phenylacetic Acid, Methyl pyruvate, α- Keto-butyric acid, sodium bromate	+ -
Antiobiotic sensitivity	Guanidine HCl, Troleandomycin, Rifamycin SV, Minocyclin,	+ -
GP-GN	Linomycin, Niaproof 4 Vancomycin Nalidixic Acid	- +
Key, '+' Utilized substrate; '-'Not Utilized substrate		

Figure 4: Phylogenetic tree



These peaks indicate the presence of alkyl chains in the biosurfactant. Characteristic carbonyl stretching was confirmed by the presence of a peak at 1645.19 cm^{-1}. Therefore, from the above findings, it can be said that the chemical nature of the produced biosurfactant was lipopeptide. FTIR spectra of the crude biosurfactant showed very close similarity with the IR absorption pattern reported by other lipopeptides produced from *Bacillus sp*. (Sivapathasekaran et al., 2010).

Identification of bacterial isolate by 16S rRNA gene sequencing: The 16S rDNA analysis showed that the sequence of the strain aligned with the sequence of *Bacillus pumilus* from the database with a probability of 99–100%. Complete sequence (1500 bp) of the 16S rDNA of the strain was determined and a similarity search by BLASTn was done. Ventura and Zink (2002) stated that 16S rRNA genes sequencing and analysis has become one of the cornerstones of modern microbial taxonomy. Therefore, these sequences are used to define genus specifics for rapid detection of bacterial species. However, 16S rRNA gene sequences are normally not sufficient to differentiate strains within a species, which the Biolog®, however, sometimes can. Whereas, this molecular tool was beneficial in the assessment of the overall phylogenetic relationship between the most typical bacteria.

Metabolic profiling of isolate PK2WM3 using Biolog® GEN III microplate: The marine bacterial isolate PK2WM3 was identified as *Bacillus pumilus* by the Biolog® system with a similarity index of 0.569. Characteristics of biochemical reactions obtained from GEN III are described in Table 2. The Biolog® identification and 16S rRNA gene sequencing gave a close evolutionary relationship with *B. pumilus*. In a previous study, it has been demonstrated that Biolog® could be a valuable complement to other methods used for strain verification. However, using it as a single method for identification could be misleading. The major advantage of the 16S rRNA gene sequencing is that the 16S rRNA gene is present in all bacteria, and it is a universal target for bacterial identification and provides high accuracy, reliability and reproducibility for identification of any bacterial organism (Drancourt et al., 2004).

CONCLUSIONS

The main focus of the present study is to screen out the potential novel strain of biosurfactant production. Biosurfactant increases the availability and emulsification of hydrophobic substances. Here, about 120 marine bacterial isolates were screened out for biosurfactant production capacity. Out of them one potential strain PK2WM3 was further analysed and identified by 16S rRNA and Biolog® GEN III microplate. The strain was identified as *Bacillus pumilus* with 96% similarity with the NCBI sequence database and similarity index 0.569 with and Biolog® assay. Characterization of produced biosurfactant showed lipopeptide nature based on TLC and FTIR results. Further optimization and use in environmental bioremediation of oil-contaminated sites can be explored.

Conflict of interest: There is no conflict of interest.

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Identifying Plant Derived Natural Antibiotic Against Multidrug Resistant Microbes Using Molecular Docking

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ABSTRACT

Multidrug-resistant microbes have become one of the most important current alarming threats to public health. Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most prevalent of multidrug-resistant microbes as it is causing primary infections of the skin and soft tissue such as folliculitis, furunculosis, and impetigo. This resistivity in MRSA is accredited to PBP2a, a type of Penicillin Binding Protein required for the synthesis of peptidoglycan during cell wall formation but PBP2a is susceptible to Ceftobiprole which is fifth generation Cephalosporin. Ceftobiprole possessing anti-MRSA activity through inhibiting the PBP2a mediated cell wall biosynthesis is commonly used to treat MRSA infections. However, the usage of this drug was limited due to the adverse side effects. So to combat this problem, the exploration of antimicrobials that are safe and nontoxic has become the need of the hour. In this present study, the potent plant bioactive molecules were docked into the binding pocket of PBP2a and compared with the standard antibiotic Ceftobiprole. Amongst all the plant active molecules it was found that Rutin was able to occupy the same binding pocket as of Ceftobiprole with better interactions and binding energy (-10.2 kcal/mol) was also found to be lower than Ceftobiprole. Hydrogen interactions were responsible for maintaining the stability of ligand-protein complexes. The aim of the study was to suggest the potent plant bioactive molecules that inhibit PBP2a which are safe, nontoxic and less problematic to overcome the adverse effects of the drug and the complications of multiple drug resistance that is experienced by most of the currently used antibiotics..

KEY WORDS: DOCKING, MULTIDRUG RESISTANT MICROBES, PENICILLIN BINDING PROTEIN (PBP), *STAPHYLOCOCCUS AUREUS*.

ARTICLE INFORMATION

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INTRODUCTION

Multidrug-resistance microbes are currently persisting as one of the most important public health problems. Due to selective pressure created on microbes by the hasty and indiscriminate usage of different antibiotics over the years has resulted into the emergence of the multidrug-resistance microbial strains. One of the classical examples of multidrug-resistance microbe is of Methicillin-resistance *Staphylococcus aureus* (MRSA) which is resistant not only to methicillin but usually also to aminoglycosides, macrolides, tetracycline, chloramphenicol, and lincosamides. MRSA causes severe skin and soft tissue infections such as folliculitis, furunculosis, and impetigo in hospitalized patients (Kusuma and Susilowati, 2018). In addition to the production of β-lactamases, the resistance of MRSA is also accredited to the alteration in the active site of Penicillin Binding Proteins. Production of β-lactamases catalyze the hydrolysis of the β-lactam rings present in β-lactam antibiotics. PBPs plays a vital role in cell wall peptidoglycan synthesis by initiating the transpeptidation reaction which is responsible for catalyzing the cross linking between the peptide side chains of neighboring NAM-NAG peptidoglycan strands by forming pentapeptidic chain (Ghuysen, 1994; Sauvage et al., 2008).

Methicillin resistance requires Penicillin Binding Protein (PBP2a), a 78 kDa protein which is encoded by gene *mecA* (Hartman and Tomasz, 1984, Chambers, 2001). The encoded PBP2a by gene *mecA* of MRSA has low affinity for all β-lactam antibiotics and it enables *Staphylococcus* to survive on high exposure to such agents and thus rendering it resistance against multiple drugs. PBP2a blocks the binding of β-lactam antibiotics in its active site and thus allow the transpeptidation reaction leading to the synthesis of peptidoglycan chain required for cell wall formation (Lim and Strynadka, 2002). Cell wall peptidoglycan plays a crucial role in maintaining cell shape and its internal pressure and thereby preventing sudden bursting of cell (Contreras-Martel et al., 2009). As PBPs are critical in the peptidoglycan synthesis of the bacterial cell wall, it can be exploited as one of the high specific target sites for designing and development of the new antibiotics as they are important for the survival of the cell (Jeonge et al., 2013).

Ceftobiprole, a fifth generation cephalosporin β-lactam shows anti-MRSA activity by inhibiting PBP2a (Bush et al., 2007). Although PBPs are inhibited by the β-lactam antibiotics, the usage of them may cause dreadful effect on host including allergies, hypersensitivity and immunosuppresion (Noel et al., 2008). Therefore, there's an urgent need to develop safer antimicrobials to avoid the side effects. As a bio-resource, plants produce diverse range of biomolecules that are can serve as an alternative sources of different antimicrobial agents (Rates, 2001). The plant metabolites serves as antimicrobial agents which are having enormous therapeutic potential and may work as potent inhibitor molecules (Cunha, 2001). For the drug discovery and development using traditional methods, screening and identification of various active drug molecules is a time consuming process as it requires highly refined target molecules to perform *In vivo* and *in vitro* assays. But with the advancement in the computational strategies, the molecular docking approach can be used to characterize and elucidate the behavior of small molecules in the binding site of the target proteins (McConkey et al., 2002). This strategy is highly useful to fasten up the drug screening process.

The aim of the present study was to identify and suggest potent plant active molecules targeting crucial amino acid residues present in the binding pocket of Penicillin Binding Proteins that are essential for bacterial cell wall synthesis. The present study represents essential *in silico* study regarding binding efficacy of Ceftobiprole and plant derived active molecules with PBP2a which will be useful for the development of new active molecules that can interact at the active site and inhibit the formation of peptidoglycan and thus target bacterial pathogens.

MATERIALS AND METHODS

Protein Receptor Preparation for In silico studies: PBP2a is essential for the synthesis of the bacterial peptidoglycan and its 3D structure with PDB entry of 4DKI was retrieved from Protein Data Bank (Lill and Danielson, 2010). The preliminary step before proceeding for the docking studies is to identify and remove co-crystallize ligands and water molecules. The next step is to minimize and assign the charges to the protein from which co-crystallize ligands and water molecules had been removed. For quick and efficient generation of high quality atomic charges for the protein molecule, AM1-BCC method was used and the charges were computed using ANTECHAMBER algorithm (Wang et al., 2001). The energy minimization step was carried out using 500 steepest descent steps with 0.02Å step size and an update interval of 10. Then the protein file was saved in PDB format. This was done using AutoDock Vina which is an add on in UCSF Chimera (Trott and Olson, 2010).

Ligand structure preparation for in silico studies: In the present study, *in silico* screening of 19 plant active molecules was carried out in the active site pocket of PBP2a. Ceftobiprole, a potent β-lactam antibiotic was used as reference molecule. All the plant active molecules that are used as ligands are retrieved from NCBI PubChem Database (Berman et al., 2000). Prior to docking studies, all the ligands were optimized by the addition of hydrogen and energy minimization using Gasteiger algorithm (Gasteiger and Jochum, 1979). Then all the ligand files were saved in mol2 format which is suitable to use in virtual screening. This is done using AutoDock Vina which is an add on in UCSF Chimera v1.13.

Active Site Identification: Recognizing the catalytic binding site residues in the protein structure is of high importance as the active site is a small region, a cleft or pocket where lead molecules can bind to stimulate the target protein and produce the desirable effect. In the present study, active site identification was done using

Qsite finder which locates the possible ligand binding sites using the Vander Waal's probe and interaction energy (Laurie and Jackson, 2005).

In silico docking studies: Once the active site of the protein has been identified, the docking of ligands with PBP2a receptor protein was carried out in AutoDock4 (version 4.2) with the Lamarckian genetic algorithm (Morris et al., 2009).

RESULTS AND DISCUSSION

The binding affinity of Ceftobiprole and plant active molecules against PBP2a were evaluated using AutoDock Vina. The binding poses for each ligand molecule into PBP2a was determined and different conformations of ligand poses were generated using AutoDock Vina algorithm. Out of all the possible poses for each ligand represented in ViewDock window, the poses showing maximum hydrogen bonds and minimum binding free energy change (kcal/mol) were chosen. The resulting files were saved as PDBQT format and they were further analyzed in Discovery Studio for precise study of hydrogen bond formation by the functional groups of ligands with amino acids residues of PBP2a. The active site of PBP2a is shown in Figure 1. The interaction of Ceftobiprole and Rutin with PBP2a is shown in Figure 2 and Figure 3 respectively. The enlarge view of comparison of the binding of Ceftobiprole and Rutin in the active site of PBP2a is as shown in Figure 4a and 4b respectively. Interactions of amino acid residues within binding pocket of PBP2a forming hydrogen bonds with Ceftobiprole and Rutin is shown in Figure 5 and Figure 6 respectively. The post docking results of potent plant active molecules and Ceftobiprole, a reference molecule with PBP2a are presented in Table 1.

Figure 1: Active site (White colour) of PBP2a (Blue colour)

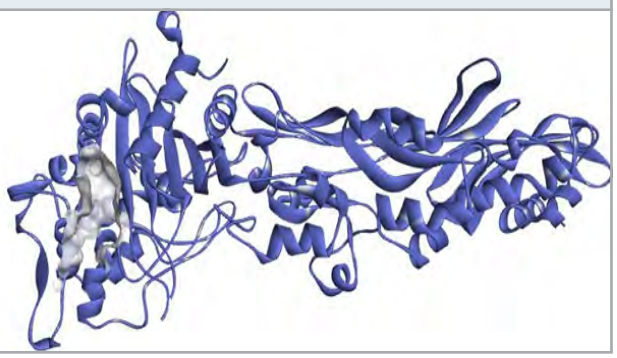


Figure 2: Ceftobiprole (Green colour) interacting with PBP2a (Blue colour)

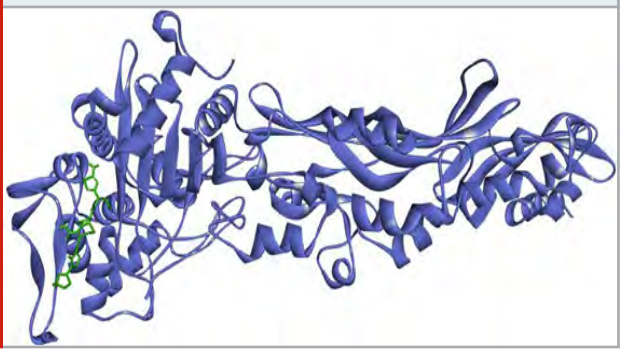


Figure 3: Rutin (Pink colour) interacting within binding site of PBP2a (Blue colour)

Figure 4a and 4b: Enlarged view of Ceptobiprole (Green colour) and Rutin (Pink colour) interacting with PBP2a within the same binding pocket respectively

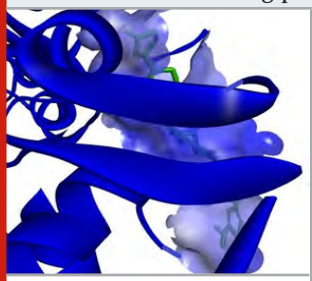


Figure 4a

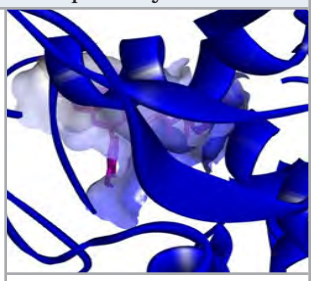


Figure 4b

Figure 5: Hydrogen bond interactions of Ceftobiprole with amino acid residues within the binding pocket of PBP2a. H-bonds are represented by dotted green colored lines. Red dotted line represents unfavorable donor or acceptor interactions

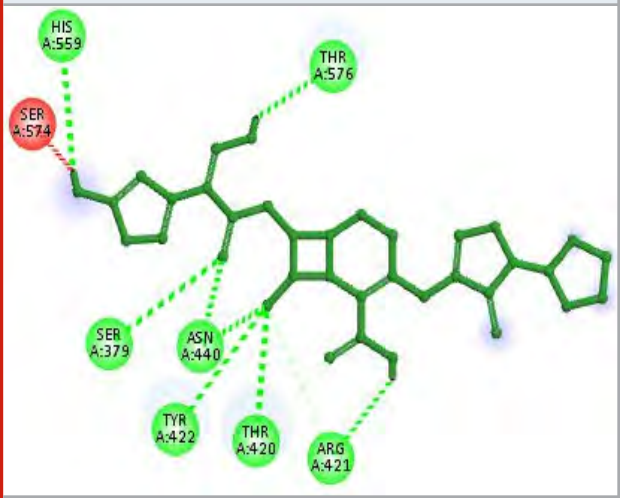
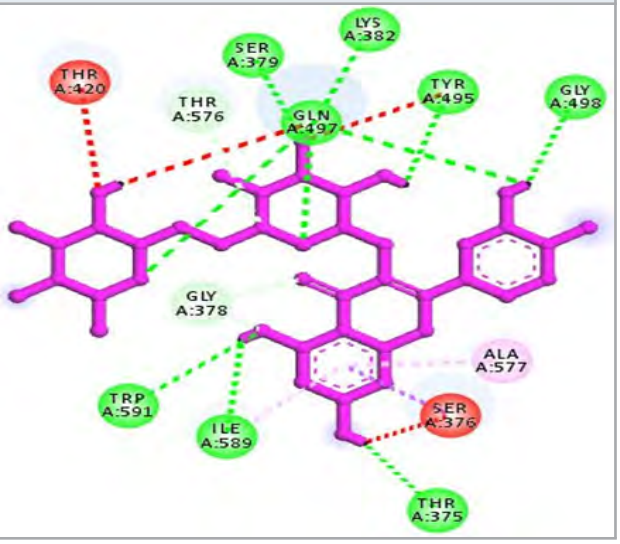


Figure 6: Hydrogen bond interactions of Rutin with amino acid residues within the binding pocket of PBP2a. H-bonds are represented by dotted green colored lines. Red dotted line represents unfavorable donor or acceptor interactions while pink colour dotted line indicates Pi-alkyl interactions



Ceftobiprole is a potent inhibitor of PBP2a which is responsible for the resistance of *Staphylococcus aureus* (Dauner et al., 2010). High affinity of Ceftobiprole to PBP2a of MRSA has also been reported by Lovering et al, 2012. For the initiation of transpeptidation step required for bacterial cell wall synthesis, the active site region of PBP2a has the deacylating acceptor moiety of an adjacent peptidoglycan strand. Cross linking between adjacent peptidoglycan will only take place if the acceptor moiety in PBP2a deacylates and thus results in cell wall formation. The presence of nucleophilic serine in active site plays a crucial role in the β-lactam mediated inhibition of PBP2a. The lactam ring present in β- lactam antibiotic interacts with nucleophilic serine and forms a strong inhibitory covalent acyl-enzyme complex in the active site and inhibiting the deacylation step (Rani et al. 2014). This will result into the disturbance in cell wall cross linking formation leading to cell lysis. The post docking results of this study also showed the interaction of Ceftobiprole, a fifth generation Cephalosporin containing β-lactam ring with serine residue present in the active site of PBP2a (Figure 5) with the binding energy of -9.1 kcal/mol.

Although many reports are available for the use of Ceftobiprole and other β-lactam antibiotics for the

Table 1: Post docking results of potent plant active molecules and a reference compound, Ceftobiprole with PBP2a

Sr. No.	Ligand	Total No of H-Bond	Interacting Amino Acid residues in Active site of PBP2a	Energy kcal/mole
1	Rutin	10	Thr375, Ser379, Lys382, Tyr 495, Gln497 (3), Gly 498, Ile589, Trp591	-10.2
2	Hesperidin	7	Ser376, Ser 379, Gln497 (2), His559, Lys573, Glu578	-9.7
3	Fisetin	6	Ser438, Asn440 (2), His559, Ser574, Ala577	-8.3
4	Myricetin	6	Asn440, Ser438, Cys578, His559, Ser574, Gln588,	-8.5
5	Luteolin	6	Thr420, Glu423, Ser437, His559, Ser574, Thr576	-8.3
6	Theaflavin	5	Ser379, Lys382, Ser438, Asn440, Glu578	-9.9
7	Kaempferol	5	Arg440 (2), His559, Ser574, Ala577	-7.9
8	Naringenin	4	Lys382, Ser438, Asn440 (2)	-7.9
9	Eriodictyol	4	Thr420, Glu423, Thr576	-8.4
10	Diosmetin	4	Thr375, Asn 440, Trp495, Trp591	-8.2
11	Epicatechin	4	Thr420, Glu423, Gln497, Ser574	-7.6
12	Scopoletin	3	Ser379, Asn440 (2)	-6.8
13	Taxifolin	3	His559, Thr576 (2)	-8.3
14	Tricin	3	Glu423, Gln497, Thr576	-8.5
15	Biochanin	3	Asn440, Ile589, Trp591	-7.6
16	Macluraxanthone	2	His559, Glu578	-9
17	Genistein	2	His559, Glu578	-7.4
18	Daidzein	2	Asn440, His559	-7.4
19	Apigenin	2	Thr375, Asn 440	-8
20	Ceftobiprole*	8	Ser379, Thr420, Arg421, Tyr422, Asn440, His559, Thr576	-9.1

*Reference molecule

inhibition of PBP2a, less reports have been reported for the exploitation of amino acid residues present in the binding site of PBP2a that can show the similar interaction of amino acid residues using plant derived molecules. From the post docking results, it was found that Rutin, a plant derived molecule is also interacting with serine residue in the active site of PBP2a which might inhibit PBP2a. From the docking results, variation is observed in the binding energy of different ligands with PBP2a. As per the reports of Syahputra G, 2014, the lower binding energy leads to more stable ligand interaction with the receptors. Based on these results, interaction of PBP2a with Rutin showed the lowest binding energy (-10.2 kcal/mol) resulting into more stable ligand-protein interaction than Ceftobiprole (-9.1 kcal/mol), a fifth generation cephalosporin commonly used as potent β-lactam antibiotic. Also, total hydrogen bond interactions within binding pocket of PBP2a for Rutin was found to be 10 while for Ceptobiprole it was found to be 8, again rendering more binding stability of Rutin with PBP2a.

Other potent ligands for PBP2a were found to be Theaflavin and Hesperidin showing binding energy of -9.9 kcal/mol and -9.7 kcal/mol more than Ceftobiprole and also interacting with serine residue of active site. However, Theaflavin and Hesperidin showed less number of Hydrogen bond interaction i.e. 5 and 7 respectively with PBP2a as compared to Ceftobiprole rendering them to be less stable. Thus, Rutin can be a promising molecule for designing and developing a natural antibiotic against MRSA. The *in silico* molecular docking analysis revealed that how various ligands interacts with PBP2a at same active site with different interactions among amino acids residues present in the binding site. Thus, exploiting use of plant derived molecules in the binding site as agonist and antagonist can play a crucial role in inhibiting the biological activity of PBP2a and opens up new avenues to design and synthesize plant derived molecule based antimicrobial agents to combat against serious infections.

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Removal of Iron from Aqueous Solution by Novel Bacteria Isolated from Marine Macro Algae

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ABSTRACT

Iron is the most abundant metal element on earth, and iron-bearing minerals are everywhere and that are reactive element of water, soils, and sediments. Iron is a beneficial when it is in permissible limit when excess amount of iron is present it becomes toxic. Current technologies for removal and recovery of toxic elements are the industrial interest and metals usually produce wastes with high concentrations. Biologically with the help of bacteria iron can be convert toxic element to less toxic element. In our study bacteria were collected from marine macroalgae and then DNA were isolated and identified as *Bacillus siamensis*, and sequence were submitted to the gene bank by Bankit online portal of NCBI. These bacteria were then use for biosorption of Iron from aqueous solution containing 200 ppm of iron using batch adsorption method and collected bacteria were then tested for growth of bacteria in the presence of 25 to 200 ppm of iron concentration and bacteria were remove 38% of iron from the aqueous solution.

KEY WORDS: BIOSORPTION, IDENTIFICATION, IRON, METAL REMOVAL, 16S RRNA.

INTRODUCTION

The creation of metals has quickly increased since the industrial uprising. The metals generally found in a complex compounds that can be toxic, carcinogenic or mutagenic even in very low concentrations. Iron is normally found in rocks and soil. Iron will leach into the water resources from rock and soil construction. The iron concentration is greater than 0.3 mg/L are the reason for the water stains that unfavourably involve in plumbing fixtures and produce a yellow to reddish surfacing in water. These levels may also report use test and smell of drinking water. Iron is the most abundant metal element on earth, and iron-bearing minerals are everywhere and that are reactive element of water, soils, and sediments. The iron cycle has a reflective effect on the geochemistry of other elements and contaminants (Acemioglu, 2004). Iron bearing minerals have large surface areas and thus can easily attach with trace metals, nutrients, and organic molecules (Chi et al. 2010, Mishra et al., 2018).

Current technologies for removal and recovery of toxic elements are the industrial interest and metals usually produce wastes with high concentrations. These wastes are the source of environmental pollution like water, air and soil. The heavy metals are one of the most significant environmental trouble, and of the most difficult to resolve (Mishra et al., 2019). Heavy metals generally form compounds that can be toxic, carcinogenic or

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mutagenic, even in very low concentrations. Toxic organic compounds can be degraded completely by CO2, water and salts by biological and chemical way. Metals are permanent and the only way of diminishing their toxicity is by changing their chemical or physical state by means of oxidation/reduction reactions, solubilization, precipitation, etc(Mishra et al., 2019).

After reduction by organisms, the biogenic Fe adsorbed to the host iron hydroxide minerals in groundwater, soils, and sediments. This absorbance of iron may reason for changing of surface structure of host minerals and lead to the formation of simple state of Fe which is the component of secondary minerals such as Fe (II), Fe (II)–Fe (III) minerals (Yang et al. 2010), cell density (Zhang et al., 2014), bicarbonate concentrations (Acemioglu, B. (2004).

MATERIALS AND METHODS

2.1 Bacterial Culture and cultivation method: The bacteria was obtained from macro algae which were collected from Veraval Sea in Arabian Sea Western Sea coast of Gujarat (20° 54' 21.1896'' N and 70° 23' 15.0180'' E.), these bacteria were first time isolated from macroalgae. These bacterial cultures were Fe resistance and all were preserved in nutrient agar at 4°C temperature. So in this study the bacterial isolates were identified and used for iron resistance and iron sorption by bacteria. Other chemicals were used in the practical were analytical grade.

2.2 Molecular Identification of isolates: Overnight incubated cultures were harvested by centrifugation for 10 minutes. 875 µl of TE buffer was added to the cell pellet and the cells are resuspended in the buffer by gentle mixing. 100 µl of 10% SDS and 5 µl of Proteinase K were added to the cells and this mixture was mixed well and incubated at 37° C for an hour in an incubator. 1 ml of phenol-chloroform mixture was added and incubated at room temperature for 5 minutes. These mixtures were centrifuged at 10,000 rpm for 10 minutes at 4° C and supernatant was collected in a fresh tube. The process was repeated once again with phenol-chloroform mixture and the supernatant was collected in a fresh tube. 100 µl of 5M sodium acetate and 2 ml of isopropanol was added and mixed gently by inversion till white strands of DNA precipitates out. These contents were centrifuged at 5,000 rpm for 10 minutes and supernatant removed. 1ml 70% ethanol was added and ¾ contents were centrifuged at 5,000 rpm for 10 minutes. After air dried for 5 minutes 200 µl of TE buffer or distilled water was added and 10 µl of DNA sample was taken and diluted to 1 or 2 ml with distilled water.

The concentration of DNA was resolute by a spectrophotometer at 260/280 nm and remaining samples were stored for further experiments. Then this DNA was analyzed on a 0.7% agarose gel with adding of ethidium bromide in TAE buffer (Dhiman et al., 2019). The amplified products were purified with Agencourt AMPure (Beckman Coulter), The products were then labelled with the Big Dye Terminator v3.1 Cycle Sequencing Kit and were analyzed on the ABI 3500 Genetic Analyzer with using Analysis protocol of BDTv3-KB-Denovo_v 5.2. The Data was obtained by Seq Scape_ v 5.2.

2.3 Determination of bacterial growth under metal stress condition: Three bacterial isolates were inoculated and grown in metal-supplemented nutrient agar media which contain 50 mL of nutrient broth in the 250 ml flasks with getting higher concentrations of respective metals of Fe ranging from 25 to 200 ppm and flasks were inoculated with 0.5 ml of overnight culture and agitated on a rotary shaker (150 rev/min) at 35˚C (Qi et al, 2018).The growth was noted After 48 h and analysed in spectrophotometrically by measuring the optical density measurements at 600 nm (Shimadzu UV-1800 UV/ Vis Spectrophotometer). Cell numbers of bacteria were counted as per the optical density measurements and growth rate was calculated.

Growth rate =1/G where G is the
Generation time of bacteria

2.4 Preparation of iron solution: From the Stock solutions of iron different concentrations such as 25 ppm, 50 ppm, 75 ppm, 100 ppm, 125 ppm, 150 ppm, 175 ppm, were 200 ppm were prepared. Standard 50 ml nutrient broth was prepared and autoclaved at 121°C for 15 minutes, in 250 ml Erlenmeyer flasks. Under aseptic conditions, the three bacterial cultures were inoculated individually into these flasks. The flasks were incubated at shaking incubator 140 rpm, 37°C temperature Uninoculated control flasks were also maintained in the same manner. After 96 hours, samples were taken from each flask and centrifuged at 8,000 rpm for fifteen minutes. The supernatants were analysed with ICP-MS (Inductively coupled plasma mass spectrometry) for iron concentration adopting standard methods. Percentage reduction in iron concentration was calculated for each iron concentration based on the initial and final readings (Ahirvar et al, 2015).

2.5 Batch adsorption experiments: Erlenmeyer flasks were filled with different concentration of iron solution and inoculated with activated bacterial biomass to 100 mL volume. Erlenmeyer flasks filled with iron solution and deionised water and a control flask containing only iron with no bacterial cultures were used to determine the iron. The control flasks contained similar solutions, but no bacterial cultures. The flasks were allowed to shake for 48h and the samples were withdrawn interval after 48h incubation and their iron concentration was analyzed by ICP-MS (Inductively coupled plasma mass spectrometry). The effect of different retention times with 3ml of bacterial concentration at constant pH and temperature on removal of iron by bacterial culture were considered in batch system. All experiments were carried out at 7±0.2 pH and 35±0.2°C temperature.

2.6 Analytical methods: For separation of bacterial from the solution, samples were centrifuged (Model-301 Sigma) at 1000 rpm for 15 min. The iron concentration in the supernatant was measured using a by ICP-MS

(Inductively coupled plasma mass spectrometry). The performance of DAS adsorption was evaluated in terms of its removal efficiency as RE (%), estimated by the following equation:

$$RE(\%) = \frac{C0 - Ct}{C0} \times 100$$

Where Ct is the iron concentration at time t (Xing et al., 2008).

RESULTS

3.1 Bacterial culture and cultivation method: The five bacteria were obtained from macro algae which were collected from Veraval Sea in Arabian Sea Western Sea coast of Gujarat (20° 54' 21.1896'' N and 70° 23' 15.0180'' E.). The optimum growth pH and temperature of bacteria were 7 to 8.5 and 30 to 35°C. Bacterial cultures were preserved in nutrient agar at 4°C temperature. In this study the bacterial isolates were identified and used for iron resistance and iron sorption by bacteria. Other chemicals were used in the practical were analytical grade. Primary screening of the isolated bacterial cultures were also studied and all five isolated bacteria were gram's positive and their physical characterization are shown in Table: 1. The present study was focused that isolated bacterial strains SUN3 from marine macroalgae can uptake and reduces the iron from the iron containing solution. Piccin et al (2019) noted the macroalgae was an excellent technique for remediation of industrial waste water by removing heavy metal contaminants. Different marine macroalgae have the capability to removing the heavy metal from aqueous solutions and are ecofriendly reported by Christobel and Lipton, (2015). The Marine algae are capable of removing the heavy metal reported by the Abdel- Raouf (2012) and Kumar and Gaur (2011) reported that heavy metal reduction by Seaweed was also reported by Williams & Edyvean (1997). Macroalgae are cost effective method for removal of heavy metal noted by Christobel and Lipton, (2015).

3.2 Molecular identification of isolates: Fast growing bacteria SUN 3 was harvested after 48 hr of incubation and DNA were isolated from it. These DNA were analysed in Agarose gel and amplified gene were in purified with Agencourt AMPure. The products were then labelled with the Big Dye Terminator v3.1 Cycle Sequencing Kit and were analyzed on the ABI 3500 Genetic Analyzer with BDTv3-KB-Denovo_v 5.2. The Data was obtained by Seq Scape_ v 5.2. After all the test reaction and observation SUN 3 was identified by 16s rDNA technology. These bacterial isolate was identified as *Bacillus siamensis* and these strain was also submitted in NCBI GenBank by BankIt online portal and obtained the accession number MK587710.

3.3 Determination of bacterial growth under metal stress condition: All the three isolated bacteria were screened for their tolerance to heavy metals i.e. Fe ranges 10 to 200 ppm. Simultaneously their Minimum Inhibitory Concentration (MIC) was calculated in the method. MIC of the tested metal (Fe) was determined by calculating the amount of Fe which could more decrease growth of the bacteria even after prolonged incubation. In our studies the obtained generation time of Fe was, SUN3 (114.83) at 10 ppm and (110.25) at 200 ppm. This study was conducted from 24 h incubation to 96 h incubation and growth rate were of Fe was SUN3 (0.87) at 10 ppm and (0.95) at 200 ppm. Microorganisms take part in active or passive bio accumulation of metals. Several studied has been showed that in vitro remediation of individual metals (Kumar et al, 2016). Bacterial cell wall and the metal ions is the key of bio remediation.

The functional group such as amine, carboxyl, hydroxyl, phosphate, sulphate, and amine are available on the cell wall of bacteria and metal ions bind to this functional groups reported by the Abdi et al, (2015); Vijayaraghavan and Yun, (2008) reported that bounded metals enter in the cells (Abbas et al, 2014). Gram positive bacteria accumulate metal ion in highest quantity (Verma et al(2005) because presence of glycoprotein in their cell wall and Gram negative bacteria accumulate metal in least amount because phospholipids and LPS is present in their cell wall (Gourdon et al, 1990). Nguema and Luo (2012) reported that metal biosorption increase as time is increased. Iron reductions by microorganisms are complex as because of environment condition some bacterial strains use iron as a electron for their respiratory chain system.

Table 1. Physical Characterization of bacteria

No	Culture	Size	Shape	Margin	Elevation	Surface	Consistency	Odour	Opacity	Grams reaction
1.	SUN 3	Medium	Irregular	Entire	Raised	Smooth	Moist	No	Opaque	+
2.	SUN 8	Big	Round	Entire	Raised	Smooth	Moist	No	Opaque	+
3.	SUN 12	Medium	Round	Entire	Raised	Smooth	Moist	No	Opaque	+
4.	SUK 17	Medium	Round	Entire	Raised	Smooth	Moist	No	Opaque	+
5.	SUK 18	Big	Round	Entire	Raised	Smooth	Moist	No	Opaque	+

(+ for positive; – for negative)

Metal removal was directly proportional to the time of incubation. However metal accumulation takes place in the initial stage of log phase by their own observed by Oves et al (2013). Our studies have also point out the maximum biosorption of Fe at primary stage of bacterial growth rate.

Figure 1: Growth of isolates in the presence of Fe

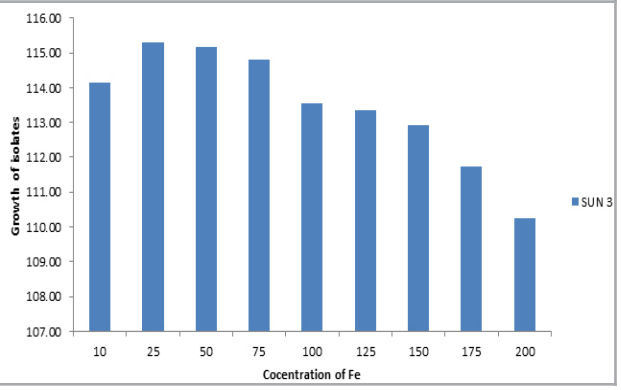
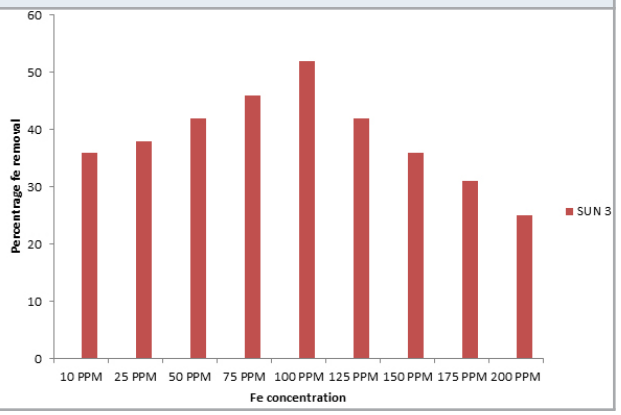


Figure 2: Percentage of Fe (ppm)



3.4 Batch adsorption experiments: From the Stock solutions of iron different concentrations ranges from 25 ppm, to200 ppm, were prepared and add in the Standard 50 ml nutrient broth. Under aseptic conditions, the three bacterial cultures were inoculated individually into these flasks. The flasks were incubated at shaking condition 140 rpm, 37°C temperatures. Uninoculated control flasks were also maintained in the same manner. After 48 hours, samples were taken from each flask and centrifuged at 8,000 rpm for fifteen minutes. The supernatants were analysed with ICP-MS (Inductively coupled plasma mass spectrometry) for chromium concentration adopting standard methods (APHA, 2012). Percentage reduction in iron concentration was calculated for each iron concentration based on the initial and final readings and SUN3 removed the 25% iron nutrient broth containing 200 ppm of iron in 48 hours. Kostka et al(2002) noted that for identification of bacteria, extracted genomic DNA was used as a template for PCR amplification of nearly the entire 16S rRNA gene (1,400 bp) with the bacterium-specific primers 8F and 1392R.16sRRNA sequencing for identification of unknown organisms was also observed by Janda and Abbott(2007).

Pichler et al, 2017 also noted the16srRNA identification method of bacteria. In our research study maximum growth was obtained at 7 pH, 350 C temperature and 2.5 % and 3% inoculum size. Similarly Pei et al (2009) reported the chromium reduction at 7.2 ph and at 37° C temperature. Wang, & Xiao (1995) reported that environmental factor effect the remediation or removal of chromium. Chuan et al (1996) reported the redox potential and pH were found to greatly affect heavy metal solubility in the soil and also reported the when pH was 6.9 to 7.0 metal solubility is maximum. Liu et al (2006) noted the chromium reduction by *Bacillus sp.* at 5 to 6 pH and 37° C temperature. Chai et al (2009) also observed the high ability for chromium reduction in liquid bacterial culture.

CONCLUSION

Isolated novel bacteria SUN 3 from marine macroalgae is efficient for removal of iron were identified as *Bacillus siamensis* by biochemical method and also confirms with 16s rRNA sequencing method. Bacterial gene sequence were deposited to the NCBI gene bank and got the accession number MK587710 for SUN 3. The study also showed that these isolated bacteria from marine macroalgae also survive up to200 ppm of iron concentration and also efficient for iron biosorption up to 38%.

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Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflict of interests.

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Screening and Characterization of PHA Production From Marine Bacteria

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ABSTRACT

Majority of the plastic products are widely used in routine life for a short period and their continuous usage leads to rapid disposal. As these products are partially degradable or non-degradable, they keep on accumulating for decades and cause various environmental hazards. Recycling and proper management of plastic waste are the few solutions which can help against plastic pollution. Another way is to use biodegradable biopolymers such as Polyhydroxyalkanoates (PHA) are seen as future alternatives of conventional plastics. The present study concentrates on the screening of marine bacteria for their ability to produce PHA and its characterization. Total 23 isolates were screened by qualitative and quantitative method for their ability to produce PHA. Among these, 16 isolates showed bluish-black coloured colony in Sudan Black B plate screening method in which 3 isolates were graded as +, 8 as ++ and 4 as +++. Out of which 13 isolates which graded as + and ++ were further screened quantitatively. From these, K3S8 was selected as potential isolate for production and recovery of PHA. Various other factors namely cell lysis method, incubation time and carbon source for maximum PHA production as well as extraction by the selected marine bacterial isolate were tested. The produced PHA was characterized by using the crotonic acid assay and FTIR spectral analysis.

KEY WORDS: BIODEGRADABLE BIOPOLYMER, MARINE BACTERIA, PLASTIC POLLUTION, POLYHYDROXYALKANOATES, SCREENING.

INTRODUCTION

Plastics are petrochemically derived synthetic polymers and their diversity has led to their use in producing a vast array of products. In today's world plastic has become an important part of our day to day life due to its desirable characteristics such as inexpensiveness, durability, corrosion resistance, high thermal and electrical insulation and moldable nature (Venkatesh and Kukreti, 2019). Today the majority of plastic products produced are for short term use, which is being rapidly disposed of in the environment. Plastic requires long periods for complete degradation as it is inert and non-biodegradable. Moreover, owing to their non-degradable nature, plastic keeps on accumulating for decades and thus cause various environmental hazards (Thompson et al., 2009). Plastic waste accumulation leads to various problems such as soil pollution, water pollution and groundwater contamination. One of the major issues caused by plastic waste is leaching of toxicants and entanglement and ingestion of plastic by wildlife. Accumulation of plastic

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waste in their natural habitat leads to ingestion of plastic waste by domestic as well as wild animals (Browne et al., 2010). Dumping of plastic waste at various places like land sites, freshwater and marine water bodies is a routine process and contaminates a wide range of habitats (Barnes et al., 2009). Apart from the above mentioned environmental issues, weathering of plastics by various environmental factors has led to the formation of micro-plastics having a diameter less than 5 mm and nano-plastic particles (Eriksen et al., 2013).

Ingestion of such micro- or nano plastics by animals have the risk of transferring toxic substances into the food chain eventually leading to biomagnification of higher trophic levels. Recycling of plastic products and plastic waste management are some of the few solutions which can help against plastic pollution. Landfilling is one of the ways used for managing the plastic waste but no availability or less availability of land resources have become a major problem. Also plastic take hundreds of years to degrade in a landfill. Thus leading to environmental pollution, limited land resources and the decline of petroleum reserves are the reasons that necessitate finding alternatives for petro-chemically derived plastics (Bomgardner et al., 2015). So, one such alternative is the use of biodegradable polymers. Polymers which get broken down into simpler compostable products by the catalytic actions of microorganisms without any external treatment are known as biodegradable polymers (Sathya et al., 2018).

The synthesis of biodegradable polymer is cost-effective so this study mentioned the ability of marine bacteria for the production of Polyhydroxyalkanoates (PHA) by fermentation process (Babu et al., 2013). As the fermentation process requires energy-consuming sterilization, high freshwater consumption and discontinuous fermentation to avoid microbial contamination. Marine bacteria can grow in high pH and high NaCl containing medium under higher temperature, allowing fermentation processes to run contamination-free under unsterile conditions (Jin yin et al., 2015). Therefore, the use of marine bacteria has the advantage of low energy and less freshwater consumption. This study aimed to screen marine bacteria to find potential PHA producer which could be further exploited for the fermentative production of PHA at a larger scale.

MATERIALS AND METHOD

Qualitative screening by Sudan Black B plate method: Total 23 marine bacterial isolates were procured from departmental preserved cultures which were revived on Zobell Marine broth/agar (HiMedia, India). All the actively grown isolates were then transferred on PHB agar medium plates (Atlas, 2010) and allowed to incubate at 30±2 °C for 7 days. After the incubation, the plates were flooded with 0.3% Sudan Black B dye solution and allow the organism to absorb it for 30 min. After that, the plates were rinsed to remove the unabsorbed dye. Then the plates were observed for the bluish-black coloured colonies and marked qualitatively based on the intensity of the dye absorbed by the isolates (Jendrossek, 2009).

Qualitative screening by Sudan Black B spectrometric analysis: The isolates showing positive results in the Sudan Black B plate screening method were allowed to grow in PHB broth. After incubation of 5-7 days, 3 mL culture was withdrawn and incubated with 1 mL Sudan Black B dye for 30 min. Then the system was centrifuged at 10,000*g* for 10 min. The obtained pellet of the bacterial biomass was separated by discarding the supernatant and the pellet was washed with distilled water for complete removal of the free dye present in the biomass followed by resuspending the resultant biomass in distilled water. The absorbance of blue colour was measured at 600 nm using a spectrophotometer (Porras et al., 2017).

Fermentative production of PHA: The most potential bacterial isolate was selected based on qualitative and quantitative screening for PHA production. If otherwise mentioned, all the experiments were carried out in triplicates. The selected marine bacterial isolate was inoculated (~ 10^8 cells/mL in each) in series of sterile 250 mL Erlenmeyer flask containing 100 mL PHA production medium and allowed to grow for 7 days at 30±2 °C in an orbital shaker at 150 rpm.

The fermentative production of PHA was optimized for incubation period and carbon source. To check the incubation time required for maximum PHA production, at different time interval the broth samples were removed and PHA extraction and further estimation was done to determine the PHA produced. To determine the most suitable carbon source by the selected isolate, 21 different carbon sources were screened. For this, the fermentation broth was prepared devoid of carbon source and various sugars were added in the form of discs containing 25 mg/disc (HiMedia, India) in the PHA fermentation broth.

PHA extraction process: As PHA accumulates as intracellular granules it requires cell lysis for extraction of PHA (Kourmentza et al., 2017). After incubation, 30 mL of culture broth was withdrawn and centrifuged at 10000*g* for 10 min. The cell pellet was collected and lysed by using 5 mL each of 1% SDS, 0.1 M NaOH and 13% NaOCl under shaking condition for 2 h. Then the systems were pelleted down and washed with a 6 mL solvent mixture containing methanol:acetone:distilled water in a ratio of 1:1:1 and resuspended in 5 mL chloroform and mixed well. The whole content was then poured in Petri plates and allowed to dry at 60 °C. The dried PHA was collected and used for further analysis.

Crotonic acid assay for quantification of PHA: The 5 mg of collected PHA powder after the extraction process was dissolved in 5 mL hot chloroform in boiling water bath for 15 min. After complete evaporation of chloroform at room temperature, 10 mL concentrated H_2SO_4 was added and kept in boiling water bath for 10 min followed by cooling at room temperature. Then the absorbance was measured at 235 nm against sulfuric acid as blank in UV –vis spectrophotometer (Law and Slepecky, 1960).

FTIR analysis: The presence of a functional group in extracted PHA was analyzed using FTIR spectroscopy in the range between 400-4000 cm^{-1}.

RESULT AND DISCUSSION

Qualitative screening of organism by Sudan Black B plate method: All 23 isolates were screened and graded as +, ++ and +++ based on blue colour intensity absorbed by the organism the isolates that unable to absorb dye blue colour were labelled as –ve. As shown in Table 1 and Figure 1, isolate 25, BD-17, K3S8, GHT-12 showed high potential for PHA production followed by isolate 1, 8, 17, BD-5, BD-11, BD-15, K3S3, NST-6. Out of 23 cultures, 8 marine bacterial isolates showed no absorbance of the dye used indicating no PHA production.

Table 1. Screening of isolates by Sudan Black B plate method

Gradation of Sudan Black B plate assay	Isolate	Total
-ve	3, 4, 11, 16, BD-6, BD-7, BD-11, BD-18,	8
+	12, 20, BD-14	3
++	1, 8, 17, BD-5, BD-11, BD-15, K3S3, NST-6	8
+++	25, BD-17, K3S8, GHT-12	4

Figure 1: Screening of isolates by Sudan Black B plate assay

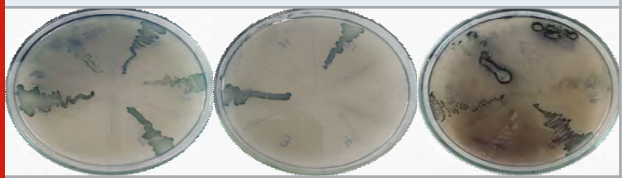
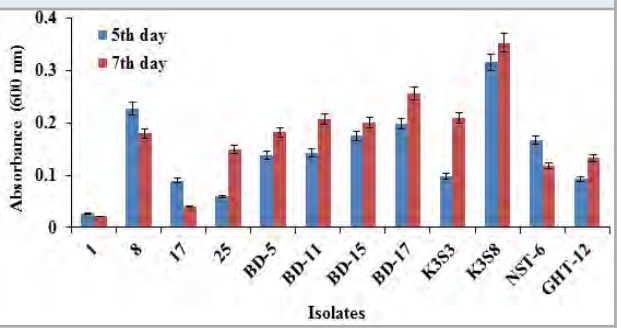


Figure 2: Quantitative analysis showing absorbance of Sudan Black B by the marine bacterial isolates



Qualitative screening by Sudan Black B spectrometric analysis: Positive isolates from the qualitative analysis showing ability to absorb blue colour that was quantified at 600 nm by neglecting growth optical density (O.D.). Figure 2 shows the quantification of 12 positive isolates from qualitative analysis. From the above analysis, isolate K3S8 was found as a highly potential bacterium among all the screened isolates and so was used for the optimization of other parameters.

PHA extraction process using K3S8 isolate: Cell lysis was done by using different chemicals and as shown in Figure 3, 13% NaOCl was found very potent under the experimental conditions as compared to other 2 chemical agents used.

Figure 3: PHA extraction by using different chemicals

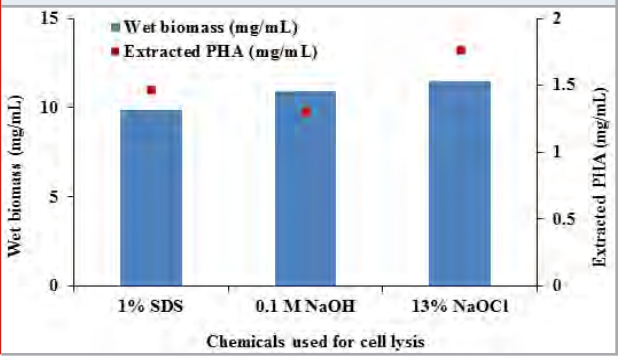


Figure 4: PHA extraction at different time intervals

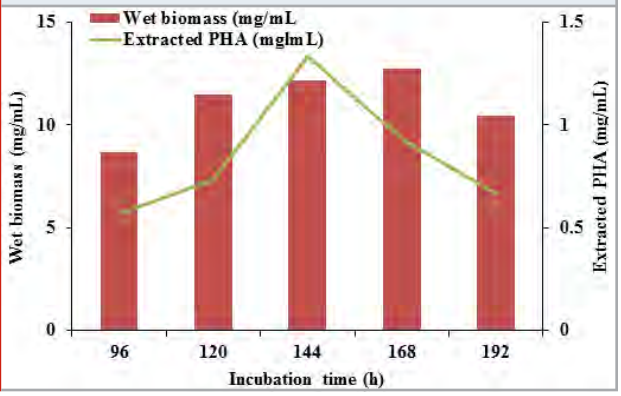


Table 2. Qualitative analysis to check the ability of the isolate to utilize sugar as a carbon source

Sugar utilization	Sugars	Total
-ve	Adonite, Dulcitol, Galactose, Insulin, Lactose, Melibiose, Raffinose, Rhamnose, Xylose	9
+	Arabinose, Cellobiose, Inositol, Maltose, Mannitol, Salicin, Sorbitol, Trehalose	8
++	Dextrose, Fructose, Mannose, Sucrose	4

Incubation time for maximum PHA production: The culture broth (30 mL) was collected at different time intervals and PHA was extracted which shows the highest product extracted at day 6 in the amount of 1.34 mg/mL. PHA production increased significantly till 6th day and

after that, it decreased. This indicated that due to lack of carbon source the organism itself started consuming PHA granules for their survival.

The carbon source for PHA production: As shown in Table 2, different sugar (21 in number) utilization tests were carried out. From the above sugar utilization test it was seen that Dextrose, Fructose, Mannose and Sucrose were the preferred carbon sources for growth and PHA production by the selected isolate and on 6th day extraction were carried out to find out potential carbon source for high PHA production (Figure 5). Fructose was found to be the carbon source of choice among the tested all the 4 sources.

Crotonic acid assay: Reaction between dried PHA and standard concentrated H_2SO_4 forms brown colour crotonic acid indicated the production of PHA. Further, it requires comparison with the standard PHA for quantification (Law and Selepecky, 1960).

Figure 5: Carbon source used for the highest PHA extraction

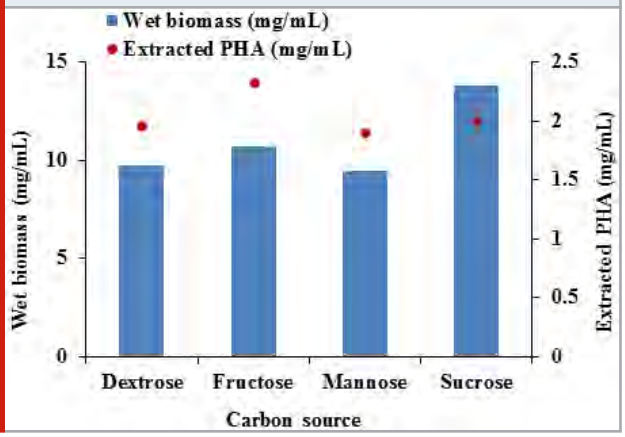
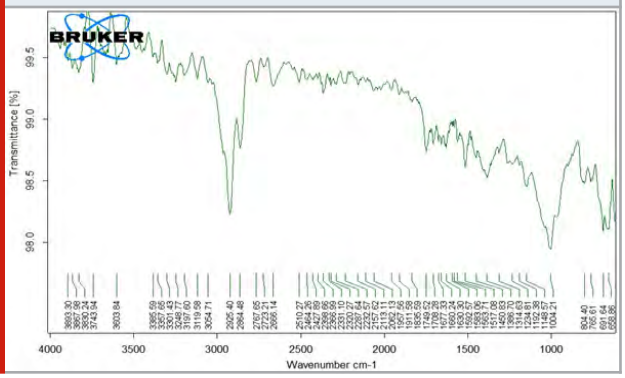


Figure 6: FTIR spectra for extracted PHA from isolate K3S8



FTIR analysis: FTIR spectra results show that absorbance peaks at 2925 cm^{-1}, 2864 cm^{-1} indicating the presence of C-H group, peaks at 1835 cm^{-1}, 1749 cm^{-1}, 1708 cm^{-1}, 1677 cm^{-1} indicates the presence of C=O group and peaks at 1004 cm^{-1} indicates the presence of C=C. When this data was compared with the reported data of other researchers it confirmed that the extracted compound is PHA (Deepa and Vidhya, 2017; Getachew and Woldesenbet, 2016; Gumel et al., 2012).

CONCLUSIONS

Total 23 marine bacterial isolates were screened for PHA production. Among them, isolate K3S8 was found the most potential PHA producer as compared to other isolates screened and for that extraction method, incubation time and carbon source analysis were carried out. Based on the crotonic acid assay and FTIR analysis, it was confirmed that extracted compound as PHA. In future studies this potential bacterial isolate can be used for PHA production using cheap carbon sources like waste material for lowering the production cost and further characterization of the PHA may lead to its application.

Conflict of interest: There is no conflict of interest.

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Study of Bacteria Isolated From Biodegradable Polythene

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ABSTRACT

Plastics are vital hydrocarbons occurring both in natural as well as in synthetic forms. The present study describes the isolation of bacteria from soil and sludge with the ability to degrade biodegradable plastics. Biodegradable plastic was buried in the glass bottle with three layers of different soil like Rhizospheric soil, Sludge and Garden soil for 4 months. The bacteria were then isolated and identified on the basis of biochemical studies as *Sphingomonas* sp. VC1, *Bacillus sp*. VC2, *Providencia sp*. VC3, *Micrococcus sp*. VC4. The identified bacteria were inoculated in modified media Bushnell's broth having biodegradable plastic for the further study of environment-friendly plastic degradation. Fourier Transform Infrared Spectroscopy (FTIR) showed certain changes on the surface of biodegradable plastic piece and formation of some new intermediate products after polymer breakdown.

KEY WORDS: HYDROCARBONS, BIODEGRADABLE PLASTIC, FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR), BUSHNELL'S BROTH.

INTRODUCTION

Plastic is a synthetic polymer that consists of carbon, hydrogen, silicon, oxygen, chloride and nitrogen. It is derived from different sources such as oil, coal and natural gas. Plastics are used abundantly because of their stability and durability. They are different varieties of plastic such as polyethylene (PE), Poly Ethylene Terephthalate (PET), nylons, Poly-Propylene (PP), Polystyrene (PS), Polyvinyl Chloride (PVC), and Polyurethane (PUR) (Strong, 2006).

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The use of plastics has transformed our life in many ways. The usage and production of plastics is ever increasing due to their versatility. They are lightweight, strong, inexpensive, duration, corrosion resistant and have high thermal and electrical insulation properties. The production of plastic increased from 0.5 million tonnes in 1950 to over 260 million tonnes now (Thompson et al., 2005).

Biodegradable plastics are fully degraded by microorganisms, without leaving visible toxic remainders. The term "biodegradable" refers to materials that can disintegrate or break down naturally into biogases and biomass (mostly carbon dioxide and water) as a result of being exposed to a microbial environment and humidity (Jain et al., 2010). Due to the absence of effective methods for safe disposal of these synthetic polymers, they often end up accumulated in the environment, posing an ever-increasing ecological threat to flora and fauna (Bhardwaj

et al., 2012). The annual production of plastics has doubled over the past 15 years to 245 million tones. During the past three decades, plastic materials are widely used in transportation, food, clothing, shelter construction, medical and recreation industries, fishing nets, packaging, food industry and agricultural field (Vatseldutt, 2014).

Plastic can be degraded by a variety of mechanisms such as chemical, thermal, photoxidation and biodegradation, all of which take an extremely long time depending on the molecular weight of polymer, it could take up to 1000 years for some types of plastics to degrade (Pramila et al., 2011). Microorganisms can also play a vital role in this process, as over 90 genera of bacteria, fungi and actinomycetes have the ability to degrade plastic (Mahdiyah et al., 2013). Generally, the biodegradation of plastic by microorganisms is a very slow process, and some microorganisms cannot degrade certain plastics (Singh et al., 2014). Biodegradable plastics are materials designed to degrade under environmental conditions or in municipal and industrial biological waste treatment facilities, and thus open the way for new waste management strategies (Gouda et al., 2012).

Many animals are dying because of waste plastics either by being caught in the waste plastic traps or by swallowing the waste plastic debris to exert ruinous effects on the ecosystem (Usha et al., 2011). Some of the plastic products cause human health problems because they mimic human hormone. Vinyl chloride is classified by the International Agency for the Research on Cancer (IARC) as carcinogenic to humans (Rudel Ruthann et al., 2007). It has also shown to be a mammary carcinogen in animals. The present study aimed to isolate polythene degrading bacteria from the soil after soil burial of biodegradable plastic and analysis of its degradation through FTIR.

MATERIALS AND METHOD

Materials: Polyolefin's collective term for the kinds of plastics that include polyethylene, namely low-density polyethylene (LDPE), linear low-density polyethylene (LLDPE), high-density polyethylene (HDPE) and polypropylene (PP) free plastic was procured from commodity selling bags of JABONG having 0.8 g/ml density was used in the present study. Nutrient agar and Nutrient broth were also during this study. Bushnell's broth (g/l: $MgSO_2.7H_2O$ 0.2, $CaCl_2$ 0.02, KH_2PO_4 1, K_2HPO_2 1, NH_4NO_3 1, $FeCl_3$ 0.05 pH adjusted to 7.0) devoid of any carbon sources, was used for the degradation experiments.

Isolation of biodegradable polythene degrading bacteria: Biodegradable plastic was buried in the glass bottle with three layers of different soil like Rhizospheric soil, Sludge and Garden soil for 4 months at room temperature amended with Bushnell's broth (g/l: $MgSO_4.7H_2O$ 0.2, $CaCl_2$ 0.02, KH_2PO_4 1, K_2HPO_4 1, NH4NO3 1, $FeCl_3$ 0.05 pH adjusted to 7.0) to maintain the availability of trace elements and moisture. Soil suspension was prepared from the three layers and diluted as per the requirement. The soil suspension was spreaded on the Nutrient agar plates by spreading method. The bacteria were incubated at room temperature, the isolated bacteria were then purified and proceed further for degradation study.

Identification of selected isolates: The bacterial isolates were then identified macroscopically (colony morphology, surface pigment, shape, size, margin, surface), microscopically (Gram staining, shape, cell

Table 1. Colony and cell morphology of biodegradable polythene degrading bacterial strain

Characteristics	VC1	VC2	VC3	VC4
Shape	Round	Round	Round	Round
Size	Small	Big	Medium	Small
Colour	Cream white	Light Peach	NIL	White (gives orange pigment at low temp.)
Margin	Even	Uneven	Even	Even
Surface	Convex	Flat	Raised	Flat
Straight rod	+	+	+	-
Cocci	-	-	-	+
Gram stain	-	+	-	+
Cell arrangement	Single/Chain	Single/Chain	Chain/Cluster	Cluster
Spore	-	+	-	-

Note: -, negative; +,positive;

arrangement, granulation, presence of spore, motility) and biochemically on the basis of Bergey's Manual of Determinative Bacteriology.

Bacterial degradation of biodegradable polythene: The isolated bacteria were then purified and inoculated in Bushnell's broth (g/l: $MgSO_4.7H_2O$ 0.2, $CaCl_2$ 0.02, KH_2PO_4 1, K_2HPO_4 1, NH_4NO_3 1, $FeCl_3$ 0.05 pH adjusted to 7.0) with a piece of biodegradable plastic (1.5 cm x 0.002 cm x 5 cm). The bacteria were then allowed to grow at room temperature devoid of any carbon source for 45 days in anaerobic condition.

Fourier Transform Infrared Spectroscopy (FTIR): After incubation of biodegradable plastic piece in liquid medium (Bushnell's broth) for 45 days, Biodegradable plastic piece were analyzed by FTIR to detect the degradation on the basis of changes in the functional groups.

RESULTS AND DISCUSSIONS

The present study deals with the isolation of eco-friendly degrading bacteria from the soil and sludge, analysis of biodegradation by FTIR. In our study out of 6 isolates, 4 were obtained through enrichment technique utilizing environment-friendly plastic as sole carbon source. According to previous study *Bacillus cereus* was able to degrade polyethylene, LDPE and polythene carry bags with the efficiency of 7.2%, 17% and 12.5% respectively (Sowmya et al., 2012) Environment-friendly plastic degrading ability of 4 different enriched bacterial isolates was conducted in liquid broth devoid of any carbon source. The bacterial isolates were identified as *Sphingomonas sp. VC1, Bacillus sp. VC2, Providencia sp. VC3, Micrococcus sp.* VC4 on the basis of colony and morphological characteristics are shown in (Table 1) and there biochemical test are shown in (Table 2).

Micrococcus luteus and *Masoniella Sp.* was isolated from forest soil degrading Plastic cup with the efficiency of 38% and 27% respectively. (Sivasankari et al., 2014). PLA-degrading Actinomycete and *Amycolatopsis sp.* Strain isolated from the sample, reduced 100 mg of PLA film by ~60% after 14 days in liquid culture at 30°C (Masaki et al., 2005). FTIR spectroscopy was performed to analyze the hydrolysis of bonds and formation of new bonds as a result of biodegradation of environmental-friendly plastic. As per previous biodegradation study, Polyurethane (PU) plastic, bacteria consortia (*Bacillus sp., Pseudomonas sp., Micrococcus sp., Arthrobacter sp.* and *Corynebacterium sp.*) was used. FTIR analysis of PU films at the end of Sturm test, showed decrease in peak from wavelength 2963 cm-1 (control) to 2957 cm-1 (test).

Table 2. Biochemical test of biodegradable polythene degrading bacterial strain

Biochemical Test	VC1	VC2	VC3	VC4
Ala-Phe-Pro-ARYLAMIDASE	-	+	+	-
ADONITOL	-	-	+	-
L-Pyrrolydonyl ARYLAMIDASE	-	-	-	-
Alanine ARYLAMIDASE	-	-	-	+
Tyrosine ARYLAMIDASE	-	+	-	+
BETA-GALACTOSIDASE	+	+	-	-
GROWTH IN 6.5% NaCl	-	+	-	-
D-GLUCOSE	-	+	+	-
BETA-GLUCOSIDASE	+	+	+	-
D-MANNITOL	-	+	+	-
D-MANNOSE	-	+	+	-
BETA-XYLOSIDASE	-	-	-	-
BETA-Alanine Arylamidase pNA	-	-	-	-
L-Proline ARYLAMIDASE	-	-	-	+
Tyrosine ARYLAMIDASE	+	+	+	+
UREASE	-	-	+	-
D-SORBITOL	-	-	-	-
SUCROSE	-	-	-	-
D-TAGATOSE	-	-	-	-
D-TREHALOSE	-	+	-	-
L-LACTATE alkalinisation	-	-	-	+
Identified as	*Sphingomonas sp.*	*Bacillus sp.*	*Providencia sp.*	*Micrococcus sp.*

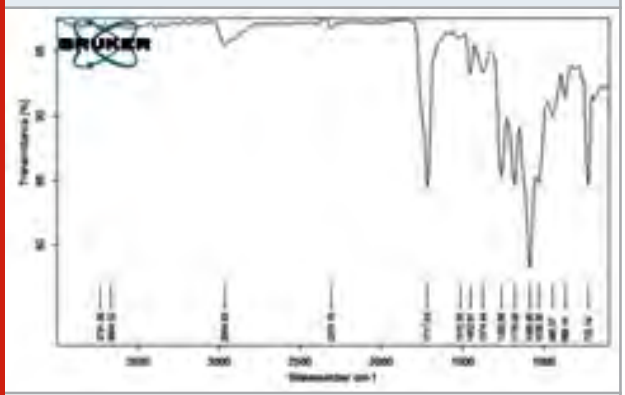
Note: -, negative; +, positive;

The appearance of some new peaks (C=C) and increase in already existing peaks, at the region of 1400-1600 cm-1, indicating the formation of new intermediate products (Aamer et al., 2008).

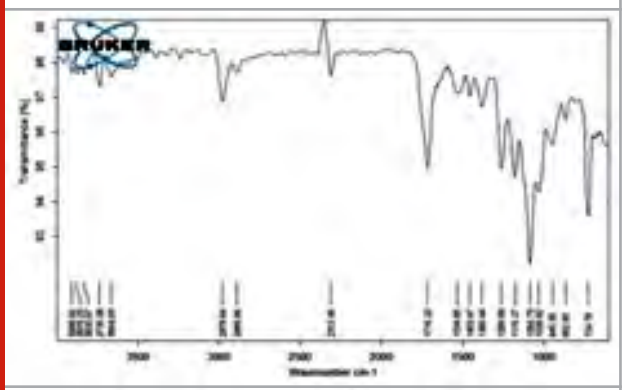
In this present study, FTIR analysis graph of control and test biodegradable plastic is mentioned in Figure 1,2,3 and 4 treated with bacteria *Sphingomonas sp. , Bacillus sp. , Providencia sp. and Micrococcus sp.*, respectively. Comparing with control biodegradable plastic there is decrease in the peak at wavenumber 1717 cm-1 of C=O of functional group carboxylic acid. Between wavenumber 3900 cm-1 to 3500 cm-1 there is formation of other functional group such as secondary amine (N-H) and alcohol (O-H). After treating with *Sphingomonas sp.* there was decrease in the intensity of IR absorption band at peak wavenumber 1717 cm-1 which changed from 85% to 95% and at peak wavenumber 1085 cm-1 changed from 80% to 92% , indicating the breakdown of C-H and C=O bond at first. There is a stretch in the bond at peak wavenumber 2310 cm-1 (S-H) thiol bond giving the transmittance percentage (%) 97.5% after treating with bacteria *Sphingomonas sp.*

When treated with isolated *Bacillus sp.* there was sudden decrease in the peak at wavenumbers 1717 cm-1, 1260 cm-1 1178 cm-1 and 1085 cm-1. The decrease in the intensity of IR absorption band, the bonds first to breakdown were C-H, S=O, C-O at above mentioned peaks. Stretch was also noticed at peak wavenumbers 2964 cm^{-1} (C-H), 2310 cm^{-1} (S-H) and 1515 cm^{-1} (N-O). The intensity of IR absorption band decreased from 85% to 96% at peak wavenumber 1717 cm^{-1} and 80% to 95% at peak wavenumber 1085 cm-1. The formation of some new functional group such as amine (N-H), alkane (C-H), aldehyde (C-H), thiol (S-H) and carboxylic acid (O-H) can be seen between peaks wavenumber 3962 cm^{-1} and 2500 cm^{-1}.

Figure 1: FTIR Spectra of biodegradable plastic treated with *Sphingomonas sp.* A. Control B. Test

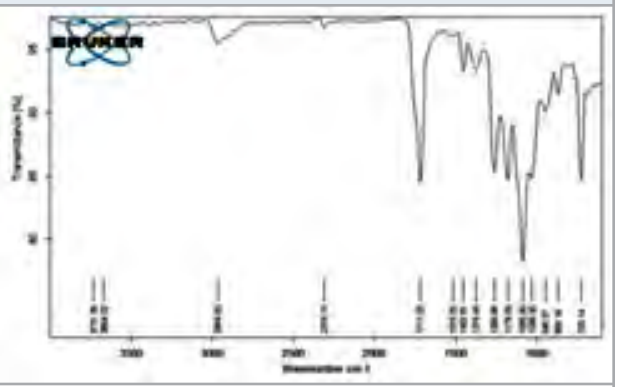


A

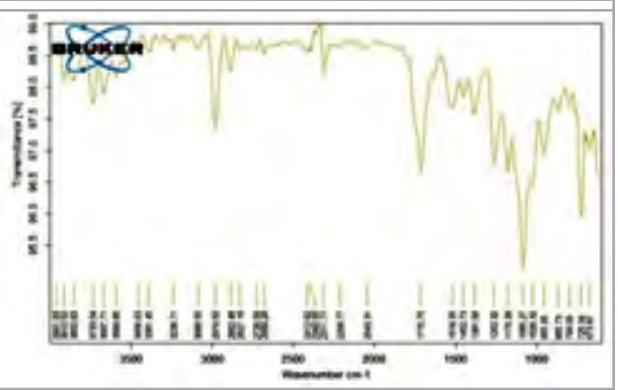


B

Figure 2: FTIR Spectra of biodegradable plastic treated with *Bacillus sp.* A. Control C. Test



A

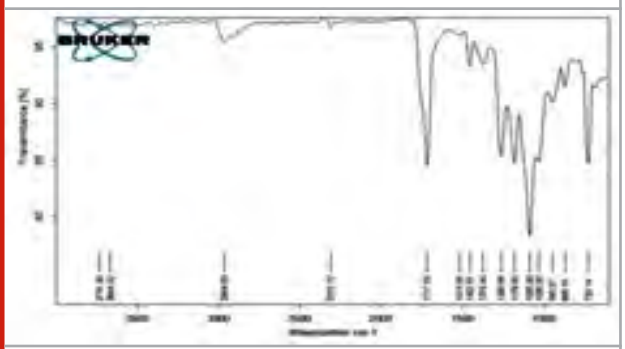


C

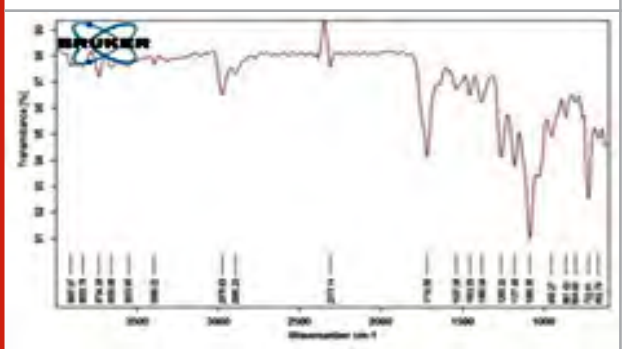
The same biodegradable plastic was treated with another bacteria *Providencia sp.* there was stretch in the functional group; alkane, alcohol, alkene and halo compound at peak wavenumbers 2964 cm^{-1}, 3731 cm-1, 804 cm^{-1} and 662 cm^{-1} respectively. The length of the peak wavenumbers 1717 cm^{-1}, 1268 cm^{-1}, 1178 cm-1, 1085 cm-1 was decreased as compared to control biodegradable plastic when analysed by FTIR. The formation of new functional group such as aliphatic primary amine (N-H) can be seen between peaks wavenumber 4000 cm-1 to 3300 cm-1.The intensity of IR absorption band decreased from 85% to 94% at peak wavenumber 1260 cm-1 and 84% to 93% at peak wavenumber 1178 cm-1.

Biodegradable plastic was treated with isolated *Micrococcus sp*. there was sudden decrease in the peak at wavenumbers 1717 cm-1, 1452 cm-1 1374 cm-1 and 1085 cm-1. The decrease in the intensity of IR absorption band, the bonds first to breakdown were C=O, C-H, S=O and C-O at above mentioned peaks. Stretch was also noticed at peak wavenumbers 2964 cm-1 (C-H), 2310 cm-1 (S-H), 785 cm-1 (C-H) and 687 cm-1 (S-Br). The intensity of IR absorption band decreased from 85% to 97% at peak wavenumber 1717 cm-1 and 80% to 95.5% at peak wavenumber 1085 cm-1. The formation of some new functional group such as amine salt (N-H), alkane (C-H), aldehyde (C-H), ketenimine (C=C=N), isothiocyanate (N=C=S) and carboxylic acid (O-H) can be seen between peaks wavenumber 3962 cm-1 and 2000 cm-1. In previous biodegradation study, PET (Poly Ethylene Terephthalate) biodegradation was stuided in marine environment, five main peaks were identified at

Figure 3: FTIR Spectra of biodegradable plastic treated with *Providenia sp*. A. Control D. Test



A



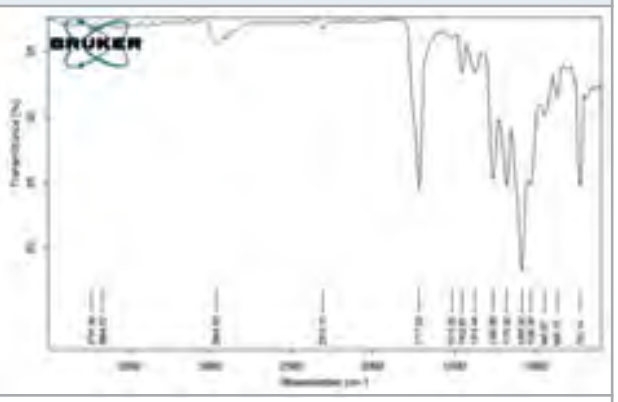
D

wavenumbers 1715 cm-1, 1245 cm-1, 1100 cm–1, 870 cm–1 and 730 cm–1, corresponding in ketones (C = O), ether aromatic (C-O), ether aliphatic (C-O), aromatic (C-H) and aromatic (C-H) bond (Ioakeimidis et al., 2016).

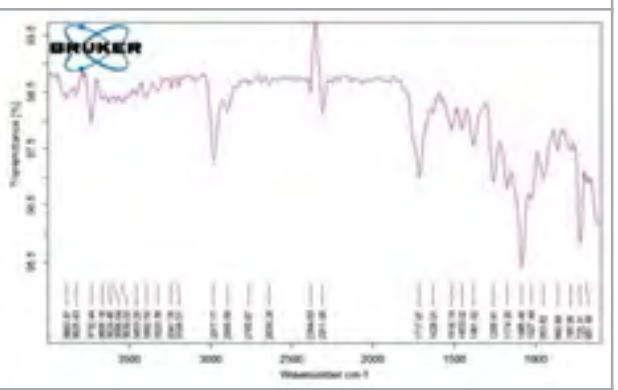
CONCLUSION

Bacteria isolated from soil and sludge have the ability to degrade plastic. The present study implicates the ability to degrade environmental-friendly plastic and

Figure 4: FTIR Spectra of biodegradable plastic treated with *Micrococcus sp*. A. Control E. Test



A



E

utilizing it as sole carbon source is different in different species. *Bacillus sp*. and *Micrococcus sp*. can degrade faster as compared to other two isolates Providencia sp and *Sphingomonas sp*.. Genome based studies can be done and strategies can be developed using bacteria to degraded plastics. It can be concluded that the soil and sludge contains potential candidates for bioremediation of plastic waste and it will reduce solid waste which causes environmental issues.

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BBRC Bioscience Biotechnology Research Communications

To Determine the Minimum Inhibitory Concentrations and Minimum Bactericidal Concentrations of the Effective Plants Extract and Endophytes of *Citrus limon*

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ABSTRACT

Endophytes are to be found inside of the plant which truly matters all around on earth. These microorganisms live in the living tissues of the host plant and do as such in an assortment of connections, going from harmonious to somewhat pathogenic. It occupies such biotopes, to be specific, higher plants, which is the reason they are presently viewed as a wellspring of novel auxiliary metabolites offering the potential for therapeutic, horticultural, as well as mechanical abuse. Planting organic product trees likewise have numerous accommodating ecological advantages, from cleaner air to decreased vitality expenses and green occupations. *Citrus* variety is the most significant natural product tree crop on the planet and lemon is the third most significant Citrus species. To decide of least inhibitory focuses (MIC) and least bactericidal fixations (MBC) of the endophytes of viable plant and detached endophytes from various pieces of Citrus limon against most defenseless bacterial strains of *E.coli, Staphylococcus aureus, Salmonella typhi, Proteus vulgaris, Pseudomonas aeruginosa, Serratia marcescens, Micrococcus luteus* and *Enterobacter aeruginosa*. This plant extract and endophytes having bioactive compounds which end up being conceivably successful can be utilized as a normal option in contrast to anti-microbials and preventives to control food contamination sicknesses and protect nourishment stuff keeping away from solid risks of synthetically antimicrobial operator applications.

KEY WORDS: *BACILLUS SPP*, CITRUS, ENDOPHYTES, MIC, MBC.

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INTRODUCTION

Plants have been utilized as a wellspring of new therapeutic mixes from the beginning of time and keep on filling in as the reason for a large number of the pharmaceuticals utilized today. (Cragg GM, Grothaus PG, et. al., 2009) Antibacterial action of Citrus remove against nourishment borne decay microbes was explored by Verma et al. (2012). Citrus Limon separates showed a strong antimicrobial activity against *Salmonella enteritidis, E. coli* and *S. aureus* yet in factor degree and with different MIC depending on the plant isolated and pathogenic living thing. (Ahmad et al., 1998; Akinyemi et al., 2006) Basic oils are important common items utilized as crude materials in numerous fields, including aromas, beautifiers, fragrance based treatment, phytotherapy, flavors and nourishment (Buchbauer, 2000).1.).

Fragrant healing is the remedial utilization of scents or if nothing else negligible volatiles to fix, moderate or forestall illnesses, diseases and indispositions by methods for inward breath (Buchbauer et al., 1993a). A few analysts detailed that ethanolic clove separate was possibly dynamic against *S aureus*, *Vibrio parahaemolyticus* and *P. aeruginosa* while it was dormant against *E. coli* and *Salmonella enteritidis* (Mahfuzul_Hoque et al., 2007). Various researchers learned development of clove oil against all attempted pathogenic microorganisms while *Vibrio cholera*, *S. typhi* and *Klebsiella pneumonia* were viewed as impenetrable to watery clove evacuate (Saeed and Tariq, 2008; Saeed et al., 2013). Basic oils are mind boggling blends involving many single mixes. Every one of these constituents adds to the valuable or unfriendly impacts of these oils.

In this way, the cozy information on basic oil structure takes into consideration a superior and uncommonly coordinated application (Buchbauer, 2000). Considering all the previously mentioned contrasts in essential oil synthesis, just a misconception on the constituents of basic oil will prompt an appropriate use in beautifying agents by perfumers and restorative scientific experts. In any case, such point by point information must be gotten by methods for deliberately performed slender GC tests (Buchbauer, 2000). The impact of plant development at the hour of oil creation and the presence of chemotypic contrasts can likewise definitely influence this structure (Lahlou and Berrada, 2003). These varieties are of unmistakable significance in the investigation of natural and pharmacological exercises of these items, as the estimation of essential oil in fragrance based treatment must be identified with its concoction synthesis (Lawrence, 2000).

Endophytes may deliver various kinds of bioactive auxiliary metabolites. The achievement of screening programs for anti-microbial generation is intensely subject to the distinguishing proof of segregates to the right taxa. (Stackebrandt E, Rainey FA, 1997; Baltz RH. 2008) A fundamental stage for the effective generation of a particular bioactive compound, data from ethnobotany/ conventional prescription has been seen valuable for a few wellbeing complexities other than irresistible sicknesses. Presently a day requirement for new, viable and moderate medications to treat microbial sicknesses in the creating scene is one of the significant issues confronting worldwide wellbeing today. (Omura, et al., 2001).

The present study done to determine the minimum inhibitory activity and minimum bactericidal activity of the effective plants extract that were treated with different bacterial culture as endophytes of *Citrus Limon* undertaken to examine the antimicrobial compound production and to purify extra cellular compound from the indigenous endophytes and to determine the bioactive compound.

MATERIALS AND METHODS

2.1. Sample: The whole *Citrus limon* (Lemon) plant was collected from the Scientific Nursery, Department of Horticulture, AAU, Anand, Gujarat, India latitude of 22.56°N and longitude of 17.95°E by a random sampling method in the sterile zip lock poly bags.

2.2. Endophytes: Endophytes MSZLd, MSZLe and MSZPse were isolated from stem, leaf and seed of *Citrus limon* plant on Nutrient agar and Tryptic soy agar medium. (Welington L. Araújo et al., 2002).

2.3. Chemicals and reagents: Sodium hypochlorite, tween 20, sodium bicarbonate, sodium nitrite, hydrogen peroxide, hydroxide, hexane, chloroform, methanol, and ethanol all chemicals used from Sigma and SRL brand.

2.4. Instruments used: Electric microscope (Lawrence and Mayo), Laminar air flow (Bright), Kjeldahl distillation unit (SENTWIN), rotary vacuum evaporator (Nutronics DTC-201), Cooling centrifuge (Remi 421LAG), Refrigerator (Kelvinator Master cool Deluxe), Deep freezer (Blue star), Orbital shaker (Ssi) and Autoclave (EQUITRON).

2.5. Pot study: Pot experiment on *Citrus limon* plant were carried out with three endophytes of total 10 different treatments in which control as untreated plant. All study performed in triplicates in which MSZLd, MSZLe, MSZPse given as in single treated, with combination of each other and with consortium of all three isolates. Pot study also treated with biofertilizer from RHBP growth plus brand and humic acid from Nutri-98% Nature Agrocare. Plant extract and essential oil collected from leaves of 120d treated *Citrus limon*.

2.6. Preparation of Plant extracts: After 120d of pot study fresh leaves were collected from bacterial endophytes treated *Citrus limon* plant. Surface sterilized (Welington L. Araújo et al., 2001) leaf powder was collected and yield percentages were calculated (Ashraf A. Mostafa et al., 2018).

2.7. Extraction of essential oil: Essential oil extracted from shadow dried leaves of *Citrus limon* by using

Kjeldahl of the steam distillation method (Mouhssen Lahlou, 2004).

2.8. Preparation of Bacterial extracts: Bacterial extraction preparation were done by a Chloroform methanol cold extraction method from fermented culture using Nutrient broth medium on orbital shaker having 250rpm at 37°C ±2 for 4d (Andre´ B. Canelas et al., 2009).

2.9. Test Bacterial strains: The following microorganisms were used during the investigation as test microorganisms. *E.coli, Staphylococcus aureus, Salmonella typhi, Proteus vulgaris, Pseudomonas aeruginosa, Serratia marcescens, Micrococcus luteus* and *Enterobacter aeruginosa*.

2.9.1. Inoculums preparation: Each bacterial strain was sub cultured overnight at 37oC ±2 in Nutrient agar slants. The bacterial growth was harvested using 5 ml of sterile saline water, its absorbance was adjusted at 580 nm and diluted to attain viable cell count of 107 CFU/ml using spectrophotometer.

2.10. Determination of MIC: The minimum inhibitory concentration (MIC) has been determined by broth dilution methods in which nutrient broth was used to it. (Wiegand I et al., 2008).

2.11. Determination of MBC: The minimum bactericidal concentration (MBC) of each formulation of each strain to a given formulation or active ingredient by suspension testing. (Laura Knapp et al., 2015)

RESULTS AND DISCUSSION

3.1. Endophytes from Citrus limon: Morphological, cultural and physiological characteristics of the strain were studied. The isolates MSZLd, MSZLe and MSZPse had been identified as *Bacillus Spp*. from 16 s rRNA bacteriological identification.

3.2. Bacterial extraction and plant extraction: Extractions of three endophytes were done by a Chloroform methanol cold extraction method (Andre´ B. Canelas et al., 2009). Ethanolic Plant extracts were done by steam distillation method (Ashraf A. Mostafa et al., 2018) mention in Figure 1.

3.3. Pot experiment and yield: The ethnobotanical data of *Citrus limon* plant listed in Table 1. The pot study results, extract percentage yield and oil extractions are summarized in Table 2. The extract of 50g of shadow dried leaf with ethanol yielded plant extract residues ranged from 3.93g to 6.73g and the height range of pot experiment from 11cm to 40cm. The highest results obtained in encoded DEF pot treated with consortium yield was 6.73g and height was 40cm followed by E pot treated with isolate MSZLe yield was 5.34g and height

Figure 1: Plant and Bacterial Extract

Figure 2: Maximum growth observed in consortia treatment

Figure 3: Yield of *Citrus limon* Plant experiment

Figure 4: Extraction of Essential oil from treated *Citrus limon* plants by Kjeldahl distillation

was 36cm while Control plant give the lowest extract yield respectively (Ashraf A. Mostafa et. al., 2018). Figure 2 described in the pot experiment maximum results obtained in plant given by consortium bacterial treatment with compare of control. Figure 3 described the yield of *Citrus limon* plant obtained from pot experiments.

Figure 5: Essential oil from treated *Citrus limon* plants

extract, bacterial extracts and essential oil. An overnight broth culture of each strain was standardized to $1x10^8$ CFU/ml, and 0.1ml volumes of this culture were added to the Nutrient broth and gradient plate too. All were incubated for 24 h at 37°C±2. Results observed under spectrophotometer shown in Table 3. The methanolic

3.4. Essential oil extraction: Using Hexane as solvent in Kjeldahl instrument with the steam distillation method oil extraction done. 1:3 proportions of leaf powder and hexane, further hexane evaporated under rotary evaporator at 68°C temperature. Average of essential oil extracts (%) mentioned in Table 2, Figure 5 and Figure 5.

3.5. Determination of MIC: The MIC was defined as the lowest concentration of a formulation or microbicide at which no bacterial growth was observed with plant

Table 1. The ethnobotanical data of *Citrus limon* plant

Plant species	*Citrus limon*
Family	Rutaceae
Local name	Lemon
Common name	Lemon
Plant part used	Leaves
Extract pH	4.8

clove extract was reported to be potentially effective against *S. aureus, P. aeruginosa* and *E. coli* with MIC ranged from 100 to 2310 µg /ml (Pandey and Singh, 2011). Antimicrobial activity of cumin seeds (*Cuminum cyminum*) extract was accounted for to be conceivably successful against a few strains of Gram positive and Gram negative microscopic organisms embroiled in food contamination with variable MIC's (Arora and Kaur, 1999; Shan et al., 2007; Chaudry and Tariq, 2008). MIC's of cumin extract effective against *E.coli, P. aeruginosa, S. aureus* and *B. pumilus* were ranged between 6250 and 25000 µg /ml. (Dua et al., 2013) *Citrus limon* was seen as incapable in controlling the other bacterial strains and these outcomes were diverged from conceivably viable with MIC ranged from 120 to 500 µg/ml.

3.6. Determination of MBC: The minimum bactericidal concentration (MBC) of each formulation or microbicide was also determined before and after the exposure of

Table 2: Results of pot experiments and extract percentage yield of *Citrus limon* plant

Sr no.	Code of pot	Bacterial treatment	Shoot length (cm)	Root length (cm)	Extract yield (%)	Extract of essential oil (%)
1	C	Control	11	5	3.93	2
2	H	Humic acid	12	6	4.94	4
3	BF	Biofertilizer	10	4	2.46	3
4	D	MSZLd	30	12	5.31	6
5	E	MSZLe	36	15	5.34	7
6	F	MSZPse	32	12	5.24	8
7	DE	MSZLd+MSZLe	34	14	5.60	8
8	EF	MSZLe+MSZPse	35	16	5.80	9
9	FD	MSZPse+MSZLd	35	15	5.75	8
10	DEF	MSZLd+MSZLe +MSZPse	40	19	6.73	12

Table : 3 MIC and MBC estimations of treated *Citrus limon* plants and streptomycin as the positive control against microorganisms

Bacteria	MIC and MBC values by Bacterial extracts and ethanol extracts of Citrus limon (µg/ml)																										Streptomycin (µg/ml)	
	B1D		B2E		B3F		P1C		P2D		P3E		P4F		P5DE		P6EF		P7FD		P8DEF		P9BF		P10H		O1C		O2DEF		SS	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E.coli	80	40	75	35	70	35	185	145	80	40	80	40	75	35	80	40	80	40	75	35	60	30	85	45	85	45	80	50	–	–	180	180
S.aureus	25	25	25	25	20	20	135	130	30	20	30	20	25	25	30	20	30	30	25	25	20	20	35	30	35	30	30	20	16	10	460	400
S.typhi	450	300	450	300	400	250	500	300	450	300	450	300	400	200	450	200	450	250	400	200	350	180	500	250	500	250	80	60	50	40	370	300
P.vulgaris	80	40	80	40	70	35	90	80	85	40	85	40	80	40	85	45	85	45	80	40	60	30	90	50	90	40	80	60	–	–	180	160
P.aeroginosa	100	80	100	80	90	40	225	180	110	80	110	80	100	80	110	80	100	80	100	80	90	40	125	50	125	50	125	40	70	30	930	400
S.marcesence	25	10	25	10	20	10	150	125	30	16	25	10	20	10	30	16	25	10	20	10	30	16	50	30	50	30	30	16	16	10	150	120
M.leutius	200	100	200	100	150	80	350	120	200	100	200	100	150	80	200	100	200	100	150	80	150	80	250	100	250	150	250	150	100	120	150	120
E.aeroginosa	50	20	55	25	40	20	170	135	50	25	50	25	50	25	50	25	50	25	50	25	60	30	70	35	70	35	–	–	–	–	70	65

Note: Values are measurements of MIC and MBC due to ethanol extracts. "–" sign denotes no activity; Streptomycin was used as dilutions from 50 µg/ml

each strain to a given formulation or active ingredient by suspension testing. 0.1ml volumes of this culture were added from MIC's tube in Nutrient agar plates and broth incubated at 37°C±2 for 24 h. The MBC was defined as the lowest concentration of the formulation or active ingredient at which no bacterial growth was observed on the agar plate results shown in Table 3. *P. granatum* extract showed potentially bactericidal activity against the tested pathogenic bacteria (*S. aureus* and *P. aeruginosa*) with MBC of 5 mg/ml while MBC of *S. aromaticum* extract reached to 10 mg/ml except *P. aeruginosa* which was less sensitive and its minimal bactericidal concentration reached to 12500 µg /ml. (Ashraf A. Mostafa et, al., 2018) These experiments results are compared with Verma et al. (2012), Qader et al. (2013) and Mahboubi et al. (2015). *Citrus limon* was found to be ineffective in controlling the other bacterial strains and these results were contrasted with potentially effective with MBC ranged from 10 to 120 µg/ml.

CONCLUSION

The present study shows that the screened *Bacillus spp*. as bacterial endophytes and plants extract from *Citrus Limon* determine MIC and MBC against most susceptible plant pathogen strains due to presence of bioactive compound. They show Biocontrol activity with different bacterial treatment. Isolates MSZLd identified as *Bacillus subtilis* MK592771, MSZLe identified as *Bacillus vallismortis* MN533948 and MSZPse identified as *Bacillus licheniformis* MN160317 shows good results with comparing of control in *Citrus limon* plant. This plant extract and endophytes having bioactive mixes which end up being conceivably compelling can be utilized as regular option in contrast to antimicrobial and preventives to control food contamination infections and safeguard nourishment stuff maintaining a strategic distance from sound perils of the synthetically antimicrobial operator applications.

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Talaromyces pinophilus Strain M13: A Depiction of New Pioneering Fungal Strain for Phytointensification

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ABSTRACT

The aim of current research was to explore different fungi as Plant Growth Promoting Fungi (PGPF). Strains of *Trichoderma* are well explored till now. But there are few other fungal strains that are better than *Trichoderma* strains. The study involves the isolation of different fungi from the rhizosphere of various agriculture farms. After isolation, 18S rRNA identification it was carried out and fungi belonging to genus *Penicilluim, Talaromyces*, *Trichoderma* and *Aspergillus*. Isolate M13 belonging to genus *Talaromyces* was screened for its PGP activity as it was a novel strain that is still to be explored. Isolate M13 is *Talaromyces* pinophilus (MG011365). Indole Acetic Acid (IAA) estimation was carried out using Salkowski reagent. Isolates were allowed to grow in cultivation media (Potato Dextrose Broth, PDB) in which one was supplemented with tryptophan (TRP) and one without TRP. Phosphate solubilization was assessed in pikovskaya's media and latter estimated using stannous chloride method, showing decent solubilization of phosphate. Siderophore production was also assessed using CAS assay that indicated good extent of siderophore production. Further for biocontrol, enzymatic assay for β-glucanase and chitinase were carried out. For cellulase enzyme production 1% carboxymethylcellulose (CMC) and for chitinase enzyme production 10% v/v colloidal chitin (as a sole carbon source) was supplemented in solid minimal-medium-9 (MM9). Antagonism effect of the test fungal isolate was carried assessed against *Aspergillus niger*. As the stain M13 showed several PGP traits, liquid bioformulation was prepared and seed germination assay in pot trials was performed followed by well as field trails on chick-pea and banana. All the experimental data of biochemical assessment with pot and field trials suggest *T. pinophilus* M13 as a novel fungus that can be used as biointensifier.

KEY WORDS: *TALAROMYCES PINOPHILUS*, PGPF, BIOCONTROL, BIOFORMULATION.

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INTRODUCTION

Today in the green revolution era, biofertilizers are preferred over chemical fertilizer and biocontrol agents are preferred over chemical pesticides. Biofertilizer consist of biological formulation that can contain bacteria, fungi, plant extracts etc. Postulates of plant growth promotion by PGPF have been put forward as hormone production (Abscisic acid, Gibberellin, Indole acetic acid), substrate degradation (mineralization), suppression of deleterious microorganisms by production of various enzymes (Celluase, Chitinase, etc.) (Bang et al., 2018: Mahmood et al., 2019). Many fungi are used as PGPF depending on the type of soil and crop. Few examples of PGPF include species of *Aspergillus, Chaetomium, Eupenicillium, Fusarium, Phoma, Penicillium, Rhizoctonia Trichoderma* etc. (Zheng et al., 2016). PGPFs activities can be determined by inspecting IAA production, phosphate solubilization along with bio-control activities attributed by production of cellulase, chitinase siderophores and phytopathogen antagonism.

Auxins constitute a group of hormones which are responsible for root formation, wound response, apical dominance etc. (Anilkumar et al 2017). IAA is the most abundantly found auxins which drives most of the effect but, there are other compounds as well such as IBA, Phenylacetic Acid and 4-Chloroindole-3-acetic acid. The focus in recent years has shifted to IAA and other auxins secreted by microorganisms in soils which can naturally provide the hormone in more concentrations thus acting as a bio-enhancer. For this reason, many species of microbes are extensively studied for their bio-fertilizing and bio-control properties which help in increasing the overall agricultural yield of crops (Singh et al 2019: Kalia and Kaur 2019). After nitrogen, phosphorous is the most limiting nutrient for plants. Despite of profound abundant reserves of phosphorous it is not available in form suitable for plant uptake (Gilliam et al 2019: Mehta et al 2019).

Plants are only able to absorb mono and dibasic phosphate which are the soluble forms of phosphate. Fungi mineralize organic phosphorous in soil by solubilizing complex structured phosphates which turns organic phosphorous to inorganic form ultimately aiding the phosphate availability to plants (Ali et al., 2019; Etesami 2018). Siderophores are another type of compounds that facilitate iron uptake in a variety of environments from terrestrial soils to the ocean surface. Siderophore and their derivative have huge application in agronomy as to the escalation of soil fertility along with the bio-control for fungal pathogen (Harish et al., 2019). Many fungi are known to be plant pathogens (Jaber and Ownley 2018). They generally exhibit this activity by excreting metabolites, colonizing in plant tissues and causing interferences in plant physiology which ultimately lead to a disease (Arya and Harel 2019; Bang et al., 2018). The most commonly known fungal pathogens belong to the genus of *Fusarium, Sclerotina, Rhizoctonia, Curvularia, Aspergillus* and others (Skiada et al., 2019: Moreno-Velandia et al., 2019).

Trichoderma strains are one of the most explored fungi till now as bio-fertilizer and as a bio-control agent (Singh et al., 2019). Strains of genus *Talaromyces* produce useful biomass-degrading enzymes and secondary metabolites (Kun et al., 2019; Fujii et al., 2018). However, these enzymes and secondary metabolites are still poorly understood and have not been explored in depth because of a lack of comprehensive studies. However, strains of Talaromyces are not even explored as PGPF (Verma et al., 2019: Mahmood et al., 2019). So, our current study is about the plant growth promoting attributes as well as biocontrol activity of *Talaromyces pinophilus* (MG011365) along with the preparation of bioformulation and assessing it in the field for the enhancement of crop productivity.

MATERIALS AND METHOD

Isolation of Fungi: Soil sample was self-assured from the rhizosphere of healthy *Musa acuminate* plant (Banana plant) from Anand District (22˚53'N, 72˚96'E). Serial dilutions were prepared from the collected soil sample and were set up to 10^{-5}. Spread plate technique was rummage-sale for isolating fungi (Teng et al., 2018). Media used for isolation was Rose Bengal Chloramphenicol Agar (RBCA). Fungal isolates that were obtained on RBCA media were sub-cultured on Potato dextrose agar (PDA) for preservation.

Strain used for current research: Fungus *Talaromyces pinophilus* (MG011365) isolate M13 was used in the current study, which was isolated from the rhizosphric soil of banana plant.

18S rRNA identification of the fungal isolates: 18S rRNA gene sequence analysis was carried out from the genomic DNA of the isolated fungus strain M13 as to recognize the organism. Partial sequencing of 18S rRNA region of fungal isolate was carried out at GSBTM. Universal primer ITS2 was used to prepare amplicon in polymeric chain reaction (PCR). ITS2 have a forward primer 5'-GGAAGTAAAAGTCGTAACAAGG-3' and reverse primer 5'-TCCTCCGCTTATTGATATGC-3' (Patel et al., 2018). Once amplicon was obtained its sequencing was carried out and obtained sequence was deposited in GenBank and accession number was obtained. Phylogenetic analysis based on 18S rRNA gene sequences available from GenBank library constructed after multiple alignments of data by ClustalW. Distances and clustering with the neighbor-joining technique were accomplished by means of MEGA 5.0 software to construct phylogenetic tree. The Bootstrap values based on 1000 replications was the paramater chosen for construction of tree (Thakor et al., 2019).

Abstraction and assessment of IAA bent by fungal isolate: Fungal isolate M13 was cultivated in the PDB. Two different compositions of PDB were prepared, one that was supplemented with tryptophan (TRP) in concentration of 1.0 mg/ml and another without TRP. After inoculation of the fungal isolate, the culture flasks were incubated at 25±2 °C in dark at static condition.

At the interval of every 48 h (up to 240 h) the culture supernatants were recovered and were acidified to pH 2.5 (using 1 N HCl). This acidified broth was then extracted with equal volume of ethyl acetate. Ethyl acetate was air dried overnight and the excerpt was re-dissolved to methanol (Patel et al., 2018). Salkowski reagent was prepared as per Patel et al. (2019) and was assorted with culture supernatants in 1:1 ratio. IAA extant in the supernatant progressively develop pink color on reacting with Salkowski reagent that was measured at 530 nm spectrophotometrically. Linear curve of standard IAA (Hi-Media) in the concentrations extending from 10–100 µg/ml was prepared against which IAA produced by this fungal isolate was computed.

Phosphate Solubilization: For qualitative analysis Pikovskaya's agar medium (containing insoluble tri-calcium phosphate) was used with supplementation of bromophenol blue as an indicator. The fungal isolate M13 was allowed to grow on it. After 72 hrs of incubation zone of phosphate solubilization was perceived, solubilization of phosphate was achieved by the release of organic acid by the fungi from sugar digestion (Adhikari and Pandey 2019). Due to this the pH of the surrounding lowers down and as a result the color of bromophenol changes from royal blue to yellowish transparent. The quantitative estimation of phosphate solubilization by this fungal isolate was assessed in the liquid Pikovskaya's medium by means of a technique defined by Pikovskayas (1948) and modified by Goswami et al. (2015). The concentration of the soluble phosphate was quantitatively estimated from the supernatant using stannous chloride method and was estimated an interval of 48 h from the culture supernatant (up to 240 hrs) after the inoculation in liquid Pikovskaya's broth.

Siderophore production: Siderophore production was measured by using chrome azurol S (CAS) shuttle assay defined by Goswami at el. (2014), Fungal isolate was cultivated in media that was iron defected, that is MM9 minimal medium (G013 Hi-Media) supplemented with 1% (w/v) PDB. For qualitative analysis CAS assay agar plate method was used and the fungal isolate M13 allowed to grow on it. After 72 hrs of incubation zone of siderophore production was observed, presence of siderophore removes the iron from the dye complex and causes reduction in the intensity of blue color to orangish color. For quantitative estimation, CAS assay solution was mixed with culture supernatant with culture grown for different time intervals (from 48 to 240 h) after the inoculation in an equal proportion and allowed to stand for 20 min (Kumar et al., 2018). Presence of siderophore is recorded at 630 nm. For the measurements, minimal medium with 1% (w/v) PDB was used as blank and % siderophore units were premeditated by succeeding formula

– [(Ar–As)/Ar] 100 = % siderophore units, where Ar = absorbance of reference (minimal media + CAS assay solution) and As = absorbance of sample.

Antagonism against pathogen: RBCA media was used for studying antagonism. On center of each half of the plate, loop-full culture of fungal strain and pathogen were inoculated. Antagonistic activity of fungi can be observed by the growth pattern and the margin of test fungal isolate and the pathogen. Antagonism bring up to the action of our test organism that quash or hinder the typical growth and activity of a plant pathogen. Enzymes for instance chitinase, glucanase released by this biocontrol agent are liable for destruction of the plant pathogens by flouting down the polysaccharides, chitin, and β-glucans that lead to the rescinding cell wall integrity (Thakor et al., 2019).

Bioformulation: Liquid bioformulation was used for assessing pot and field trail. Fungal isolate was grown on media for 10 days and then spores were used seed germination assay and field trial. For liquid bioformulation 2 % glycerol (MB060 Hi-media) and 0.5 % trition-X100 (MB031 Hi-media) was used along with 10^5 spores (Sarwar et al., 2018). Spores of fungi was enumerated with the help of Neubauer chamber (Sampaolesi et al., 2019).

Seed Germination: For seed germination, seed of chickpea (*Cicer arietinum*) was taken for study during the month of March where the normal temperature is 30 ± 2 °C and humidity is around 20%. Chickpea was chosen owing to its rapid germination and growth and on which the impact of bioformulation can be easily gauged. The seeds were surface sterilized by washing them with 70% alcohol for 2 minute and the washed with sterilized distilled water thrice. Two batches of seeds were kept for study, one treated with fungal isolate M13 and another were kept as control. The batch was treated fungal liquid bioformulation that contain fungal isolate M13's spores and the other was kept in distilled water for four hours (Yobo et al., 2019). After incubation the seeds were transferred to petri-plate containing cotton. Sterilized distilled water was used to sprinkle on them at regular intervals to maintain optimum moisture.

RESULTS

Isolation of fungi and molecular identification using 18s rRNA gene sequencing: Around sixteen fungi were isolated from the soil and the isolates belong to *Penicilluim spp., Talaromyces spp., Trichoderma spp. and Aspergillus spp*. All the isolates that were obtained were identified using 18S rRNA gene sequence analogy. Identification of the fungal isolates Isolate fungal strain M13 was used for current research out of isolates accomplished. On partial sequencing of fungal isolate M13, it was identified as *Talaromyces pinophilus* (MG011365) as it showed maximum similarity with the strains of Talaromyces (99.99% using blast). As displayed in the Figure 1. The reason behind electing M13 was that it belongs to *Talaromyces spp*. a novel strain which is still not explored and among primary screening it was observed that isolate M13 showed more amount of IAA production and good amount of Phosphate solubilization. While the strains of Trichoderma are well exposed and

we need to find an alternative hence, *Talaromyces* strain was selected for the current study.

Extraction and Quantification of IAA produced by M13: Spectrophotometric analysis depicted that isolate M13 under scrutiny could deliver indolic auxin by TRP digestion in the cultivation medium that is supplemented with TRP and even without TRP. Amount of IAA produced by isolate M13 after each 48 hrs is summarized in Figure 2. As there are two different pathways of IAA production, one TRP dependent and second is TRP independent and based on the observation it was found that there was no signification difference observed in the yield of IAA. As the incubation time increased the production of IAA also increased with the passage of time and it was detected that isolate M13 was able to yield maximum of 73.64 ± 1.38 µg/ml of IAA ($P= 2.72 \times 10^{-6}$, ANOVA) and 64.04 ± 1.56 µg/ml of IAA ($P= 2.28 \times 10^{-6}$, ANOVA) in media that was supplemented with and without TRP respectively after 192 hrs of incubation.

Figure 1: Display of (A) Morphological, (B) Microscopic and (C) Phylogenetic tree of *Talaromyces pinophilus* M13 (MG011365)

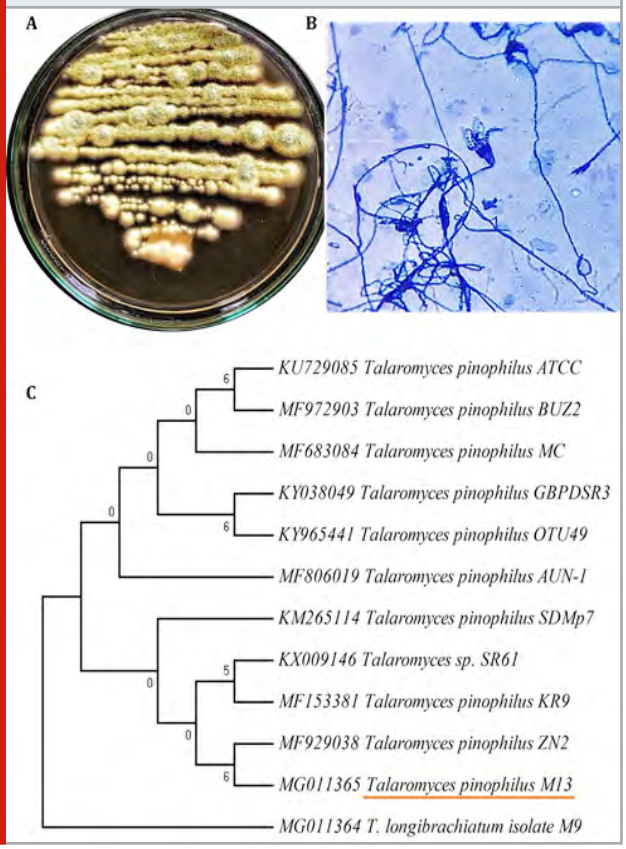


Figure 2: Display of (A) IAA production in µg/ml by *Talaromyces pinophilus* on successive days after inoculation and (B) Confirmation of IAA by TLC where track 1 is loaded with std IAA, track 2 is loaded with std IBA, track 3 is loaded with std IPyA, track 4 is loaded with Talaromyces pinophilus extract with TRP and track 5 is loaded with *Talaromyces pinophilus* extract without TRP.

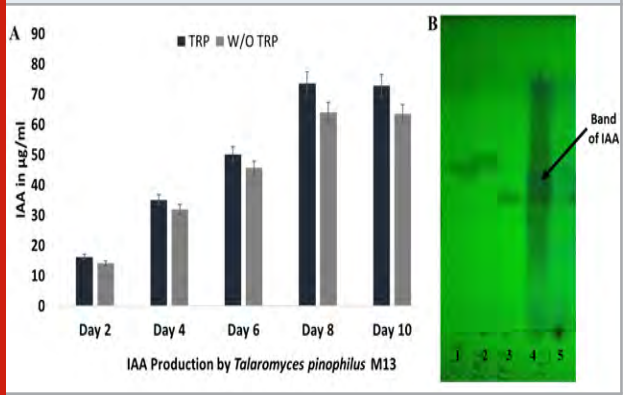


Figure 3: Display (A) *Talaromyces pinophilus* M13 grown on Pikovskaya's Media containing bromophenol blue dye and (B) Phosphate Solubilization in ppm by *Talaromyces pinophilus* M13 on successive days after inoculation.

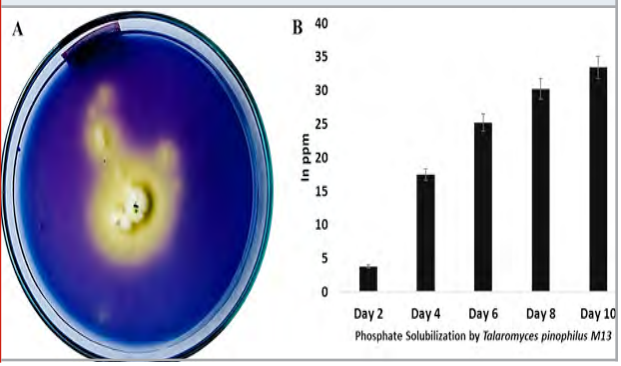
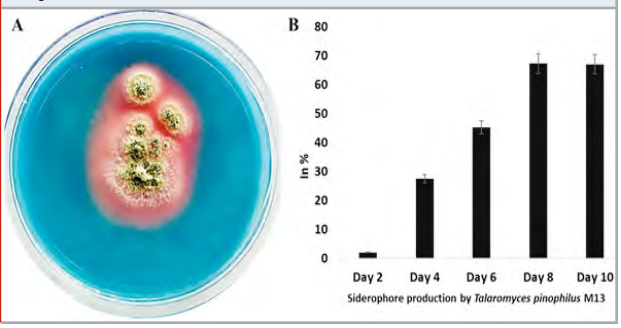


Figure 4: Display (A) *Talaromyces pinophilus* M13 grown on Minimal salt medium 9 media along with chrome azurol S (CAS) dye and (B) Siderophore production in percentage by *Talaromyces pinophilus* M13 on successive days after inoculation.



Phosphate Solubilization: On performing qualitative analysis 34 × 30 mm (10 × 6 mm of fungal growth) of zone was observed. Yellowish zone was observed around the fungal isolate showing the amount of phosphate solubilization. Quantitative estimation of phosphate solubilization was carried out after each 48 hrs and based on the observation it was found that solubilization of phosphate also increased over the time period after incubation. There was no significant solubilization of phosphate for 96 hrs and the amount of phosphate solubilization by isolate M13 after each 48 hrs is

summarized in Figure 3. Fungal isolate M13 was able to solubilize maximum of 33.42 ± 0.46 µg/ml of phosphate (P= 7.8 × 10^{-6}, ANOVA) after 196 hrs.

Siderophore production: The fungal isolate on qualitative analysis showed 44 × 58 mm (37 × 48 mm of fungal growth) zone of siderophore formation. Quantitative estimation of siderophore production was carried out after each 48 hrs and based on the observation it was found that formation of siderophore also increased over the time period after incubation as shown in Figure 4. Similarly, like phosphate solubilization the formation of siderophore occurred after 96 hrs after incubation and maximum formation of siderophore was observed after 196 hrs after the inoculation, after that the formation of siderophore was stable. Maximum siderophore formation isolate M13 was able to produce 67.23 ± 1.38 percent of siderophore (P= 4.2 × 10^{-3}, ANOVA).

Fungal Antagonism: *Talaromyces pinophilus* M13 was able to successfully inhibit the growth of *Aspergillus niger*, a test phytopathogen used under present study. The depiction of inhibition of pathogen studied using dual culture method is shown in Figure 5 where it is evident the dominance of M13 clearly visible.

Figure 5: Antagonism of *Aspergillus niger* by *Talaromyces pinophilus* M13

Seed Germination: On comparing the length of the germinated root, it was found that over a period of time there was a significant difference in the treated seeds compare to the control ones. Initially there was no major difference but after four days the percent of germination and the rate of sprouting was increased as well as the length of germinated root was also boosted as compared to non-treated control as shown in Figure 6.

DISCUSSION

Biofertilizers are live formulations of agriculturally beneficial microorganisms. There are various ways of application i.e. seed, root or soil. Biofertilization improve nutrient status of the plant by various means including associative nitrogen fixation, phosphorus solubilization, siderophores production, altering the permeability and transforming nutrients in the rhizosphere resulting in the increasing their bioavailability (Mohamed et al., 2019). Biofertilizers can mobilize the nutrients availability to improve the soil health by their biological activity. *T. pinophilus* strain M13 was isolated from agricultural rhizosphere, belonging to the Charotar region of Gujarat State, India. Charotar area is well known for its agricultural soil and its outmost yield. Most of the area is covered by farms and main occupation of the people belonging to this region is farmer. About 70% of income comes from agriculture and farmers nowadays tends to move towards organic farming instead of using artificial fertilizers. Till now *Trichoderma spp*. are well known that are used as biofertilizer or as biocontrol agent, but there are still a lot more fungi that have similar or are more effective then *Trichoderma spp*. (Moreno-Velandia et al., 2019: Jaber and Ownley 2018). In current study we tried to isolate different fungal isolate and to check their potential as PGPF.

Figure 6: Seed germination bioassay by *Talaromyces pinophilus* M13.

To portray any rhizo-fungi as PGPF it ought to be ascribed to the production of assorted metabolites including phytohormone (IAA), metal chelating compounds (siderophores) and mineralization (phosphate solubilization). IAA production is the most bulbous peculiarity detected by *T. pinophilus* strain M13. IAA is the phytohormone that impacts numerous parameters in plant, explicitly elongation in addition to cell division, differentiation of vascular tissues and apical dormancy (Kolachevskaya et al., 2019: Singh et al., 2019). Patel et al., (2019) described IAA production of IAA production from three different Aspergillus spp. which showed a good amount of IAA. But few studies showed that *Aspergillus spp*. are pathogenic to some crops so they can't be used as biofertilizer. Patel et al., (2018b) displayed IAA production by *Trichoderma longibrachiatum* 40.17 ± 0.42 µg/ml (ANOVA P = 3.6 × 10-9) that longibrachiatum which was isolated from the healthy plant's rhizosphere. Here in current study *T. pinophilus* strain M13 was able to produce almost twice then the *Trichoderma strains*. Anam et al., (2019) carried out PGP traits of *Trichoderma asperellum* strain CHF 78 and it was able to produce 8.59 mg/ml of IAA

T. pinophilus strain M13 was able to solubilize around 33.42 ppm of phosphate. Normal requirement of phosphorus is about 0.2% of a plant's dry weight and in soil more than 80% of the phosphorus becomes immobile and unavailable for plant uptake because of adsorption, precipitation, or conversion to the organic form so its' solubilization is necessity. Tandon at el. (2019) isolated 33 endophytic *Trichoderma* isolates and qualitative estimation was carried out for phosphate solubilization. Based on qualitative estimation 22 isolates were further screen for quantitative estimation. All the isolates were able to solubilize phosphate in single digit while there was one exception isolate PR-5 showed maximum solubilization around 70.8 ppm (almost eight to ten fold more). Aman et al., (2019) also carried out phosphate solubilization of same strain i.e. *Trichoderma* asperellum strain CHF 78 and a clear radiance zone of solubilization around fungal colonies was formed on Pikovskaya's medium ($Ca_3(PO_4)_2$ as phosphate source) but didn't quantify.

On qualitative analysis *T. pinophilus* strain M13 showed good amount of siderophores production on the bases of zone observed. Siderophore actually are an organic compound (low molecular masses) produced by microorganisms and plants growing under low iron containing soil (Numan et al., 2018). Siderophores function as biosensors, bio-controls, bioremediation agents and chelation agents, as well as their important role in weathering soil minerals to enhance plant growth. After the PGP activities another crucial factor in farmer's mind is protection of their crops against any diseases. *T. pinophilus* strain M13 showed promising results against phytopathogens suggesting its potent mycoparasitic activity. Bioformulation can be used either in solid or liquid state. In the current study liquid bioformulation was used for seed germination assay. On assessing the seed germination assay it was found that the treated seeds root lengths were more than the controlled one (~40 %).

CONCLUSION

The results obtained herein show that *T. pinophilus* strain M13 promote plant growth, which may be attributed to its ability to produce IAA, solubilize phosphate, formation of siderophores and along with the biocontrol activity by the means of producing enzyme of class cellulase i.e , β-glucanase and chitinase. Even positive results were achieved for seed germination assay. Therefore, *T. pinophilus* strain M13 can be served as new Phytoaugmentor (Biofertilizer and Biocontrol Agent).

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A Review on Microbial Decolorization and Degradation of Dyes

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ABSTRACT

Dye is any colored organic compound that absorbs light strongly specifically in the visible region and can firmly attach to the fibers by the virtue of physico-chemical bonding between the dye and fiber. For commercial usage a dye should be fast to light, rubbing and water. Dyes and dyestuffs are most widely used in textile manufacturing. Wastewater from the textile industry is generally full of complex mixture of various polluting substances including dyes and certain heavy metals. Thus, textile industry too gets foremost attention by environmentalists due to consumption of huge volume of water, dyes and chemicals for diverse processing of textiles and pose problems like other industrial pollutants. Textile effluents mainly comprise of carcinogenic aromatic amines, dyes, organic and inorganic materials. Elimination of dyes from textile industrial effluents by various physico-chemical as well as biological methods is presently operational. The physico- chemical methods are expensive and less effective for complete removal of colored effluent, whereas the biological decolorization of textile effluents is getting much importance due to cost effective process but on the contrary is a bit time consuming. Recent promising research on biological decolorization and degradation of textile effluent has showed that diverse group of microorganisms are capable of decolorizing and degrading broad range of dyes. The factors affecting decolorization and biodegradation of dye compounds such as pH, temperature, dye concentration, effects of carbon and nitrogen sources need optimization. Some of these parameters involved in microbial decolorization of dyes have been discussed.

KEY WORDS: DYE, DECOLORIZATION, DEGRADATION, EFFLUENT, TEXTILE MANUFACTURING.

INTRODUCTION

There are more than 100,000 commercially existing dyes with over 7107 tons of dyestuff produced per annum worldwide. These dyes are extensively used in several industries, such as food, cosmetics and paper printing, while the textile industries are the prime consumer of dyes. (Silveira, 2009). Textile processing is water-intensive, releasing an enormous amount of wastewater. Unfortunately, imperfect exhaustion of dyes onto textile fiber from an aqueous dyeing process leads to a major part of dyestuff being released with the wastewater. (Hassaan, 2017).

The textile industry accounts for the largest consumption of dyestuffs at nearly 70 percent. Reactive Dyes, Vat Dyes and Azo Dyes are mainly required for dyeing and printing of cotton fibers. Disperse dyes constitute the largest market with about 21% share followed by direct

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dyes and reactive dyes with 16% and 11% respectively. Textile dyes are generally classified either in accordance with their chemical constitution or their application to textile fibers and other coloring applications. (Walters, 2005). However, with the increasing needs and demands, industries became dependent on dyes manufactured from petrochemicals, i.e., synthetic dyes. These dyes are soluble in water, easily absorbed, and very fast in coloration as compared to the natural dyes and provide a large versatility in colors. (Jamee & Siddique, 2019).

Harmful chemicals can be acquired through the skin, bronchially or through digestion. Textile materials can cause allergic reactions and sometimes can even be carcinogenic and mutagenic. Textile products, which affect human and environmental health, should be taken into consideration. Chemical analysis of fabrics and production materials should be carried out, for production of healthy fabric, free from harmful substances. (World Health Organization, 2010). The effluent of textile is not completely free from metal contents. There are mainly two sources of metals. Firstly, the metals may come as impurity with the chemicals used during processing such as caustic soda, sodium carbonate and salts. For instance, caustic soda may contain mercury if it is produced using mercury-based catalyst. Secondly, the source of metal could be dye stuffs like metalized mordent dyes. The metal complex dyes are mostly based on chromium. Even a small concentration of chromium in the water is not acceptable. (Nergis, 2009)

Physical and chemical purification methods, including the advanced oxidation processes (i.e. application of ozone, hydrogen peroxide, and ultraviolet light), are not always applicable. They always involve high costs and therefore their use is restricted in scale of operation and pollution profile of the effluent. (Kurade, 2012). Biological methods are currently viewed as specific, less energy intensive, effective and environmentally harmless since they outcome in partial or complete bioconversion of organic pollutants to steady and nontoxic end yield. Many bacterial, fungal and algal species have the capability to degrade dyes. Bacterial decolorization of dyes is either aerobic or anaerobic. (Pandey et al., 2007). Several studies on the biological decolorization and degradation of dyes are focused on the isolation of efficient dye decolorizing and degrading microorganisms from samples collected at the waste disposal sites which representing the natural adaptation of these microorganisms to stay alive in the presence of such toxic chemicals. (Moosvi et al., 2007).

2. Dyes and their classifications: Dyes are compounds that absorb light with wavelengths in the visible range i.e. 400 to 700 nm. It's make up an abundant class of organic compounds characterized by the presence of unsaturated groups (chromophores) such as -C=C-, -N=N- and –C≡N-, a delocalized electron system with conjugated double bonds which are responsible for the dye colors, and of functional groups responsible for their fixation to fibers as for example, -NH2, -OH, -COOH and -SO3H. Dyes are the major class of synthetic dyes. (Molinari et al., 2004) About 70% of all the dyes used in industry are dyes. They are generally used in textile, cosmetic, leather, pharmaceutical, paper, paint and food industries. (Lucas, 2007)

There are two ways to categorize a dye:
1. According to its chemical structure: - Manufacturers classify dyes according to their chemical structure.

2. According to how it is applied to materials: - Dyers classify dyes according to the way the dyes are applied

2.2.1 Azo dyes: Azo dyes are the major group of synthetic aromatic dye used in the textile industry for dyeing intention and are extremely water soluble in nature. (Sudha, 2014). Commonly, azo dyes contain one, two, three, or more azo linkages; linking phenyl, naphthyl rings regularly substituted with some functional groups like triazine amine, chloro, hydroxyl, methyl, nitro, and sulfonate. (Bell, 2000) Monoazo dyes contain one nitrogen=nitrogen bond (N=N); likewise, diazo dyes contain two N=N bonds, triazo dyes contain three N=N bonds, and polyazo dyes contain more than three N=N bonds. According to hydrophobicity, azo dyes are mainly of two types: (i) hydrophobic azo dyes that are taken up by bacterial cell and reduced inside the cell and (ii) hydrophilic ones which are reduced outside the bacterial cell. (Barragan et al., 2007).

2.2.2 Reactive dyes: The reactive dyes are the only textile colorants designed to outline covalent bond with the substrate during the application process, reactive dyes furnish a wide shades of good light fastness and admirable wash on cotton. Such properties place this class of dyes at the quality end of the market. The reactive dyes used for cellulose and a rising amount is used on wool and nylon. (Farouk & Gaffer, 2013).

2.2.3 Vat dyes: The vat dyes are deliberate for application to cellulosic fibers, notably cotton, on which they typically display beyond compare levels of fastness to a range of agencies (e.g. washing, bleach, light, etc.), this being attributable, in the most part, to their water insolubility. Vat dyes have the benefit of little, if any, usage on other types of fiber, for which alternative dye classes are as an alternative preferred; indeed, in the context of synthetic fibers, the typically low substantively displayed by vat dyes attached with the generally pale depths of shades that arise from their inadequate diffusional behavior within the fibers, results in them being very little used on such substrates. (Burkinshaw, 2013).

2.2.4 Sulphur dyes: Sulfur dyes represented 9.1% of total US dye production and 15.8% of the dyes made for use on cellulosic fibers, and the world production was estimated at 110,000–120,000 tons per year. (Nguyen, 2013) Half of the volume of all dyes used on cellulosic fibers are sulfur dyes37, of which approximately 80% are black sulfur dyes. Sulfur dyes with a constitution number are defined in the Color Index 53185, 53228, 53810, etc. by the starting materials and the type of sulfurization. (Wu & Wang, 2001).

2.2.5 Acid dyes: These dyes can be applied to nylon, wool or silk in the pH range 3.0–7.0. The wet-fastness of these dyes varies from sensible to excellent and their light fastness is commonly in the blue-scale range 5.0–6.0. The dyes are generally applied under acidic conditions (using formic or acetic acid) with the degree of acidity depending upon individual dye properties. Acid dyes are normally bright with a variable fastness to washing. Structurally the dye molecules vary significantly and include some metal complexes. The defining feature of the group is the presence of sulphonated groups, these provide water-solubility. Bonding to wool occurs partially due to interaction between these sulphonate groups and ammonium groups on the wool fiber. Additional bonding interaction is provided by Van der Waals forces. The degree of interaction, and hence, the degree of color fastness varies directly with dye molecule size. (Marsh, 2009).

2.2.6 Disperse dyes: The disperse dyes are synthetic colorants for hydrophobic substrates and are frequently applied as commercial mixtures in textile coloration. They are repeatedly used in great quantities and due to the vast amount of water involved in the associated dyeing processes and the high quantity of the dye that remains in the water bath, huge volumes of wastewater can be generated. These dyes are frequently insoluble or sparingly soluble in water, non-ionic in character and applied to hydrophobic fibers from an aqueous dispersion. (Ibrahim, 2011).

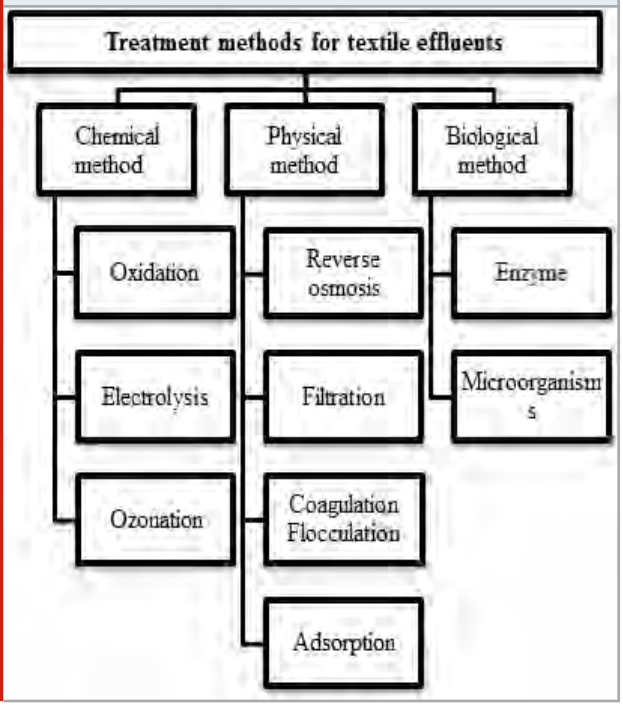
2.2.7 Direct dyes: The direct dyes are classified according to many parameters such as chromophore, fastness properties or application characteristics. The major chromophoric types are as follows: stilbene, phthalocyanine, dioxazine and other smaller chemical classes such as formazan, anthraquinone and quinolone. Although these dyes are effortless to apply and have a wide shade gamut, their wash-fastness performance is only moderate; this has led to their alternate fairly by reactive dyes which have much higher wet and washing fastness properties on cellulosic substrates. (Harfı & Harfı, 2017).

3. Decolorization and Degradation of textile Dyes: As one with industrialization, awareness towards the environmental troubles arising due to effluent discharge is of a serious consequence. A dye house effluent characteristically contains 0.6–0.8 $g/^{-1}$ dye. Pollution caused by dye effluent is mainly due to durability of the dyes in the wastewater. (Jadhav, 2007) Therefore, both color creating and the color using industries are compelled to search for novel physicochemical treatments and technologies which are intended for the most part towards the decolorization of the dyes from the effluents. There are many reports on the use of physicochemical methods for color removal from dyes containing effluents. (Singh et al., 2015) The various physical/chemical methods were used for the removal of dyes from wastewater effluent (Figure: 1).

A variety of chemical and physical processes, such as ozonation, photo oxidation, electro coagulation, adsorption, activated carbon, membrane filtration and flocculation are applied for color removal from textile. Such methods are often very costly and although the dyes are removed, accumulation of concentrated sludge creates a disposal problem. (Bumpus, 2004) Due to the financial constraints posed on the treatment of pollutants, they are discarded into the environment and it contributes to about 40% of the total industrial wastewater. There is a need to find alternative biodegradations that are effective in removing dyes from large volumes of effluents and are low in cost such as biological or combination systems. Biological methods are generally considered environment friendly as they can lead to complete mineralization of organic pollutants at low cost. Biodegradation is a promising approach for the remediation of synthetic dyes wastewater because of its cost effectiveness, efficiency, and environment friendly nature. It is now known that several microorganisms, including fungi, bacteria, yeasts, and algae, can completely decolorize many dyes. (Gupta & Tripathi, 2011).

Biological methods being cheap and simple to use are resorted to as the proposed solution. The capability of microorganisms to carry out dye decolorization has received much attention and is seen as a cost-effective method for removing these pollutants from the environment. Recently, basic work has revealed the existence of an extensive variety of microorganisms capable of decolorizing wide range of dyes. Microbial decolorization concerning suitable bacteria, algae and fungi has attracted increasing interest, these microorganisms are able to biodegrade and/or bio absorb dyes in wastewater. (Fu & Viraraghavan, 2001).

Figure 1: Treatment methods for the removal of dyes from waste water effluent.



3.1 Bacteria: Reactive orange 16 was efficiently degraded by *Enterococcus faecalis* YZ 66. (Sahasrabudhe, 2014). *Bacillus lentus, Pseudomonas luteola, Staphylococcus arlettae and Micrococcus sp*. decolorized dyes in aerobic condition to more than 90%. Two bacterial strains *Bacillus cereus* (KEB-7) and *Bacillus pumilus* (KEB-10) decolorize indigo dye. (Khelifi, 2009) Bacterial decolorization is normally more rapid and efficient. For dye degradation under anoxic conditions different studies on bacteria indicated that, bacterial strains like *P. mirabilis, P. luteola, Pseudomonas sp*. and *K. rosea* have shown extremely promising results reported. Citrobacter sp. decolorizes several recalcitrant triphenylmethane and dyes by adsorption mechanism. (Parshetti, 2006) The more details for decolorization of dyes using bacteria represented in Table 1.

Table 1. Decolorization of dyes using bacteria

Dyes used	Bacteria used	Remarks	References
Alizarin Red S	*Pseudomonas sp*	72% color removal in 48 h	Selvaraju & Bakar, 2017
Acid Orange	*Shewanella sp.*	98% color removal in 6 h	Wang, 2012
Methyl Red	*Sphingomonas paucimobilis*	98% color removal in 10 h	Ayed, 2011
Sudan III	*Staphylococcus aureus*	97% color removal in 48 h	Cho, 2011

3.2 Fungi: Fungal systems appear to be most suitable in the treatment of dyes due to the possibility of complete degradation of dyes. *Phanerochaete chrysosporium* is the most studied fungus in the field of bioremediation and the other frequently applied fungi are *Bjerkandera adusta* and *Trametes versicolor*. Although constant operation of continuous fungal bioreactors for the treatment of synthetic dye solutions had been achieved application of white-rot fungi for the removal of dyes from textile wastewater faces numerous problems. As waste product isn't the natural atmosphere of white-rot fungi, the protein production is also unpredictable and therefore the biomass growth and retention in bioreactors are a matter of concern. (Stolz, 2001) The more details for decolorization of dyes using fungi represented in Table 2.

Table 2. Decolorization of dyes using fungi

Dyes used	Fungi used	Remarks	References
Textile effluent	*Leptoshaerulina sp*	Above 90% color removal in 72 h	Placido, 2016
Amido black	*Phanerochaete chrysosporium*	98% color removal in 72 h	Senthilkumar et al.,2014
Direct brown	*Trametes versicolor*	100% Color removal in 3 h	Solis, 2012
Congo Red	*Aspergillus niger*	99% color removal in 36 h	Karthikeyan, 2010

3.3 Yeast: Yeast strains have the ability to adsorb a wide variety of dyes and heavy metals and have been used as a tool in bioremediation. They have advantages over filamentous fungi for their quicker growth and capacity to survive under adverse conditions. The mechanism of decolorization by yeast strains includes adsorption and enzymatic action or a combination of two processes. Yeast has mainly been studied with regard to biosorption. Compared to bacteria and filamentous fungi, yeast has many advantages; they not only grow rapidly like bacteria, but like filamentous fungi, they also have ability to refuse to accept unfavorable environments. More recently, some studies have shown that yeast species acted as a promising dye adsorbent capable to uptake higher dye concentration, such as *Galactomyces geotrichum, Saccharomyces cerevisiae* and *Trichosporon beigelii*, etc. (Jadhav, 2008) The more details for decolorization of dyes using yeast represented in Table 3.

Table 3. Decolorization of dyes using yeast

Dyes used	Yeast used	Remarks	References
Sudan Black	*Paraconiothyrium variabile*	84% color removal in 3 h	Aghaie-Khouzani,
Reactive Blue	*Candida rugopelliculosa*	90% color removal in 48 h	2012 Liu, 2011
Reactive Black	*Trichosporon akiyoshidainum*	100% color removal in 16 h	Pajot et al., 2011
Basic violet	*Candida tropicalis*	85.3% color removal in 48 h	Charumathi, & Das, 2010

3.4 Algae: Algae have been found to be potential biosorbents because of their accessibility in both fresh and saltwater. (Wu & Jean, 2012) The biosorption capability of algae is credited to their relatively high surface area and high binding affinity. Cell wall properties of algae play a key role in biosorption; electrostatic attraction and complexation are known to occur during algal biosorption. Functional groups such as hydroxyl, carboxylate, amino and phosphate found on the algal cell surface are considered to be responsible for sequestration of contaminants from wastewater. (Srinivasan & Viraraghavan, 2010). Color removal by algae was due to three fundamentally different mechanisms of assimilative utilization of chromophores for production of algal biomass, CO_2 and H_2O transformation of colored molecules to non-colored molecules, and adsorption of chromophores on algal biomass. (Chary & Raj, 2005) The more details for decolorization of dyes using algae represented in Table 4.

3.5 Cocktail of microbes: Three bacteria identified as *Acinetobacter sp*., *Citrobacter freundii* and *Klebsiella oxytoca* were isolated from enrichment cultures of

activated sludge in 4-nitroaniline, after which the isolates and the mixed culture was studied to find out optimal conditions for biodegradation. Dyes go through degradation under anaerobic conditions environmentally toxic aromatic amines, including nitro anilines are usually generated in the dye contaminated wastewater. In HPLC analyses it showed under aerobic conditions, these mixed culture was capable of complete removal of 100 micro mol/L of 4-nitroaniline within 72h. (Khalid et al., 2009).

Table 4. Decolorization of dye by Algae

Dye used	Algae used	Remarks	References
Blue and Red	*Spirogyra sp.* and *Oscillatoria*	color removal 70% in 14 days	Brahmbhatt & Jasrai, 2016
Acid Black	*Oscillatoria curviceps*	84% color removal in 8 days	Priya et al., 2011
Methyl Red	*Chlorella vulgaris, Lyngbya lagerlerimi* and *Volvox aureus*	Methyl Red, 82% color removal in 10 days	El- Sheekh, 2009

The main idea of development of consortia for dye decolorization hold diverse strains that can attack dyes in different positions and the metabolites formed by one strain can be used by the other strain. (Joshi, 2010) This can lead to mineralization of the dyes. Consortia can be of bacteria, fungi or a combination of two. Synergistic activity of microorganisms in consortia was reported to be accountable for improved dye degradation. Proper preservation in the quantity of microbes in consortia corresponds to effective degradation. (Jadhav, 2010) The more details for decolorization of dyes using consortia represented in Table 5.

4. Factors affecting the Dye Degradation: It is very difficult to treat textile industrial effluents by commonly used physical and chemical methods mainly because of their high biological oxygen demand, chemical oxygen demand, heat, color, pH and the presence of metal ions. (Kalyani, 2008)

4.1 pH: In dye decolorization the pH has a foremost outcome on the efficiency. The optimal pH for color removal in bacteria is often between 6.0 and 10.0. The tolerance to high pH is significant in particular usually performed under alkaline conditions for industrial processes using reactive dyes. (Zhang, 2010) The pH has a major consequence on the effectiveness of dye decolorization; the optimal pH for color removal in bacteria is often between 6.0 and 10.0. The tolerance to high pH is important in particular for industrial processes using reactive dyes, which are usually performed under alkaline conditions A fungi consortium of *Schizophyllum spp.* which decolorized 73% of solar golden yellow at a pH of 4.5 after 6 days and the efficiency decreased from 59% to 8% as pH was improved from 5 to 6 while a bacterial consortium of *Acinetobacter spp.*, *Citrobacter freundii* and *Klebsiella oxytoca* decolorized under aerobic conditions with shaking 92% of 4- nitroaniline (and structurally different dyes) at a pH of 7.2 within 42 h which decreased as pH varied below 7 or greater than 7.2. (Asgher, 2008).

4.2 Temperature: Temperature is moreover a very significant factor for the remediation of water and soil in all processes related with microbial strength, including. It was also observed that the decolorization rate of dyes increases up to the optimal temperature, and afterwards there is a marginal fall in the decolorization activity. Normally, photo catalysis is not a temperature dependent. However, a raise in temperature can affect the amount of adsorption and helped the reaction to complete more proficiently with recombination. (Daneshvar, 2004). The differences observed were irrelevant by the effect of temperature at 20°C, and at 55°C. At 20°C the percentage of color removal was 88.2±1.2 whereas for 55°C it was of 87.9±2.0. In many systems within a defined range that depends on the system the rate of color removal increases with increasing temperature. (Pearce et al., 2003).

4.3 Concentration of the dye: Earlier reports show that by dye molecules with different structures increasing the dye concentration gradually decreases the decolorization rate, probably due to the toxic effect of dyes with regard to the individual bacteria and/or inadequate biomass concentration, as well as blockage of active sites. (Lavanya, 2015).

Table 5. Decolorization of dye using microbial consortia

Name of Dye	Consortia	Remarks	References
Direct Blue	Moderately alkaliphilic bacterial consortium	97% color removal in 5 days	Lalnunhlimi & Krishnaswamy, 2016
Reactive Blue	Bacterial consortium	96% color removal in 30days	Khouni et al., 2012
Blue Orange	Mixed consortia *of* Bacteria and Fungi,	94% color removal in 1 day	Ndasi, et al., 2011
Congo Red	Sphingomonas paucimobilis and Bacillus sp.	100% color removal in 1 day	Ayed et al., 2010
Reactive Black	Thermophilic strains	More than 70% color removal in 1 day	Deive et al., 2010

4.4 Effects of carbon and sources supplements: Under anaerobic conditions, carbon sources such as glucose, yeast extract, starch, fatty acids, tapioca, propionate, acetate, and butyrate act as electron donors to allow the reduction of the bond. (Mohanty et al., 2006) A mixture of four structurally different dyes (Acid Red 88, Reactive Black 5, Direct Red 81, and Disperse Orange 3) was used as sole source of carbon and nitrogen to test the ability of select six strains of bacteria to decolorize the dyes individually or in mixtures; a *S. putrefaciens* strain was identified as the most efficient decolorizer. (Khalid et al., 2008).

Dyes are deficient in carbon and nitrogen sources, and the biodegradation of dyes lacking any supplement of these sources is very tricky. Dye decolorization by mixed as well as pure cultures usually requires complex organic sources, such as yeast extract, peptone, or a combination of complex organic sources and carbohydrates. (Khehra, 2005) Glucose is the most easily accessible and effective carbon source for microbial metabolism, and it has been frequently demonstrated that its addition improves the worth of dye degradation. (Bardi & Marzona, 2010) During decolorization of dyes via reduction of bonds, reducing equivalents from various carbon sources are transferred to the dye. Some studies performed the dye decolorization in the existence of additional carbon and nitrogen sources. Besides, carbon sources seemed to be less effective in promoting decolorization, probably due to the preference of the cell in assimilating the extra carbon source in addition to using the dye compound as the carbon source. (Saratale, 2009) In contrast, addition of the organic nitrogen sources, such as peptone, beef extract, urea, yeast extract and so on, can stimulate NADH, which acts as an electron donor for the reduction of dyes by microorganisms, and thus efficient decolorization was reported. (Chang & Kuo, 2000).

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Isolation of Heavy Metal Tolerant Rhizobacteria from Zawar Mines Area, Udaipur, Rajasthan, India

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ABSTRACT

Environmental contamination by heavy metals (HMs) is a severe problem worldwide, various anthropogenic, industries and agricultural practices contributes to pollution of the environment due to their non-biodegradable nature that considerable effect all resources became increasingly contaminated, threatening land ecosystems, surface and groundwater, as well as food safety and human health. Zawar is a small mining township near Udaipur which is contaminated with heavy metals like zinc, lead, cadmium, nickel, copper, and iron etc. which offer a threat to microbial diversity. This study represents the isolation and morphology characterization of heavy metal tolerant Rhizobacteria from Zawar Mines Area, Udaipur, Rajasthan. Soil samples were collected from five different site of rhizosphere of the plants growing near Zawar mines area, Udaipur, Rajasthan, India. The rhizosphere soil sample from a north Baroi branch of the Zawar Mines area was collected and physico-chemical parameters were analysed. Rhizosphere Soil samples were enriched separately in the nutrient broth supplemented with 100 ppm of HMs (Pb^{2+}, Cd^{2+} and Ni^{2+}). From enriched broth, serial dilution tubes were prepared and followed by the spread on N-agar plates supplemented with 100 ppm of HMs (Pb^{2+}, Cd^{2+} and Ni^{2+}). Total 51 rhizobacteria were isolated from Zawar Mines Area and the morphological and colony characteristics of isolated rhizobacteria were noted. These isolates may be useful further for potential agents for bioremediation and in combination with phytoremediation of heavy metals in contaminated soil.

KEY WORDS: BIOREMEDIATION, HEAVY METALS, PHYTOREMEDIATION, RHIZOBACTERIA, ZAWAR MINES.

INTRODUCTION

Heavy metal contamination depicts an important environmental problem due to their non-biodegradable and the toxic effects of metals, and their accumulation around the food chain stores to serious environmental and health issues. Heavy Metals impurities are commonly found in water, soils and sediments. Metal pollutants can be created through various anthropogenic, industries and agricultural activities, as well as natural disasters such as hurricanes and volcanic eruptions contribute to heavy metals pollution. Removal of heavy metals from the environment can be divided into two methods: 1) biotic methods, in which plants or microorganisms are used to remediate/accumulation of heavy metals and 2) abiotic methods, in which various physiochemical processes for example precipitation, co-precipitation, ion exchange, and adsorption by suitable adsorbent are used for removal of heavy metals (Celis et al., 2000; Vijayaraghavan et al., 2008).

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In the present study we are scrutinizing the tolerance of rhizobacteria for three heavy metals such as cadmium, nickel and lead.Cadmium has no essential biological function, but it is well identified for it extremely toxicity, bio magnification and bioaccumulation through the food chain. In chronic conditions, it also aids in the body, mainly in the kidney and the liver (Williams et al., 2008). Cadmium has a wide-ranging of bases in the environment, it may appear naturally or as an impurity for example, fertilizers, sewage sludge, mining effluents and polluted groundwater are vital bases of Cd and from several industries. Occupational introduction may happen from the production of batteries, metal coatings, alloys, pigments, plastics and from welding, and smelting of lead, zinc and copper as these occur in mixed ores with cadmium. It is also occurring in cigarette (0.007 to 0.35 µg per cigarette) and vehicular fumes. Residential sites can be polluted by municipal waste or leaks from hazardous waste sites (Alam et al., 1995).

One source is from the incorporation of growing foodstuffs, particularly grain and leafy vegetables, which voluntarily absorb cadmium from the soil. It may also pollute fish (Williams et al., 2008). Acute killing from the breath of fumes and ingestion of cadmium salts can also arise and at least one death has been described from self poisoning with cadmium chloride (Baldwin & Marshall, 1999). Cadmium (Cd) is unneeded but toxic to plants, animals, and humans (Gupta and Gupta, 1998). One of the greatest toxic pollutants of the surface soil layer is cadmium, freed into the environment by smelting activities and mining, incineration of plastics, atmospheric deposition from metallurgical industries, and batteries, land application of sewage sludge, and burning of fossil fuels (Tang et al., 2010).

Figure 1: Geographical location of Zawar mines area

Lead has not one recognized biological function, is highly lethal and gathers in humans. The foremost cause of lead in the environment is earth's crust. Lead arrives the food and water source fairly naturally and is engrossed by foodstuffs (such as green leafy vegetables) climbing on soil where lead is present. Pollution from vehicle drains or wastes or from the areas naturally high in lead are extensive sources of Pb. Previously; tetraethyl lead was an additive in petrol and lead was used in plumbing (Williams et al., 2008). Today, lead is quiet castoff in batteries, some insecticides, and occurs in cigarette smoke, where there is between 0.017 and 0.98 micrograms per cigarette (Alam et al., 1995). Lead (Pb), a major toxin that is found in soil, water and air is a hazardous waste and is highly polluted to human, animals, plants and microbes.

At least since 200 BC, people have known about the toxicity of lead, when Dioscorides wrote, "lead makes the mind give way". For hundreds of years, in both industrial and domestic application lead has been used extensively (Baldwin & Marshall, 1999). The earth's crust is a major source of lead in the environment. Lead comes in the food and water supply quite naturally and is enthralled by foodstuffs (particularly green leafy vegetables) growing on lead contaminated soil. Pollution due to vehicle exhausts or wastes or from the areas naturally high in lead is substantial sources of Pb. Previously; in the petrol tetraethyl lead was used as an additive in petrol and lead was used in plumbing (Williams et al., 2008). The use of leaded petrol is being phased out, and lead pipes in households are gradually being replaced in many countries, due to serious health concerns. In paints, lead was used and some cases of lead poisoning are due to small children eating flakes of this paint (Alam et al., 1995). Today, in the various batteries lead is still used and in some insecticides, and is found in cigarette smoke, where there is between 0.017 and 0.98 micrograms per cigarette (Alam et al., 1995). Lead (Pb), a major pollutant that is found in soil, water and air is a hazardous waste and is highly toxic to human, animals, plants and microbes.

Figure 2: A. Rhizosphere soil sample collected from Zawar mines area and B. Enriched broth

(a) (a)

In the earth's crust nickel (Ni) is the 24th richest element and has been detected in diverse media in all parts of the biosphere. Ni is considered as the bordering metal ion because it has both soft and hard metal things and can bind to sulfur, nitrogen and oxygen groups. Ni has been concerned as an embryotoxin and teratogen (Lin and Chen, 1998). A vital aspect to the remediation of metals is that metals are non-biodegradable, but can be altered through sorption, complexation, methylation, and changes in valence state. Microorganisms that disturb the reactivity and mobility of metals can be used to detoxify some metals and prevent further metal contamination. Notwithstanding the toxic stress, micro-organisms that

tolerate high metal concentrations and more rapidly decompose pollutants are more likely to survive.

Bioremediation process has offered an alternative method over the conventional methods. Microbes residing in the rhizosphere of plants can be exploited in remediation processes owing to their ability of increasing nutrient uptake and reducing metal toxicity through biosorption (Sen et al., 2017; Tank et al., 2009). Therefore, microbial–based remediation and microbial-assist-phytoremediation technique is a better alternative to reduce metal pollution from contaminated sites. Dependant root colonizing organism through metal sequestration will increase metal tolerance in plants. Microbial relationship and mutualism at the rhizosphere zone of plants show a very important role within the accumulation of metals (Aransiola et al., 2019; Tank et al., 2009). The aim of this study was isolation and morphological characterization of the rhizobacteria with high heavy metals tolerance in the Zawar mines area.

MATERIALS AND METHODS

2.1. Study area: The present study area is Zawar mines situated about 45 km away from Udaipur, Rajasthan ,India ; located at 24° 22'N and 73°43' E (Figure 1).

2.2. Sampling: Soil samples were collected from six different sites of rhizosphere of the plants growing near the Zawar mines area, Udaipur, Rajasthan, India. The samples were kept in sterile zipper plastic bags in refrigerated conditions till transferred to a laboratory for further studies (Figure 2 A) and preserved at 4°C.

2.3. Measurement of physicochemical parameters of rhizosphere soil sample: Rhizosphere soil sample which was collected near north Baroi mines of Zawar mines and subjected to analysis of physicochemical parameters like pH, conductivity, total carbon and average copper, iron, potash, zinc, manganese were also determined (Arshad et al., 2019; Yu et al., 2014).

Table 1. Physico-chemical analysis of rhizosphere soil sample of near north Baroi branch of Zawar mines area

Parameters	Results
Ph	6.7
E.C. (milimhos/cm)	3.3
Organic Carbon (In %)	1.35
Average Phosphorous (In ppm)	2
Average Potash (In ppm)	<100
Average Copper (In ppm)	11.5
Average Zinc (In ppm)	84.5
Average Manganese (In ppm)	6.62
Average Iron (In ppm)	28
Average Boron (In ppm)	2.81
Average Sulphur (In ppm)	289.8

2.4 Preparation of heavy metal stock solution: The aqueous stock solutions of all the three heavy metals Ni^{2+} (5000 ppm), Pb^{2+} (5000 ppm) and Cd^{2+} (5000 ppm) were prepared by using NiCl2.7H2O, Pb(NO3)2, $CdCl_2$ respectively and double distilled water. All the metal solutions were sterilized by autoclaving at 121 ^{0}C and 15psi for 15min.

2.5 Isolation of heavy metal tolerance rhizobacteria: Isolation of rhizobacteria from the rhizosphere soil samples was carried out using the enrichment isolation procedure. In the first step, 1 g of soil sample was incubated on Nutrient broth supplemented with 100 ppm heavy metals (Pb^{2+}, Cd^{2+} and Ni^{2+}), at the room temperature for 24h on a shaker at 150 rpm (Figure 2 B). Isolation was achieved by the serial dilution method. The enriched broth was serially diluted, in which 9ml of sterile distilled water in 5 test tubes, then 1ml of enriched broth was added to the 1st test tube to have 10-1 repeated up to 10^{-5} then 0.1ml of the highest dilution was spread on the surface of the nutrient agar plates supplemented with 100 ppm heavy metals (Pb^{2+}, Cd^{2+} and Ni^{2+}), incubated at 37°C for 24 to 48h (Arshad et al., 2019; Mustapha and Halimoon, 2015). On the basis of morphological differences (e.g. shape, size and color) repeatedly streaked on nutrient agar plates to get pure cultures and preserved at 4^{0}C.

2.6 Characterization of the isolated rhizobacteria

a. Morphological characterization: Morphological characteristic was studied using gram staining method. Cells were observed after gram staining under the microscope (oil immersion, 100 X) (Arshad et al., 2019; Mustapha and Halimoon, 2015). KOH test was performed for the confirmation of bacteria whether it is showing gram positive or gram negative reaction.

b. Cultural characterization: Isolated colonies of purified strains grown on solidified nutrient agar plates were observed and data were recorded regarding the form (round/circular, irregular and filamentous); elevation (flat, slightly raised, convex and umbonate); margin (entire, erose undulate,and filamentous) and optical feature (opaque, transparent and translucent) of the colonies (Arshad et al., 2019; Mustapha et al., 2015).

c. Preservation of isolated rhizobacteria: Isolated pure cultures were streaked on an N-agar slant and preserved at 4°C (Arshad et al., 2019; Mustapha and Halimoon, 2015).

RESULTS AND DISCUSSION

Investigations were focused on the isolation, morphological and colony characterization of the rhizobacterial strains with metal tolerance to identify potential candidates for heavy metal bioremediation in further studies. The physicochemical parameters of rhizosphere soil sample from near north Baroi branch of Zawar mines area was analysed and result of physicochemical parameters are given in the Table 1. About 84.5 ppm Zinc, 28 ppm Iron <100 ppm potash and 289.8 ppm sulphur were

founded which indicates that it is a good resources for isolation of heavy metal resistant rhizobacteria. (Yu et al., 2014) evaluated the quality of the V-Ti magnetite mine tailing soil, the physicochemical characteristics were measured such as the soil pH (5.3), organic matter (16.9864.45%), the iron, titanium manganese and vanadium concentrations were high, up to 76.15, 28.19, 1.42 and 5.58 g kg-1, respectively. The concentration of chromium, zinc, copper and nickel were 98.51, 87.72, 56.75 and 48.11 mg kg-1. In addition, lead (3.87 mg kg-1), arsenic (0.94 mg kg-1) and Cadmium (0.5260.25 mg kg-1) were detected in the mine tailing soil (Yu et al., 2014).

A total about 51 morphologically different rhizobacterial colonies were isolated from different rhizosphere soils of the Zawar mines area. Gram staining was performed with all the isolated rhizobacteria to characterize their morphological features. About 33 rhizobacteria were found gram negative, in which 10 were short rod & 23 were gram negative cocci and about 18 were found gram positive, in which 2 were rod shape & 16 were gram positive cocci. (Sen et al., 2017) recovered a total of 265 isolates from rhizospheric soil of plants growing in tailing dam of Zawar mines, Udaipur, India. Out of 256 isolates 96 were found to solubilize phosphate (Sen et al., 2017).

Table 2. Morphological and Culture Characteristic of isolated rhizobacteria

Sr. No.	Culture Code	Colony Size	Form	Elevation	Margin	Pigmentation	Surface	Texture	Opacity	Gram reaction	KOH Test
1	SZHM1	Medium	Round	Flat	Entire	Milky White	Smooth	Moist	Opaque	-Ve	Test +
2	SZHM2	Large	Irregular	Flat	Filiform	Green	Smooth	Moist	Translucent	–Ve	Test +
3	SZHM3	Medium	Irregular	Flat	Filiform	Bluish Green	Smooth	Moist	Translucent	– Ve	Test +
4	SZHM4	Large	Irregular	Flat	Filiform	Green	Smooth	Moist	Translucent	– Ve	Test +
5	SZHM5	Small	Round	Flat Slightly	Entire	Pale yellow	Smooth	Moist	Translucent	– Ve	Test +
6	SZHM6	Medium	Round	Convex	Entire	Cream colour	Smooth	Moist	Opaque	– Ve	Test +
7	SZHM7	Medium	Irregular	Flat	Entire	Pale yellow	Smooth	Moist	Translucent	– Ve	Test +
8	SZHM8	Small	Round	Flat	Entire	Orange	Smooth	Moist	Opaque	– Ve	Test +
9	SZHM9	Medium	Round	Convex	Entire	Milky White	Smooth	Moist	Opaque	– Ve	Test +
10	SZHM10	Medium	Round	Convex	Entire	Off white	Smooth	Moist	Opaque	– Ve	Test +
11	SZHM11	Medium	Round	Convex	Entire	Milky White	Smooth	Moist	Opaque	– Ve	Test +
12	SZHM12	Medium	Round	Flat	Entire	Off white	Smooth	Moist	Translucent	– Ve	Test +
13	SZHM13	Medium	Round	Convex	Entire	Milky White	Smooth	Moist	Opaque	– Ve	Test +
14	SZHM14	Small	Round	Flat	Entire	Off white	Smooth	Moist	Translucent	– Ve	Test +
15	SZHM15	Medium	Round	Flat	Entire	Off white	Smooth	Moist	Translucent	– Ve	Test +
16	SZHM16	Medium	Round	Flat Slightly	Entire	Yellow	Smooth	Moist	Translucent	+Ve	Test -
17	SZHM17	Small	Round	Convex	Entire	Pale yellow	Smooth	Moist	Translucent	+Ve	Test -
18	SZHM18	Small	Round	Flat	Lobate	Off white	Smooth	Moist	Translucent	- Ve	Test +
19	SZHM19	Small	Round	Flat	Entire	Off white	Smooth	Moist	Translucent	- Ve	Test +
20	SZHM20	Small	Round	Flat Slightly	Lobate	Off white	Smooth	Moist	Translucent	- Ve	Test +
21	SZHM21	Small	Round	Convex	Entire	White	Smooth	Moist	Opaque	– Ve	Test +
22	SZHM22	Small	Irregular	Flat	Lobate	Off white	Smooth	Moist	Translucent	- Ve	Test +
23	SZHM23	Small	Round	Flat	Entire	Off white	Smooth	Moist	Translucent	– Ve	Test +
24	SZHM24	Small	Round	Convex	Entire	White	Smooth	Moist	Translucent	- Ve	Test +
25	SZHM25	Small	Round	Flat	Entire	Off white	Smooth	Moist	Translucent	- Ve	Test +
26	SZHM26	Small	Round	Flat	Entire	Off white	Smooth	Moist	Translucent	- Ve	Test +
27	SZHM27	Small	Round	Flat	Entire	Orange	Smooth	Moist	Translucent	+Ve	Test -
28	SZHM28	Small	Round	Flat	Entire	Off white	Smooth	Moist	Translucent	+Ve	Test -
29	SZHM29	Small	Round	Flat Slightly	Entire	Off white	Smooth	Moist	Translucent	+Ve	Test -
30	SZHM30	Small	Round	Convex	Entire	Off white	Smooth	Moist	Opaque	+Ve	Test -
31	SZHM31	Small	Round	Flat Slightly	Entire	Off white	Smooth	Moist	Translucent	+Ve	Test -
32	SZHM32	Small	Round	Convex Slightly	Entire	White	Smooth	Moist	Translucent	+Ve	Test -

33	SZHM33	Small	Round	Convex	Entire	Off white	Smooth	Moist	Translucent	+Ve	Test -
34	SZHM34	Small	Round	Flat	Entire	Orange	Smooth	Moist	Opaque	+Ve	Test -
35	SZHM35	Small	Round	Flat	Entire	Yellow	Smooth	Moist	Opaque	+Ve	Test -
36	SZHM36	Small	Round	Flat	Entire	light Brownish	Smooth	Moist	Translucent	+Ve	Test -
37	SZHM37	Medium	Round	Convex	Entire	Milky White	Smooth	Mucoid	Opaque	+Ve	Test -
38	SZHM38	Small	Round	Flat	Entire	Pale yellow	Smooth	Moist	Translucent	- Ve	Test +
39	SZHM39	Medium	Round	Convex	Entire	Milky White	Smooth	Moist	Opaque	- Ve	Test +
40	SZHM40	Medium	Round	Convex	Entire	Milky White	Smooth	Moist	Opaque	+Ve	Test -
41	SZHM41	Small	Round	Raised Slightly	Entire	Cream colour	Smooth	Moist	Opaque	+Ve	Test -
42	SZHM42	Small	Round	Convex	Entire	Milky White	Smooth	Moist	Opaque	+Ve	Test -
43	SZHM43	Small	Round	Flat Slightly	Entire	Cream colour	Smooth	Moist	Translucent	+Ve	Test -
44	SZHM44	Small	Round	Convex	Entire	Milky White	Smooth	Moist	Opaque	– Ve	Test +
45	SZHM45	Small	Round	Flat	Entire	Off white	Smooth	Moist	Opaque	+Ve	Test -
46	SZHM46	Small	Round	Flat	Entire	Dark brown	Smooth	Moist	Translucent	- Ve	Test +
47	SZHM47	Small	Round	Flat	Entire	Off white	Smooth	Moist	Translucent	- Ve	Test +
48	SZHM48	Medium	Round	Convex Slightly	Entire	Milky White	Smooth	Moist	Opaque	- Ve	Test +
49	SZHM49	Small	Round	Convex	Entire	White	Smooth	Moist	Opaque	- Ve	Test +
50	SZHM50	Medium	Round	Convex	Entire	Milky White	Smooth	Moist	Opaque	- Ve	Test +
51	SZHM51	Small	Round	Flat	Entire	Dark brown	Smooth	Moist	Translucent	- Ve	Test +

(Yu et al., 2014) recovered rhizobacteria from V-Ti magnetite mine tailing soil of Panzhihua, China, about 136 isolates were systematically analysed for the heavy metal resistance and plant growth-promoting activity. Out of 136 isolates, the most abundant genus was Bacillus (79 isolates), *Rhizobium sp.* (29 isolates) and *Ochrobactrum intermedium* (13 isolates) (Yu et al., 2014). (Roman-Ponce et al., 2017) isolated the plant growth-promoting rhizobacteria (PGPR) for phytoremediation of heavy metal contamination, 60 bacterial strains were isolated from the rhizosphere soil of plants growing in a heavy metal-contaminated zone in Mexico. Results of colony characteristics are given in the table 2.

Where, -Ve means gram negative reaction, +Ve means gram positive reaction Where, KOH test positive - Result with gram negative bacteria where the solution will be viscous and form a mucoid string, KOH test negative - Result with gram positive bacteria where the solution will not be viscous.

CONCLUSION

Heavy metal (Pb^{2+}, Cd^{2+} and Ni^{2+}) tolerant rhizobacteria isolated in the present study would screen out the capacity of 51 isolated bacterial species to tolerate and grow at various concentrations (maximum inhibition concentration). The bacterial species which will give maximum MIC will select and can be efficiently used as plant growth promoting rhizobacteria for bioremediation and in combination with phytoremediation for removal of Pb2+, Cd2+ and Ni2+ from contaminated sites.

Conflict of interest: The authors have no conflict of interest in preparing for this article.

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Review on Pesticides Application in Agriculture and its Effect on Environment with Brief on their Microbial Degradation

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ABSTRACT

Pesticides utilization for preventing insect pests, weeds in crops and improving production has led to application of pesticides. The superabundant utilization of pesticides has been known to be toxic to the environment and ecosystem and its collected in soil and easily moved into the ecosystem and affect soil fertility as well may impart toxicity in living organisms. The India is an agriculture basis country where pesticides utilization from last decline to protect agriculture damage for higher production. The Gujarat patronise a importance of pesticides for controlling pests and make better production. Usually microbial degradation is the powerful and active method to degrade and detoxify pesticides pollutants. These review paper is planned on the basis of adverse effects of pesticides importance on environment and overcome the problem of pesticides degradation from contaminated soils..

KEY WORDS: AGRICULTURE, BIODEGRADATION ENVIRONMENT POLLUTION, MICROORGANISM, PESTICIDES.

INTRODUCTION

The world population is wait for develop to nearly 10 billion by 2050, food production becomes the main aim of all countries. According to the evidence, world population is rising by an approximated 97 million per year (Saravi and Shokrzadeh, 2011). The India is primarily an agriculture basis country with more than 60-70% of its population dependent on its and covers greatest part of its economy (Sachdeva, 2007). The maximum portion of agricultural field is being degraded every year by urban encroachments and industries, which creates food deficiency. The increasing food demand utilization by world population, which can only be achieved by wide utilization of pesticides, because every year approximately 45% of the world total food productivity was lost by pest infestations (Abhilash and Singh, 2009).

The Pesticides have become an integral portion of our modern life and its utilized to protect agricultural land, stored grain, flower gardens and destroy the pests transmitting infectious diseases. Pesticides are a diverse group of organic and inorganic chemicals employed in agriculture as herbicides, insecticides, nematicides, fungicides and soil fumigants (Kumar et., al 2018). The pesticides are largely utilized to manage agricultural and household pests. All pesticides are biocides, their chemical substances design to destroy the organisms. They are used for destroying, controlling, preventing, repelling and

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attracting the pests, such as insects, nematodes, rodents, and fungi and are named accordingly, i.e. insecticides, nematicides, rodenticides, and fungicides (Dileep K. Singh, 2002).

Behind the green revolution in 1960, the scenario of Indian agriculture has transmuted due to promotion of high productivity varieties of crops, which marked the green revolution, has led to large scale significance of agrochemicals as pesticides. The period of this revolution a huge of agrochemical industries were established, which not only relief to rise the food grains production, but also get ready for population job opportunities. The majority of Indian population have busy in agricultural works and therefore, exhibit to large amount of pesticides utilized in agriculture field for improving productivity by the pest management practices. The rised demand of agriculture production in changing regional climate has resulted in an increase in application and consumption of pesticides (Shetty et al., 2008).

The Pesticides usage in Indian Scenario Pesticides are the chemical substances that are apply to kill pests such as weeds, fungus, insects etc. in agricultural as well as domestic field. Abhilash and Singh (2009) reported that 45% of yearly food production is consumed by pest infestations therefore efficient pest management looks one of the main strategies to rised crop productivity for rapidly growing population. During 1940s, the production and utilization of synthetic chemical substance pesticides rapidly rised. The period of 1991, there were around 23,400 pesticide products registered with the U.S. Environmental Protection Agency (EPA). In period of 1997, 1.2 billion pounds of pesticides were utilized. Approximately 4.6 million tons of chemical pesticides are every year sprayed into the environment, in which currently about 500 pesticides with mass applications are organochlorine pesticides; some pesticides containing mercury, arsenic and lead are highly poisonous to the environment. Approximate 140 organophosphate compound pesticides being used as plant growth regulators and pesticides around the world (parte et al., 2017).

The India utilizes approximate 3% of total pesticides in the world and it is yearly increasing 2-5% rate. In India, approximately 67% pesticides consumption is utilized by agriculture and horticulture field and 75% of total account insecticides. The insecticides include organochlorines (40%), organophosphosphates (30%), carbamates (15%), synthetic pyrethroids (10%) and others (5%). The remaining 25% of total pesticides used are fungicides (10%), herbicides (7%) and others (8%). The Pesticides play main role in Indian agriculture as a plant protection for improving food production. The Indian agriculture is depending on use of pesticides and agrochemicals to increase the productivity. During the green revolution period, pesticides and chemical fertilizers were used to increase the production of food grains. (Singh, 2000).

Agriculture in gujarat: The Gujarat is quickly growing states of India. It has made fast strides in economic growth in manufacturing and service sectors. The agriculture sector role remains crucial because it is the great source of employment for majority of population. The Gujarat appoint nearly 6.2 percentage of total geographical area and 4.99 percentage of total population of India. According the Census 2011, about 3.47 crores state people live in rural areas forming about 57.4 per cent of its total population (Swain et al., 2012). The Agriculture is the main detain of the people in Gujarat, create a big part of the state economy and arrange Gujarat with the required food grains, and it also bring a major share for the adjoining areas (Swain et al., 2012). The 70.5 % of total workers are belonging in the state from rural basis and for their agriculture is the primary occupation. The cotton, groundnut, tobacco, sugar cane, cumin, rice, pulses and vegetable including green chillies are main cash crop in the state. The Gujarat is the single largest cotton producing state with 36% of the total national production. In the Gujarat state, where the commercial cropping is predominant, pesticides applications provide higher production and economic benefits to farmers.

Pesticides application: The main profits of pesticides application which directly affects the benefit expected from their consumption. For example the effect of killing caterpillars feeding on the crop brings the primary profit of better quality production of cabbage. The main three effects result in 26 main benefits ranging from protection of recreational turf to saved human lives. The secondary profits are the less clear profit that results from the main profit which may be less or longer intuitively clearness and insidious. It follows that for secondary profit therefore big matter to cause and effect, but still they can be give powerful justifications for pesticide utilization. There are identified various secondary benefits, ranging from fitter people to conserved biodiversity (Aktar et al., 2009).

Improving productivity: The wonderful advantages have been derived from the utilization of pesticides in various agriculture fields, forestry, public health and the domestic, where Indian economy is mainly dependent. Pesticides have been part of the process by reducing losses from the diseases, insect pests and weeds. Food grain production, which stood in year of 50 million tons in 1948–49, had increased almost fourfold to 198 million tons by the end of 1996–97 from an estimated 169 million hectares of continuance cropped land. This result has been obtained by the use of high production varieties of seeds, advanced irrigation technologies and agricultural chemicals (Employment Information: Indian Labour Statistics, 1994). Webster et al. (1999) reported that the considerable economic losses would be suffered without pesticide consumption.

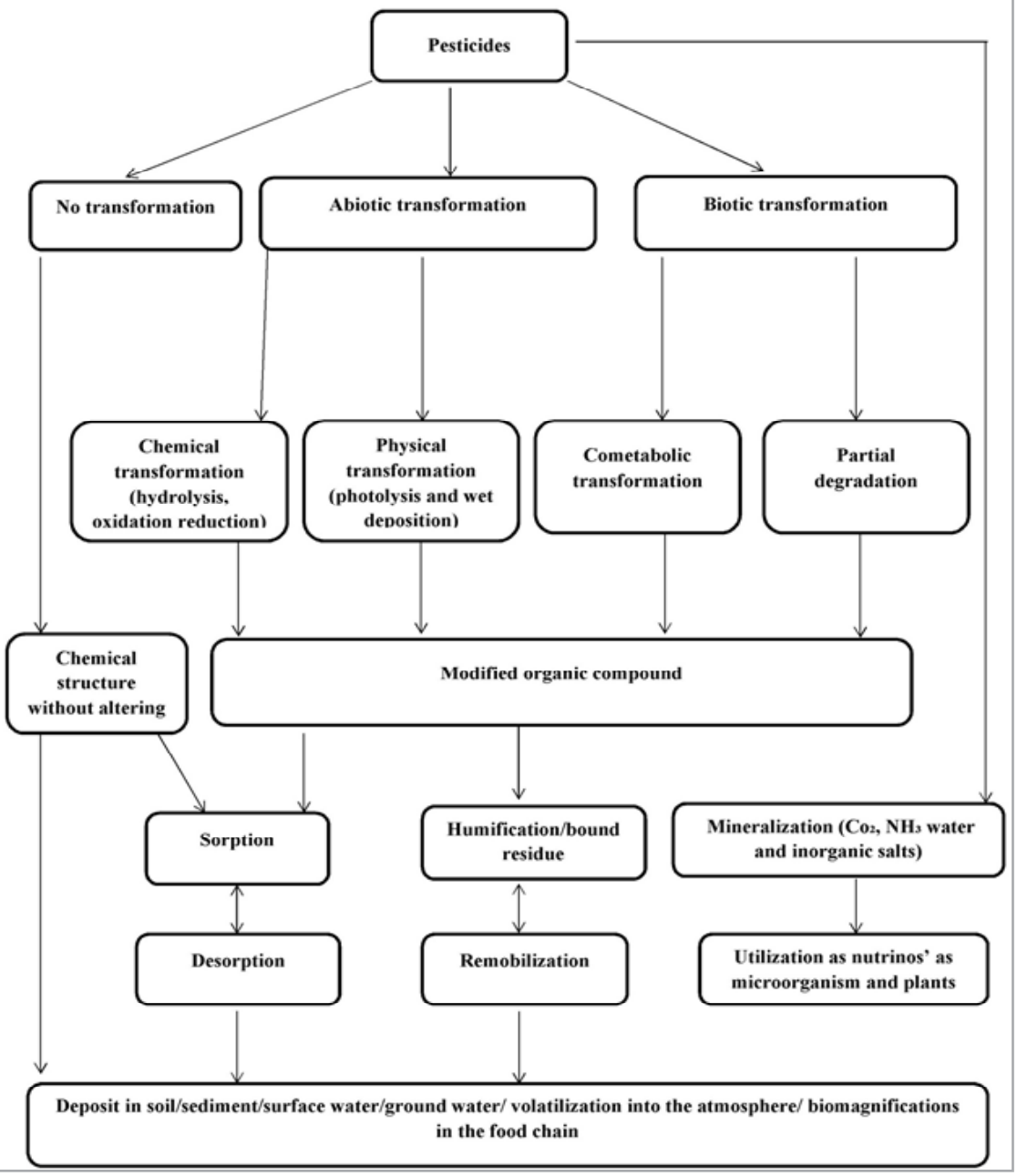
Protection from crop losses: The Pesticides play significant role in decreasing crop losses caused by pests. Aktar (2008) reported that weeds reduce agriculture production of dry land crops by 37% - 79% and it's reported that in medium land during the vital period secured an effective weed range (28% to 48%) control practice to protect in

losses in rice agriculture product. During the early stage of crop establishment 40% crop reduction were occurred by terrible infestation of weeds.

Vector disease control: The vector-borne diseases are very strongly take on by killing the vectors. Bhatia, 2004 reported that morbidity and mortality is one of the leading causes of malaria in the developing world and a main public health problem in India. Insecticides are one of the virtual ways to control the insects that disperse deadly diseases such as malaria, resulting in an approximate 5000 deaths every day and its strategies are also important for livestock.

Food quality: The Lewis et al. (2005) considered that the nutrition properties of blueberries and apple in the US diet and determined that their high concentrations of antioxidants act as protectants against cancer and heart disease. Lewis noted that doubling in wild blueberry production and subsequent rise in consumption of herbicide. It has been attention that fresh fruit and vegetables far outweigh potential risk from eating low residue of pesticides in crop. The increasing certify by the Dietary Guidelines, 2005 show that eating fruit and vegetables resolve the risk of high blood pressure, cancers, heart disease, diabetes, stroke, and other chronic diseases.

Figure 1: Microbial degradation of pesticide affected by various environmental factors



Other areas: Pesticides are applicable in different areas like sport complex, transport, buildings etc. The herbicides and insecticides are used to preserve the turf on sports pitches, golf courses and cricket grounds.. The especially herbicides use in transport sector which make an extensive use of pesticides. The insecticides protect building and wooden structure from termites and woodboring insects damage (Aktar et al., 2009).

Environmental hazards due to pesticides application: Pesticides play an important role in reducing crop losses caused by pests but their misuse can have serious negative impacts on both human health and the environment. The Pesticides can pollute the soil, water, turf, and other vegetation. In additionally to killing insects or weeds, pesticides can be dangerous to a host of other organisms including birds, fish, beneficial insects, and non-target plants. The use of pesticides is increases can result in serious negative effects on environmental and health problems like poisoning of farmers, cardiopulmonary, neurological, skin disorder, fetal deformities and miscarriage etc. (Patil and Katii, 2012).

Although consumption of pesticides in India is comparatively very low to the many other countries, but their widespread pollution of food commodities with pesticide residues, basically due to improper usage. The production and usage of some chlorinated pesticides such as DDT and lindane is still going on in the India (Abhilash and Singh, 2009; Vijgen 2011). Continues and improper application of pesticides has create the serious environmental contamination due to the dispersion into nontarget sites.

A synthetic chemical are environmentally stable and ready to bioaccumulation, and cause toxicity. Because some pesticides can persist in the environment, they can remain there for years. In our country at present day, the application method of pesticide is still based on the spraying of liquid and the spraying of powder. Many research shows that only 1% to 2% of the drug is used to control the target body, 10% to 20% on the body of the crop, and 80 to 90 percent of the pesticide is mainly scattered in the surrounding environment of the crop, such as farmland, floating in the atmosphere. Extensive use of chemicals in agriculture has contributed to build up of many hazardous compounds in air, soil and water, which cause environmental pollution. Wrong spraying and improper handling of the agrochemicals can create high risk of health hazards (Gupta, 2004). According to the Environmental Protection Agency (EPA), 30 % of insecticides 60 % of herbicides, 90 % of fungicides are known to be carcinogenic.

Effects on Human /Animal Health: The higher risk groups exposed to pesticides including in production workers, formulators, sprayers, mixers, loaders and agricultural farm workers. In certain developing countries, it has been noticed that organophosphate (OP) pesticides are liable to death in more than 70% cases and make up a large proportion of the poisonings by high toxicity of pesticides (Gupta, 2004). Toxicity of pesticides based on different factors like as the route of exposure, concentration and time of exposure. The organophosphorus class of pesticides poisoning is a global health problem with around 3 million poisonings and 200,000 deaths yearly (Sogorb, 2004).

Pyrethroids are less toxic than organophosphate, organochlorine and carbamate class of pesticides with some exception of esfenvalerate, deltamethrin, bifenthrin, tefluthrin, flucythrinate, cyhalothrin and fenpropathrin which show the highest severe oral toxicity. The toxicity of organophosphates group of pesticides is mainly associated with inhibition of acetyl cholinesterase enzyme involved in neurotransmission as acetylcholine substitutes which can interferes with muscular responses and leads to death (Shimazu, 2001) in human and other exposed organism. Singh, 2006 reported that more than 2500 pesticides are currently in use in the world. Conformably the report of UNEP and WHO, worldwide there are more than 26 million human pesticide poisonings with about 220,000 deaths annually (Richter, 2002).

The first report of organophosphorus class of pesticides poisoning was come from Kerala in 1958, where over 100 persons expire due to use of parathion contaminated wheat flour (Karunakaran, 1958) Pesticides may damage the immune system and can mimic hormones and may thus interrupt the endocrine system in both humans and animals. The period of manufacture and formulation the possibility of risk may be higher because the processes are not free from risk. In accordance with the United Nation, approximately 1 to 5 million pesticides cases poisoning occur yearly, resulting in several thousand fatalities among agriculture workers. Punjab showed various health problems including kidney failure, cancer, still birth, infertility, etc. during the survey on the basis of pesticides application in the agricultural (Abhilash and Singh, 2009). There are also observed that other clinical effects such as neck muscle weakness and diarrhea in humans.

The excessive use of pesticides can not only cause death but also stimulate various diseases. The total cancer patients in 10 % patients are resulted from pesticide poisoning. Organochlorinated pesticide, DDT and its derivative DDE are likely responsible for breast cancer. The period of 2005-2007, 16 bird species in more than 100 samples were gathered from Ahmedabad after being killed with kite flying threads. All carcasses were found with pesticide contamination for the time of study, therefore these situation make more alarming (Venugopal Dhananjayan, 2013). The organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs) were also found in blood plasma of white-backed vulture, Egyptian vulture, and Griffon Vulture collected from Ahmedabad (Dhanajayan et al. 2011).

Effects on Environment: The application method of pesticides in our country is still based on spraying of powder and liquid in which 80% to 90% of pesticides scattered in environment of the crop like as floating

in atmosphere, farmland and absorbed with dust from aerosol. After the probation period of degradation, metabolism and other effects, its content will be reduced to certain level, generally body of trace pesticides and environment residues called residual pesticides, which has a higher toxicity comparable to the parent. Organochlorine pesticides are high residue, these classes of pesticides that are currently banned from production but have been extensively used for long periods, therefore still residue present in the environment and there are treat very big. The excess use of pesticides leads to an accumulation of a large amount of pesticide residues in the environment, therefore causing a substantial environmental health hazard due to uptake and accumulation of these toxic compounds in the food chain and drinking water (Hossain, 2018).

Pesticides treated plant and soils can easily reach surface water by the runoff. Water Contamination by pesticides is widespread. Mostly 90 percent of water and fish samples from all streams contained from several pesticides. The Pesticides were exposed in all samples from greater rivers with mixed urban and agriculture land utilization influences and 99 percent of samples of urban streams (Bortleson and Davis, 1997). According to (United States Geological Survey) USGS, in general more pesticides were found in urban streams than in agricultural streams (Bortleson and Davis, 1987–1995). The herbicide 2, 4-D was the mainly found pesticide, detected in 12 out of 13 streams. The insecticide diazinon and the weed-killers such as triclopyr, dichlobenil, diuron and glyphosate were also found in Puget Sound basin streams. Groundwater contamination by the pesticides is a worldwide problem. The USGS (United States Geological Survey), 1999 that at least 143 various pesticides and 21 transformation products have been detected in ground water. The survey period in India, 58% of drinking water samples drawn from various wells and hand pumps around Bhopal were contaminated with organochlorine pesticides.

A large amount of transformation products (TPs) from a wide range of pesticides have been documented. The persistency of these pesticides and their transformation products are determined by a little parameters, like as soil-sorption constant (Koc), the octanol/water partition coefficient (Kow), water solubility and half-life in soil (DT50). These pesticides and their transformation products could be grouped into (1) Hydrophobic, persistent, and bioaccumulable pesticides that are strongly bound to soil. (2) The herbicides are represented as a polar pesticides but they also include fungicides, carbamates and some organophosphorus insecticide transformation products (Aktar et al., 2009). They can be proceed from soil by runoff and leaching. The most researched pesticide transformation products in soil are undoubtedly those from herbicides.

The various metabolic pathways have been advised including transformation through hydrolysis, methylation, and ring cleavage that make several toxic phenolic compounds. The pesticides and their transformation products are reserved by soils to different degrees, based on the interactions between soil and pesticide properties. The organic matter content has the great properties to the greater adsorption of pesticides and transformation products. The soil pH is also great important properties, including adsorption increases with decreasing soil pH for ionizable pesticides (e.g. 2,4-D, 2,4,5-T, picloram, and atrazine) (Andreu and Pico, 2004).

Effect on soil fertility: The heavy treatment of pesticides on soils can cause beneficial soil microorganisms to decay. Overutilization of pesticides and chemical fertilizers has effects on the soil microorganisms. For example, plants depend on a various variety of soil microorganisms to convert the atmospheric nitrogen into nitrates, which plants can use, general landscape herbicides interrupt this process triclopyr inhibits soil bacteria that transform ammonia into nitrite, glyphosate reduces the growth and activity of free-living nitrogen-fixing bacteria in soil, and 2,4-D decrease nitrogen fixation by the bacteria that live on the roots of bean plants, decrease the growth and activity of nitrogen-fixing blue-green algae (Mishra and Pandey, 1989). The mycorrhizal fungi develop with the roots of many plants and aid in nutrient uptake. These fungi can also be disturbing by herbicides in the soil. Triclopyr was also found to be toxic to several species of mycorrhizal fungi (Chakravarty and Sidhu, 1987) and oxadiazon herbicide decreased the number of mycorrhizal fungal spores (Moorman, 1989).

Contamination of air, soil, non-target vegetation and organism: The utilization of pesticides is increasing day by day which pollute our air, water, soil and ultimately reach to us through food chain. Pesticide sprays can directly or indirectly hit non-target vegetation or organism, or can volatilize from the treated area. Some pesticide volatile occurs during every application (Glotfelty and Schomburg, 1989). Almost every pesticide examined has been found in fog, air, rain or snow across the nation at different time period of the year (U.S. Geological Survey, 1999). Many herbicides have been exhibit to volatilize off treated plants with vapors sufficient to cause severe damage to other plants (Straathoff, 1986). Phenoxy herbicides 2,4-D can injure nearby shrubs and trees if they volatilize onto leaves. Exposure to the herbicide glyphosate can severely depress the seed quality (Zablotowicz et al., 2000).

The Ganges river heavy polluted by pesticides, fertilizers and industrial and domestic effluents (Kumari et al., 2002). In which Included to fish, other marine animals are endangered by pesticide pollution. Non-target organism like birds may also be killed if they ingest toxicant grains (US EPA, 1998). Herbicides can also adversely impact birds by destroying their habitat. Salim Ali Center for Ornithology and Natural History (SACON) had noticed that high levels of pesticides in some wetlands like Sukhbhadar and Meshwo dams and Pariej and Wadhvana irrigation reservoirs. Organochlorines were traced in the blood plasma of another 13 species of birds. (Dhananjayan & Muralidharan, 2010). The Organophosphate pesticides Chlorpyrifos, a contaminant of urban streams is highly dangerous to fish, and has

caused fish, kills in waterways near treated fields (US EPA, 2000). The environment protection agency (EPA, 1996) studies indict those trifluralin herbicides, an active ingredient in the weed-killer Snapshot, it's very highly toxic to both warm water and cold water fish.

Microbial degradation of pesticides: The application of pesticides is well understood but their effect cause serious environment problem. The scientist looking for efective ways and develop the technologies of pesticide degradation to solve the pesticides contamination problems that elimination in a safe, efficient, eco-friendly and economical way is important. Biodegradation or bioremediation technologies apply relatively low cost techniques, which generally have a high public satisfaction.The hazardous Pollutants are transformed by living organisms through various reactions such as biotransformation, biomineralization, bioaccumulation, biodegradation, bioremediation and co-metabolism (Porto et al., 2011). The degradation or detoxification rate depends on the type of pesticides, soil type, climate, the species of microbes present and the size of their population. Soil microbes play strong role in different activities including litter degradation, nutrient cyclin, promotion of plant growth, and the degradation of pollutants and pesticides (Pino and Penuela 2011).

The Biodegradation studies began in the 1940s, and it was refers to the mineralization of aerobic microbes in the soil, water and wastewater biological treatment systems for natural and synthetic organic matter. In microbial degradation microorganism play greatest role in the various biodegradation. Pesticides in the soil could be degraded by different direction of the traditional methods included chemical degradation, physical degradation and physical-chemical degradation, which basically caused secondary pollution (Zhang et al., 2017).

For example, Pseudomonas sp strain ADP utilize atrazine as the carbon source, and three enzymes (AtzA, AtzB, AtzC) were involved in the first few steps of degradation of atrazine. It is generally mentioned that biodegradation mainly refers to microbial degradation. Microorganisms perform the effective role in a variety of biodegradation therefore variety of biodegradable pesticides has been isolated, including bacteria, fungi, actinomycetes and algae. The bacteria and fungi both are the major entities for pesticide biodegradation. The pesticides degradation or detoxification may occur by biological activity of the soil microorganisms or under normal atmospheric conditions. There are various microorganisms like Pseudomonas, *Flavobacterium, Alcaligenes, Rhodococcus, Gliocladium, Trichoderma* and *Penicillium* are reported to use the pesticides as carbon source (Aislabie and Lloyd-jones, 1995).

On the current level of technology, chemical pesticides in the future for irreplaceable products so the decontamination of pesticide residues in the environment has become a hot topic in the world. The microbial degradation includes the use of effective microorganism for the enzymatic breakdown of toxic pesticides (organochlorines, organophosphates and carbamates) into a nontoxic compounds or it may be defined as complex organic compound (pesticide) into simple inorganic chemicals on the contaminated sites (Porto et al. 2011). The complete biodegradation of the pesticides into carbon dioxide and water by the oxidation of the parent compound and this process provides the energy to the microbes for their metabolism. The enzymes of the microbes play important role in the degradation of chemical compounds. The Carbamates class of pesticides was introduced in the early 1950s and is extensively applicable in prevent the crop by pest due to their effectiveness and biological activity (insecticides, fungicides and herbicides).

The chemically carbamate pesticides are esters of carbamates and organic compounds derivative from carbamic acid. The carbamates are change of form into various products by several processes such as hydrolysis, biodegradation, oxidation, photolysis, biotransformation and metabolic reactions in living organisms (Soriano et al., 2001). The Carbamate class of pesticides such as thiram is a fungicide. It is used as a to control damping off diseases (Phytophthora and Pythiumspp.) of maize, ornamentals and vegetables, seed treatment and animal repel. In the last few decades, the microbial decomposition of TMTD has been investigated by several authors. Richardson (1954) reported that more than 95% of the TMTD was completely converted to metabolites by soil microorganisms after 55 days of incubation.

The mechanism of microbial degradation of pesticides: Microorganism play main role in the biogeochemical cycles of the earth. The chemical substance degraded or transformed by microorganisms as used as a source of carbon, nitrogen, other nutrient and energy or as electron acceptor of a respiratory process. When microbial degradation of pesticides is caused by its intracellular enzymes the whole degradation process cover three steps (A) is the pesticides adsorption on the surface of microbial cells, the process is a dynamic balance, but also lead to the early degradation. It is a critical lag phase. (B) Pesticides penetrate the cell membrane into the membrane, the bacteria in a certain amount of pesticide on the cell membrane permeability determine its penetration of the cell membrane, and pesticide penetration of the cell membrane is the degradation rate limit step. This pesticides penetration rate is closely related to the molecular structure parameters of pesticides. (C) Pesticides in the cell membrane by the combination with the degrading enzyme by rapid enzymatic reaction (Singh, 2009). Microbial degradation pathway of pesticides including the process of mineralization, co metabolism and other degradation pathway.

Mineralization: The complete breakdown or transform of an organic compound into inorganic compound form, water and carbon dioxide. Mineralization is the best way to degradation process. Hou et al (2003) reported that the complete degradation of methyl parathion into nitrogen oxide (NO2-) and nitrate (NO3-) by *Pseudomonas vaginalis*.

Co-metabolism: A microbial population growing on one compound may transform a contaminating chemical that cannot be utilize as carbon and energy source its referred as a Co-metabolism but if there is another carbon matrix and energy for the presence of auxiliary matrix, they can be partially degraded (Ye et al., 2018). Some chemical substances such as insecticides, fungicides, and herbicides, etc could not be degraded easily but only by adding some organic matter such as exogenous or iso-biomass as the main energy (Huang et al., 2018). For example, Deng et al (2009) reported that *Aspergillus niger* YAT could degrade beta-CY(β-CY) and its intermediates degraded by co-metabolism and mineralization way, and the whole process was analyzed while there was rare analysis in other degrading strains of pyrethroids.

Other degradation pathway: The action of microorganism for degradation of pesticides included some other pathway such as hydrolysis, dehalogenation, oxidation, nitro reduction, methylation and demethylation (Ye et al., 2018).

Table 1. Pesticides degrading microorganism have efficacy to degrade different pesticides

Sr. No.	Pesticides	Organism	Source	References
1	Aldrin	*Trichoderma viride, Micrococcus* 204, *Bacillus sp* 458	Comparison of insecticide degradation by soil micro-organisms	Patil et al., 1970
		Pseudomonas fluorescens	Removal of Aldrin, using activatedcarbon Pseudomonas fluorescens free cell cultures	Erick et al., 2006
2	Atrazine	*Cryptococcus laurentii*	Biodegradation of atrazine by Cryptococcus laurentii isolated from contaminated agricultural soil	
		Raoultella planticola	Atrazine biodegradation by a monoculture of *Raoultella planticola* isolated from a herbicides waste water treatment facility	Swiss et al., 2014
3	Chlorpyrifos	*Pseudomonas sp.*	Effects of soil pH on the biodegradation of Chlorpyrifos and isolation of Chlorpyrifos–degrading bacterium	Singh et al., 2003
		Klebsiella sp.	Biodegradation of Chlorpyrifos by Klebsiella sp. Isolated from an activated sludge sample of waste water treatment plant in Damascus	Ghanem et al., 2007
4	DDT	*Pseudomonas aeruginosa* *Trichoderma viride*	Biotransformation of Chlorpyrifos and bioremediation of contaminated soil Degradation of DDT by a soil	Lakshmi et al.,2008 *Matsumura and Boush 1968*

			fungus *Trichoderma viride*	
		Eubacterium limosum	Reductive dechlorination DDT by human intestinal bacterium Eubacterium limosum under anaerobic conditions	Yim et al., 2008
		Azoarcus	Biodegradation of DDT by stimulation of indigenous microbial populations in soil with cosubstrate	Ortiz et al., 2013
5	Dieldrin	*Mucor racemosus* strain DDF	Biodegradation of dieldrin by a soil fungus isolated from a soil with annual endosulfan applications	Kataoka et al., 2010
		Bacillus subtilis MTCC1427	Biodegradation of soil-applied endosulfan in the presence Biosurfactant	Awasthi et al., 1999
6	*Endosulfan*	*Pseudomonas fluorescens*	Biodegradation of endosulfan isomers in broth culture and soil microcosm by *Pseudomonas fluorescens* isolated from soil	*Giri et al., 2014*
		Staphylococcus, Micrococcus, Bacillus, Pseudomonas, Mucor, Penicillium, Aspergillus fumigates, Candida	Bioremediation of Endosulfan contaminated Soil	Mohanasrinivasan et al., 2013
7	Endrin	*Trichoderma viride12, Pseudomonassp.27, Micrococcus204, Arthrobactersp. 278,Bacillussp.4*	Comparison of insecticide degradation by soil microorganisms	Patil et al., 1970
8	Methyl parathion	Stenotrophomonas maltophilia M1	Degradation of methomyl by the novel bacterial strain Stenotrophomonas maltophilia M1	Mohamed, 2009
		Cyanobacteria	Biodegradation and Utilization of Organophosphorus Pesticide Malathion by Cyanobacteria	Ibrahim et al., 2014
		Pseudomonas sp. Strain WBC-3	Bioaugmentation of a methyl parathion contaminated soil with *Pseudomonas sp.* Strain WBC-3	Wang et al., 2014
9	Monocrotophos(MCP)	*Aspergillus niger* MCP1	Isolation and characterization of Monocrotophos degrading activity of soil	*Jain et al., 2012*
		Pseudomonas stutzeri MTCC 2300	Microbial degradation of Monocrotophos by pseudomonas stutzeri	Barathidasan and Reetha 2013
10	Parathion	*Bacillus sp.* and	Degradation of parathion	Siddaramappa

		Pseudomonas sp. *Arthrobacter sp.,* *Bacillus sp.*	by bacteria isolated from flooded soil Biologically induced hydrolysis of parathion in soil :kinetics and modeling	et al., 1973 Nelson et al., 1982
11	PCBs	Desulfomonile tiedjei, Desulfitobacterium, Dehalobacter restrictus	Polychlorinated biphenyls and their biodegradation	Borja et al., 2005
12	2,4,Dichlorophenoxy acetic acid	*Aspergillus niger*	Metabolism of 2,4-Dichlorophenoxy acetic Acid ('2,4-D') by *Aspergillus niger* van Tiegh	Faulkner and Woodcock 1964
		Cupriavidus pinatubonensis JMP 134	Simultaneous assessment of the effects of herbicide on the triad: rhizobacterial community, an herbicide degrading soil bacterium	
13	Prophenofos	*Pseudomonas putida, Burkholderia gladioli.*	and their plant host Isolation and identification of profenofos degrading bacteria	Kraiser et al., 2013 Malghani et al., 2009
14	Thiram (TMTD)	*Bacillus cereus, Pseudomonas aeruginosa, Pseudomonas mendocina*	Isolation, Characterization, Identification and Potentiality of Fungicide Thiram (TMTD) Degraders under Laboratory Conditions	A.A. Elhussein, 2011
		Streptomyces and *Aspergillus flavus*	Isolation, Characterization and Identification of Thiram-degrading Microorganisms from Soil Enrichment Cultures	Sahin, n. and Tamer, a.ü., 2000

CONCLUSION AND FUTURE PROSPECTS

The access of pest management are necessary to increase sustainable agricultural yield and protect the environment therefore agriculture development, planning and policy are main aim. In agriculture field for huge amount of production pesticides play important role as a plant protection. The effective pest control using pesticides or synthetic chemicals has beneficially affected the farmers' economy to produce higher agriculture production. During these period only 2-3% of the total pesticides reached to the target area, rest remains contaminate the environment and pollute the water, soil and air, and cause many human diseases. Biodegradation process can be applied for the pesticides degradation through the microorganism. The microorganism have capabilities to degradation and detoxification of pollutants, which the most efficient and cost effective technology and generally have a high public acceptances and can be frequently carried out on affected sites. Microbial degradation processes applied for bioremediation involves use of variety of different microbes including bacteria, fungi and actinomycetes.

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