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Screening of Biosurfactant Producing Bacteria from Oil Contaminated Sites of Erode District, Tamilnadu, India

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ABSTRACT:

Biosurfactant production by three bacterial strains (PBS 10, DLS 08, and MVS 11) were isolated from various oil contaminated sites like petrol bunk, diesel sheds etc in and around Erode. In the present study, biosurfactants were produced by using Mineral salt (MS) media and the isolates were identified and screened for biosurfactant production using drop collapse, oil displacement and emulsification test. The produced biosurfactant was extracted and characterized using FT- IR. The biosurfactant producing organisms were identified as Pseudomonas aeruginosa, klebesilla pneumoniae, and Pantoea dispersa by 16SrRNA gene sequencing. The three strains have shown high effectiveness in biosurfactant productions whereas the emulsification index revealed that Pseudomonas aeruginosa having the highest index of 25 % and it can be utilized in oil recovery than chemically synthesized surfactants and it's more attractive to be applied in microbial enhanced oil recovery.

KEYWORDS: Biosurfactant; Mineral salt (MS) media; Emulsification test; FT- IR; 16SrRNA; Microbial enhanced oil recovery

INTRODUCTION:

Synthetic surfactants are components of laundry detergents, shampoos, dish washing liquids etc which pose many serious hazard to the environment (Ostroumov, 2006) these surfactants reach the aquatic ecosystem and pose great danger, anthropogenic impact on the biota is too high that it interferes with the hydrobionts which maintain the water quality and self- purification. The self- purification processes in aquatic ecosystems are of importance not only from the viewpoint of maintaining habitats appropriate for preservation of biodiversity (Ostroumov, 2004). Biosurfactants produced from natural origin especially from microorganisms can serve as an effective alternative for these environmental implications. Biosurfactant have similar properties to that of the synthetic surfactants yet ecofriendly in nature as they are a diverse group of surface- active biomolecules produced by many living organisms (Maier, 2003; Banat, 1995).These amphiphilic compounds contain a hydrophobic and a hydrophilic moiety, and have the ability to reduce interfacial tension between different fluid phases. Their uses and potential commercial applications have been reported in several fields, including surfactant-assisted flooding for enhanced oil recovery in the oil industry, emulsifiers in the food industry, and moisturizers in the cosmetic industry (Cameotra and Makkar, 2004; Desai and Banat, 1997; Banat *et al.,* 2000).

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These amphiphilic structures confer a wide range of properties, including the ability to lower surface and interfacial tension of liquids and to form micelles and micro emulsions between two different phases , biosurfactants exhibit high surface-activities together with low critical micelle concentrations (CMCs), being in some cases even lower than most of the traditional chemicals surfactants. They occur in nature and comprise molecules of glycolipids, lipopeptides, fatty acids, phospholipids and polymeric surfactants. The type and quantity of microbial surfactants depends primarily on the producer organism, factors such as carbon sources, nitrogen sources, microelements, temperature, pH, degree of aeration, can also influence biosurfactants production by microorganisms. They are mainly produced by hydrocarbon utilizing microorganisms exhibiting surface activity.

In this work we describe the isolation of biosurfactant producing bacteria from oil contaminated sites near petrol bunk, motor vehicle service station and diesel loco sheds. Different screening methods were done to identify a bacterial strain which would yield the biosurfactant. The organisms were identified using 16SrRNA sequencing and the biosurfactant produced by the organisms were extracted and analyzed using FT- IR and other techniques.

MATERIALS AND METHODS:

Isolation of microorganisms from Oil contaminated sites:

Soil from oil contaminated sites were collected from areas in and around Erode, they were kept in sterile covers and transferred to the laboratory and maintained at 4˚C until use. Serial dilution of the soil sample were done on Nutrient agar plates, morphologically varying colonies were pure cultured and maintained in NA slants.

Screening of biosurfactant-producing strains:

The different isolates were screened for extracellular biosurfactant production in mineral salt medium under aerobic conditions at 37 ˚C. The cultures were inoculated in *Mineral salt (MS) medium (g/L)*: Nacl, 10; Na_2HPO_4, 5.0; KH_2PO_4, 2.0; MgSO4.7H2O, 4.0; sucrose, 10; ammonium nitrate, 2.0. The pH level was adjusted to 7.0 Sucrose act as a carbon source and ammonium nitrate act as a nitrogen sources. Cell growth was monitored by measuring the optical density at 600 nm (Mnif *et al.,* 2009). Isolated bacterial colonies were plated onto crude oil (1%, v/v) agar basal medium. The plates were incubated at 37˚C for 1-2 days, until colony formation. Colonies surrounded by a clear zone in this medium were presumed to produce surfactants (Desai and Banat, 1997) and they were transferred to the fresh basal media containing 1% (v/v) crude oil (Kiyohara *et al.,*1982) by centrifugation at 13,000 rpm for 30 min the cells were removed from the broth and the supernatant of each strain was harvested for use in the biosurfactant activity assay like drop collapse, oil displacement, Penetration assay and emulsifying activity were taken into account.

Drop Collapse:

96 well microtitre plates were coated with 2 µl of mineral oil. The plate was equilibrated for 24 hr at 37˚C. 5µl of culture supernatant on MS at 37˚C at 200 rpm for 24 hrs were added to the surface of oil, with distilled water as control. The biosurfactant producing organisms were detected from the Drop Collapsing within a

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minute from the oil coated (Youssef *et al.,* 2004).

Oil Displacement:

15µl of crude oil was placed on surface of 40 µl distilled water in petridish and 10 µl of 24hr culture supernatant was gently placed on surface of the oil. The diameter and area of clear halo was visualized under visible light and measured after 30 seconds. (Morikawa *et al.,*1993).

Penetration Assay:

96 well plates were filled with 150µl of hydrophobic paste made up of oil and silica gel. The paste was covered with 20µl of oil.10µl of safranin was added to 90µl of supernatant and the coloured supernatant was placed on the surface of paste. This relies on contacting of two insoluble phase which leads to the penetration of the stained biosurfactant into the hydrophobic paste (Maczek *et al.,* 2007).

Measurement of Emulsification index:

A qualitative biosurfactant activity assay was performed using an emulsification test. This was carried out using 2 ml of culture supernatant with 2 ml taken in test tube and vortexed for 2 min. The height of the emulsion layer was measured after 2 h and again after 24hr. The supernatants which produced a stable cloudy appearance in the emulsion layer indicate greater surface activity (Cooper *et al.,* 1987). Emulsion index after 24 hr (E24) was calculated as follows:

E24 = (Height of Emulsion layer/ Total height) X 100

Extraction of biosurfactant:

Cultures showing high emulsion activity were inoculated into 50 ml MS broth and incubated at 25˚C for 7 days in shaking incubator (200 rpm). The cultures were centrifuged at 5,000rpm for 30 min at 4˚C. To the supernatant 1M H_2SO_4 was added to adjust the pH to 2. Equal volume of chloroform: methanol (2:1) was added to the pH adjusted supernatant and shaken rigorously for proper mixing and left overnight. White coloured precipitate at the interface between two liquid phases proved presence of Biosurfactant (Li *et al.,* 1984).

Purification of biosurfactants:

Precipitated biosurfactant observed at the interface was taken carefully using a micropipette, to which about 1 ml of distilled water was added centrifuged at 7000 rpm at 4˚C for 30 min. Supernatant was discarded and the pellet was allowed to dry for 24hrs, which will be the crude extract of biosurfactant.

FT-IR analysis of Crude biosurfactants:

The solid biosurfactant extracts recovered from the supernatants of the isolates and there the characterization was done on a Jasco FT-IR 4100 spectrometer by KBr pellet method (Gnanamani *et al.,* 2010). The FTIR spectra, with a resolution of 4 cm^{-1}, were collected from 400 to 4000 wave numbers (cm^{-1}).

RESULTS:

From serial dilution of soil samples about 15 organisms were isolated. They were given specific codes based on their location from where the samples have been collected. (Table1).

Table 1: Organisms isolated from oil contaminated site.

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S.No	Specific codes for isolates
1	PBS 10
2	OM 11
3	OM 12
4	OM 13
5	OM 14
6	MVS 15
7	MVS 16
8	MVS 17
9	MVS 18
10	MVS 19
11	MVS 20
12	DLS 06
13	DLS 07
14	DLS 08
15	DLS 09

*PBS – Petrol Bunk Soil; DLS – Diesel loco shed; MVS – motor vehicle service station ; OM – oil mill

All the organisms were screened for the production of biosurfactant by inoculating them in Mineral salt (MS) media agar plates. The colonies showing clear zone were taken as positive biosurfactant producers. 3 isolates exhibited a clear zone in MS agar plates (PBS 10; DLS 08; MVS 11).The 3 organisms were subjected to further screening of producers. Table 2 shows the results of Drop collapse, Penetration Assay, oil displacement for the 3 strains (PBS 10; DLS 08; MVS 11). The 16s rRNA sequence of the organisms showed to be *Pseudomonas aeruginosa* (PBS 10), *klebesilla pneumoniae* (DLS 08) and *Pantoea dispersa* (MVS 11).

Table 2: Results of Drop collapse, Penetration Assay, Oil Displacement of PBS 10, DLS 08 and MVS 11 for biosurfactant production.

S.No	Organisms	Drop collapse	Penetration Assay	Oil Displacement
1	PBS 10	+	+	+
2	DLS 08	+	+	+
3	MVS 11	+	-	+

The E24 value of PBS 10 was 25 % and DLS 08 was 18%.As PBS 10 & DLS 08 showed the positive results for extracellular biosurfactant production from the bacteria, they are then analyzed for biosurfactant production by shake flask method by inoculating the 2 isolates in MS broth (100 ml in 250 ml Erlenmeyer flask), after the biosurfactant has been extracted. The Dry weight of biosurfactant produced by PBS 10 (*Pseudomonas aeruginosa*) was 0.249g / 100 ml, DLS 08 (*klebesilla pneumoniae*) produced 0.14 g /100 ml of crude biosurfactant and MVS 11(*Pantoea dispersa*) was 0.10 g /100 ml.

The FT-IR results show the corresponding peaks which authenticate the production of biosurfactant by PBS 10 (*Pseudomonas aeruginosa*) (Fig 1).

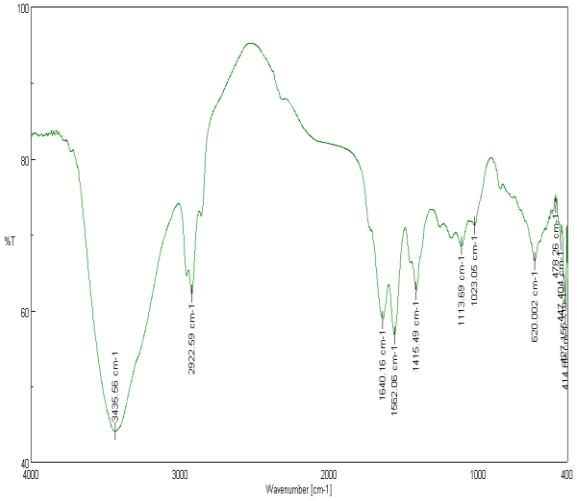

Fig 1 FTIR spectra of biosurfactant produced by PBS 10

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DISCUSSION:

Hydrocarbon utilizing microorganisms have been reported to show high surface activity as they produce surfactant compounds responsible for those activated activities. This was the reason for the selection of oil contaminated sites for isolation of bacteria for Biosurfactant production. Both Gram positive and Gram negative bacteria capable of producing surface active agents and they are amphipathic extracellular lipopeptides (Molitt *et al.,* 2000).
From 15 organisms only 3 organisms were capable of producing biosurfactant and all the three organisms belong to different genera (*Klebesiella, Pseudomonas, Pantoea).* The partial sequence of 16S rRNA genes from isolates PBS 10 and DLS 08 showed 100% similarity with *Pseudomonas aeruginosa* and *klebesilla pneumoniae.* The partial sequence of 16S rRNA genes of the remaining isolate MVS 11 showed 100% similarity with *Pantoea dispersa*.

In drop collapsing test a flat drop and in oil displacement method, a clear zone of was observed. From the above observation, it was confirmed that PBS -04, DLS-08, MVS-11 were potent biosurfactant producers. Both the techniques have several advantages such as small volume of samples was required, rapid and easy to carry out and also do not require specialized equipment (Kiran *et al.,* 2009). As PBS 10 showed higher emulsification index (25%) than DLS 08 (18 %) and MVS 11 (8%) it was considered to be the best organisms to produce biosurfactant from the rest of the isolates.

The presence of hydroxyl groups of protein was confirmed by Infrared spectrum of the purified biosurfactant, which showed broad stretching peaks at 3435 cm^{-1}. Absorption around 2922 cm^{-1} was assigned to the symmetric stretching of CH_2 and CH_3 groups of aliphatic chains respectively. Also, an intense absorption band at 1640 cm^{-1} indicates the presence of ester carbonyl groups in the biosurfactant. The ester C-O was also proved from the band at 1113 cm^{-1}. Similarly, another strong IR absorption found at 1415 $cm^{-1,}$ 1023 cm^{-1} were also observed (Joshi *et al.,* 2008). The FTIR spectra of the biosurfactant obtained indicated that the isolated biosurfactant was glycolipids in nature.

However, while considering the advantages of biosurfactant over chemically synthesized surfactants, such as lower toxicity, biodegradability and ecological acceptability the possibility of replacing the chemical surfactant in oil pollution with biosurfactant is sought and need further research with different kind of experiments.

CONCLUSION:

In this paper, biosurfactant producing strains of bacteria isolated from oil contaminated sites in and around Erode were studied for the emulsification properties. Using a previously cited methods of measurement, we found that the three strains of bacteria viz *Pseudomonas aeruginosa, klebesilla pneumonia* and *Pantoea dispersa* had comparable emulsification activity and superior emulsification stability compared to the other isolates. *Pseudomonas aeruginosa* produced the highest percentage of emulsification index and produced glycolipid biosurfactant which was authenticated by FT-IR spectrum.

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