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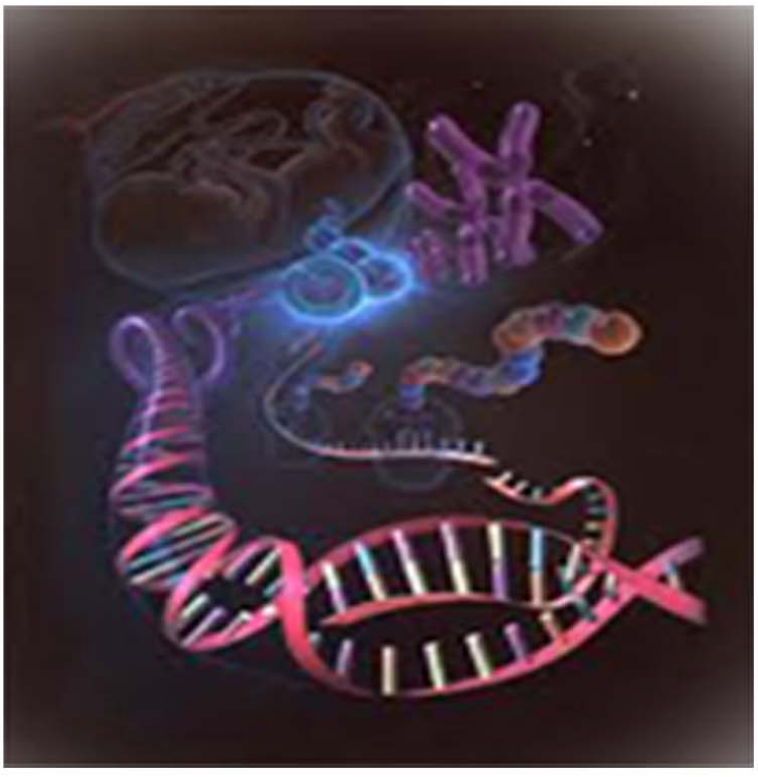

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Research Paper

BIOCHEMICAL AND MOLECULAR DETECTION OF BIOSURFACTANT PRODUCING BACTERIA FROM SOIL

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The use of biosurfactant is a promising alternative over the chemical surfactant as they are better biodegradable and do not pollute the environment. Most of the biosurfactant producing microorganisms have been isolated from the hydrophobic environment such as oily waste, sludge etc. in this study, petroleum contaminated soil was collected and enriched on oil containing media for isolation of biosurfactant producing bacteria. From these soil samples 18 different bacterial species were isolated and screened for biosurfactant production by surface tension measurement, drop collapse test, oil displacement test, emulsification index and â-haemolysis. Further all these isolates were identified on the basis of biochemical and 16S rRNA sequencing. Result of biochemical and phylogenic characterization showed that the bacterial were grouped into seven genus and species are *Pseudomonas aeruginisa, Aneurinibacillus miugulanus, Bacillus circulans, Ochrobactrum oryzae, Ochrobactrum pseudintermedium, Ochrobactrum intermedium, Achromobacter insolitus, Nocardia farcinica and Stenotrophomonas maltophilia.* The result of the phylogenic tree provided a roadmap to the future research, especially in isolation and identification of new biosurfactant-producing strains as well as the produced biosurfactant.

Keywords: Biosurfactant, Bioemulsifier, Phylogeny, 16S rRNA

INTRODUCTION

Since 1970, oil spills and an accidental leakage of oil tankers have released several million tons of oil to the environment causes coastal and offshore contamination and health problem (ITOPF, 2004). Such oil spills are often treated with synthetic surfactants to disperse oil and accelerate its mineralization which act additional source of contamination and has rate limiting steps (Bognolo, 1998). A need for the new specialty chemicals in the field of agriculture, cosmetic, food, pharmaceutical, environmental industries and bioremediation turns the attention towards the microbial world, as they has ability to produce a largely unexplored variety of chemicals, such as biosurfactant (Maier, 2003).

Biosurfactant is amphiphilic substance produced by microorganisms containing hydrophilic and hydrophobic moiety useful in bioremediation of polluted sites in petroleum

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industry, microbial enhanced oil recovery, viscosity reduction of heavy oils and oil storage clean up etc. (Makkar and Cameotra, 1997). Biosurfactant from many microorganisms have demonstrated antimicrobial and anti-carcinogenic activities (Karanth *et al*, 1999).

At present, field of biosurfactant attract the attention of research as many biosurfactant have been used on industrial scale, Ferhat *et al*, 2011, studied biosurfactant-producing bacteria from a crude-oil-contaminated soil as *Ochrobactrum* sp. 1C and *Brevibacterium* sp. 7G showed tolerance against slight variations for heat, pH, and salinity and exhibited antimicrobial activities against *P. aeruginosa* and *S. aureus.* Huang *et al*, 2010, characterized phylogenetically twenty bio-emulsifier producing strain out of six strain *Brevibacillus* sp*., Dietzia* sp*., Ochrobactrum* sp*., Pusillimonas* sp*., Sphingopyxis* sp. and *Achromobacter* sp. were firstly reported as demulsifying strains. *Ochrobactrum* species are also isolated in this study as biosurfactant producer.

Regarding the distribution in nature and microbial diversity biosurfactant producing bacteria already have been studied, however these studies mainly concentrated on the isolation of biosurfactant producing bacteria from the environment with the aid of molecular biological analysis means, their phylogenic evolution was revealed. The results of phylogenic analysis provided a roadmap to the future research, especially in isolation and identification of new biosurfactant-producing strains as well as the produced biosurfactant. The aim of this study was to isolate microorganisms with elevated potential for biosurfactant production and to study the microbial diversity and phylogenic relationship of biosurfactant-producing bacteria.

MATERIALS AND METHODS

Collection of Soil Samples

Soil samples including oil contaminated and petroleum contaminated were collected from various automobile workshops of the city in sterile zip lock bag and transported to the laboratory.

Enrichment and Screening of Biosurfactant Producing Bacteria

1 g soil was inoculated in 250 ml Erlenmeyer flask containing 100 ml Mineral Salt Medium and 2% oil as a sole source of the carbon. The flasks were incubated at 37 ^{0}C on rotary shaker at 200 rpm for 72 h. Continuous sub culturing and shaking was carried out five times for enrichment. Isolation was carried out by inoculation on nutrient agar and morphologically distinct colonies were isolated and stored as a stock culture at 4^{0}C.

Preliminary Screening for Biosurfactant Production

Selected isolates were further screened for preliminary screening test as 5ml culture broth was used as inoculums and inoculated in 250ml flask containing 100ml MSM media having composition: 2.5g/l of $NaNO_3$, 3.0 g/l of KH_2PO_4, 7.0 g/l of K_2HPO_4, 0.01 g/l of $CaCl_2$, 0.5 g/l of $MgSO_4.7H_2O$ and trace element solution containing 0.116 g/l of $FeSO_4.7H_2O$, 0.232 g/l of H_3BO_3, 0.41 g/l of $CoCl_2.6H_2O$, 0.008 g/l of $CuSO_4.5H_2O$, 0.008 g/l of $MnSO_4.H_2O$, 0.022 g/l of $[NH_4]_6Mo_7O_{24}$, 0.174 g/l of $ZnSO_4$ containing 2% engine oil as carbon source. After incubation for 72h the broth was centrifuged at 10,000 rpm for 20 min. Surface oil layer was discarded and culture supernatant was stored in sterile bottles. Surface tension measurement of cell free broth was determined in K6 tensiometer using the du Nouy ring method. Oil spreading test and Drop

collapse test of the cell free broth supernatant was carried out according to the method described previously by Youssuf *et al*, 2004. Hemolytic activity of the isolate was performed on blood agar plate. Emulsification capacity of the isolate was also performed by adding the 2 ml of hydrocarbon oil to the same amount of the culture supernatant mixing with a vortexed for 2 min and leave to stand for 24h. E24 is given as percentage of the height of emulsified layer (mm) divided by total height of the emulsified column (mm) (Abouseoud *et al*, 2007).

Biochemical Characterization

The selected cultures were examined for their morphological, cultural and standard biochemical test according to Bergey's manual of Systematic Bacteriology (Sneth, 1986).

Identification and Phylogenetic Analysis

Biochemically characterized isolates were identified by 16S rRNA sequence analysis the NCCS, Pune. The partial sequence of the 16S rRNA gene was amplified by using PCR with universal primer, 16F 27 (5'CCAGAATTGATC MTGGCTCAG-3') and 16R 1525 (5'TTCTGCAGT CTA GAAGGA GGTGWTCCAGCC-3'). The 16S rRNA sequences were analyzed using BLASTA programme. In addition, sequences were analyzed via RDPII using SEQUENCE-MATCH (Version 2.7) to identify the most closely related database sequences. Multiple Sequence Alignments of approximately 900 bp sequences was done by using CLUSTAL X version 1.8. The phylogenetic tree was constructed from evolutionary distances using the neighbor-joining method of MEGA 4 program package (Kumar *et al*, 2004).

RESULTS AND DISCUSSION

In the present study, from thirty petroleum contaminated soil samples, eighteen bio-surfactant producing bacteria were isolated out of these fourteen were identified on the basis of 16S rRNA sequencing including two spore bearing strains. Most of them are Gram negative short rod except four strains which are Gram positive.

All the strains were motile, aerobic, non sugar fermenting and oxidase positive except one *N. farcinica* (L3) was non motile and oxidase negative. Out of fourteen strains seven gives zone of β-hemolysis which is also a property of biosurfactant producing bacteria. Report of the previous study showed that bacteria isolated from sites with a history of oil contamination or its byproducts are mostly Gram-negative, and this may be a characteristic of these bacteria contributes to survival of these populations in such harsh and unfavorable environments (Bicca *et al.*, 1999).

Dendrogram of fourteen bacterial strains based on comparison of the morphological, cultural and biochemical characteristics was constructed. All these strains were found to be grouped in two main clusters. In first cluster, three sub clusters were found. In first sub cluster, were found four bacterial species and the species are *P. aeruginosa* (2), *N. farcinica* (1), *O*. oryzae (1) and *O. pseudintermedium* (1). In second sub cluster four bacteria *A. migulanus, B. circulans, O. intermedium and P. aeruginosa* were observed while the third sub cluster form a separate clad and separate species *P. aeruginosa* from others. In second cluster four bacteria were observed *A. insolitus, S. maltophilia* (2) and *P. salinarum.* The dendrogram showed cultural and physiological similarity and dissimilarity in there genus and it

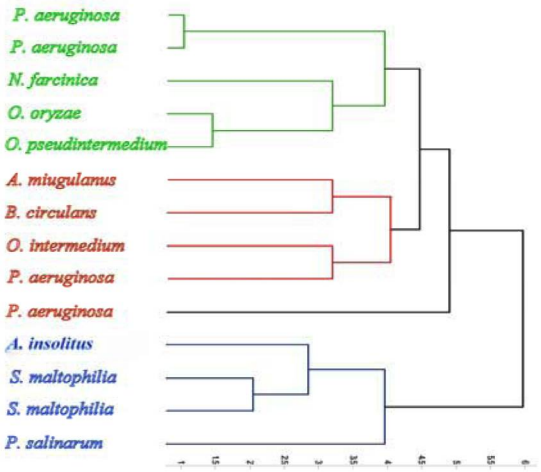
Table 1: Morphological and Biochemical Characteristics of Biosurfactant Producing Bacteria

Name of Organism	*A. migulanus* (G1)	*P. aeruginosa* (G2)	*A. insolitus* (G3)	*P. aeruginosa* (L1)	*B. circulans* (L2)	*N. farcinica* (L3)	*O. oryzae* (L4)	*O. pseudintermedium* (L5)	*O. intermedium* (L6)	*P. aeruginosa* (PVG 1)	*S. maltophilia* (PVG 3)	*S. maltophilia* (PVG 4)	*P. salinarum* (PVG 5)	*P. aeruginosa* (PVG 7)
Gram Reaction	+	–	–	–	+	–	–	–	–	–	–	–	+	–
Shape	LR	SR	SR	SR	LR	SR	SR	SR	SR	SR	SR	SR	CB	SR
Arrangement	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Motility	+	+	+	+	+	–	+	+	+	+	+	+	+	+
Spore staining	+	–	–	–	+	–	–	–	–	–	–	–	–	–
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	–	+	+	+	+	+	+	+	+
Indole	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Methyl red	–	–	–	–	–	–	–	–	–	–	–	–	–	–
VP test	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Citrate utilization	–	+	+	+	–	–	–	+	–	+	+	+	–	+
Nitrate reduction	+	+	+	+	–	+	+	+	+	+	–	–	+	+
Glucose	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Lactose	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Mannitol	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Arabinose	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Trehalose	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Xylose	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Rhamnose	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Sorbitol	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Galactose	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Salicin	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Cellobiose	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Growth at 4^{0}C	–	–	–	–	–	+	–	–	–	–	–	–	–	–
Growth at 42^{0}C	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 30^{0}C	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 40^{0}C	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 50^{0}C	+	+	–	–	–	–	–	–	–	–	–	–	–	+
β–hemolysis	–	+	–	–	+	–	+	–	–	+	+	+	–	+

Note: LR= long rod, SR=short rod, CB=coccobacilli, S=single.

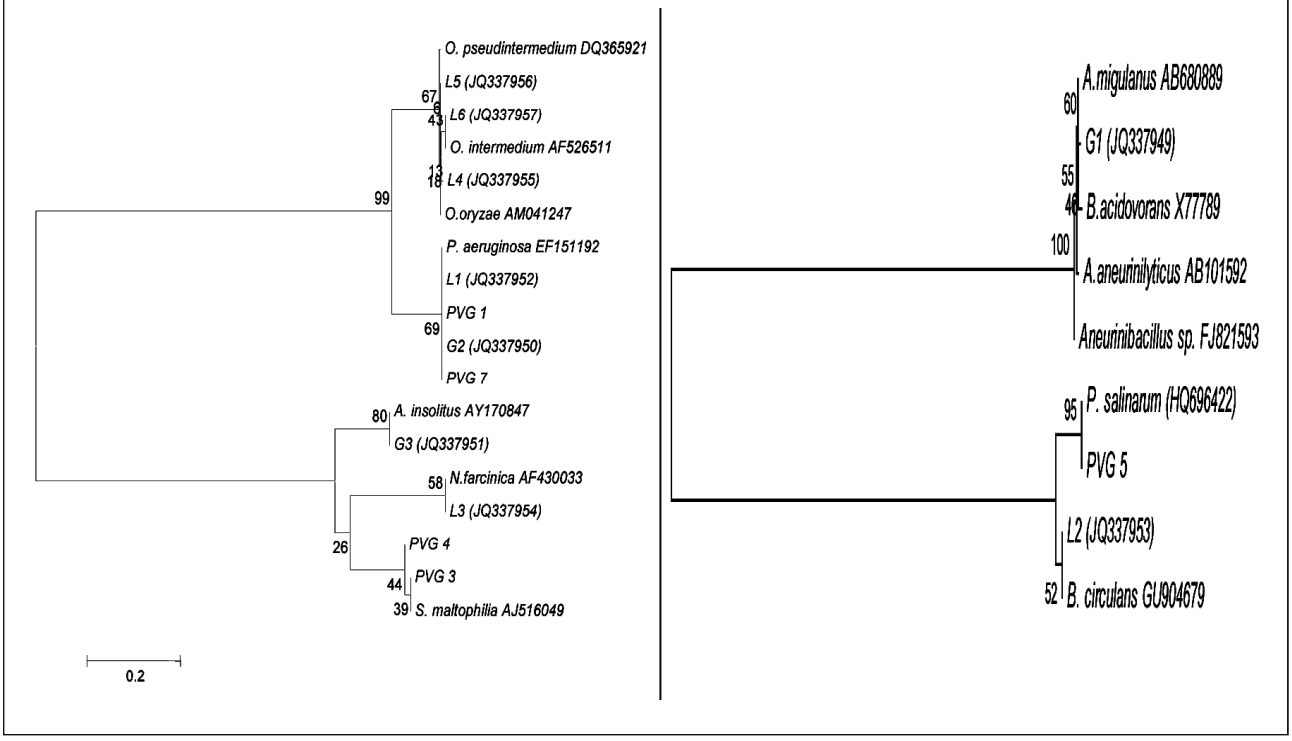
was elucidating for taxonomical position based on their physiological characteristics (Figure 1).

Figure 1: Dendrogram Based on Similarity of Morphological, Cultural and Biochemical Characteristics of Isolates Using Average Linkage



Biochemically characterized bacterial strain were identified by 16s rRNA sequencing and these sequences were analyzed by CLUSTAL W alignment. Two separate phylogenetic trees were constructed depending upon the Gram character. The results of the phylogenetic position of biosurfactant producing bacterial strains isolated from petroleum contaminated soil were related to phylum Proteobacteria, Firmicutes and Actinobacteria, but most of them are Gram negative belonging to phylum Proteobacteria including α-proteobacteria, β-proteobacteria and γ-proteobacteria. According to 16S rDNA gene sequences, out of fourteen strains six strains showed a high level of similarity with the Genus *Pseudomonas* (6 strain), three with Genus *Ochrobactrum* (3 strain), three with Genus *Bacillus* one of *Achromobacter* and *Nocardia* each, while the species was *P. aeruginosa* (6), *O. oryzae, O. intermedium, O. pseudintermedium, A.migulanus, B.circulans, P. salinarum, A. insolitus* and *N. farcinica* respectively (Figure 2).

Figure 2: Phylogenetic Tree Based on a Comparison of the 16S Ribosomal DNA Sequences of the Isolates, the Tree was Created by the Neighbor-Joining Method by Using MEGA 4 Package



All the identified bacterial strains were characterized for their biosurfactant production capability using oil displacement test, drop collapse test, surface tension measurement, â-hemolysis and emulsification index determination by using 2T grade engine oil as sole source of carbon in mineral salt medium. Out of these fourteen strains thirteen strains shows positive oil displacement test drop collapse test (when it compare with distilled water and media without inoculums used as control) except isolate L2 (*B. circulans*) gives negative oil displacement test. But the strain G2, L1, PVG1, PVG4, PVG7 gives prominent zone of oil displacement test as they displaces the oil layer up to 90mm, 30mm, 35mm, 40mm, 55mm respectively (Table 2).

Tong *et al*. (2011) studied the biosurfactant producing bacteria from Daqing oil-contaminated sites showed an obvious haemolytic zone on the blood agar plate and also shows the oil displacement zone of 50 mm and this isolate was identified as *Pseudomonas* species by 16S rRNA sequencing. Tambekar *et al*, (2012) also reported biosurfactant production from bacteria isolated from petroleum contaminated sites.

The surface tension and emulsification activity of the culture supernatants of 14 bacterial strains was determined in the preliminary screening. According to Cooper (1986), a microorganism considered to be promising for biosurfactant production if it is able to reduce the surface tension to values lower than 40mN/m^{-1}. Out of fourteen strains five strains reduce the surface tension of the supernatant below 40mN/m^{-1} and three isolates reduces the surface tension of the broth below 30mN/m^{-1} Table 2. The strain G2 and PVG1

Table 2: Biosurfactant Production Property of the Bacterial Isolates

Isolates	Oil Displacement Test (mm)	Drop Collapse Test	Surface Tension (mN/m)	Emulsification Index (%)	β-Hemolysis test
A. migulanus (G1)	10 mm	-	36.07	16.92	-
P. aeruginosa (G2)	90 mm	+	28.88	32.25	+
A. insolitus (G3)	10 mm	-	48.08	19.04	-
P. aeruginosa (L1)	30 mm	+	42.02	8.33	-
B. circulans (L2)	00mm	-	53.04	6.45	+
N. farcinica (L3)	10 mm	-	63.18	13.33	-
O. oryzae (L4)	10 mm	-	52.24	5.00	+
O.pseudintermedium (L5)	10 mm	-	59.35	6.66	-
O. intermedium (L6)	5 mm	-	52.24	8.19	-
P. aeruginosa (PVG 1)	35 mm	+	28.43	22.22	+
Ste. maltophilia (PVG 3)	5 mm	-	60.72	5.00	+
Ste. maltophilia (PVG 4)	40 mm	+	29.62	11.47	+
P. salinarum (PVG 5)	5 mm	-	50.35	8.33	-
P. aeruginosa (PVG 7)	55 mm	+	31.89	16.39	+

reduces the surface tension of the supernatant up to 28.88 and 28.43mN/m^{-1} and identified as *P. aeruginosa.* Also these strains produce extracellular emulsifier able to stabilize oil water emulsion. The results showed that the strain reducing surface tension of broth below 30mN/m shows emulsification capacity greater than 11% while the isolates reduce maximum surface tension showed higher emulsification index. Biosurfactant and bioemulsifier producing bacteria were isolated and characterized by Batista *et al*, (2006), the results of the their studies supports the outcomes of the present work and helps in providing the suitable platform to the further study regarding the bioremediation of the oil contaminated environment and heath issues of environment.

CONCLUSION

Screening of biosurfactant-producing bacteria from soils contaminated by hydrocarbons constitutes a powerful tool for the selection of strains with high emulsifying capacity. Understanding the interactions between oil-degrading micro organisms is essential, not only to predicting the fate of hydrocarbons in the environment but also for development of successful bioremediation strategy. In this direction, we characterized good biosurfactant-producing strains isolated from a region that has been exposed to hydrocarbon pollution. We particularly focused on the interactions between oil water mixtures. This feature could be advantageous for hydrocarbon waste treatment. The results of the evolutionary analysis showed that the phylogenic tree provided a roadmap to the future research, especially in isolation and identification of new biosurfactant-producing strains as well as the produced biosurfactant.

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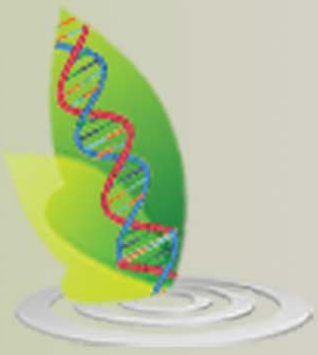

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