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RESEARCH ARTICLE

Isolation and screening of biosurfactant producing microorganisms from oil contaminated soil

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Abstract

Isolation and screening of microorganisms from oil contaminated soil samples with biosurfactant producing ability was investigated. The biosurfactant producing ability of the microorganisms isolated from the soil samples was investigated by hemolytic assay, drop collapse test, emulsification index and methylene blue agar plate method. The best isolate was identified as *Pseudomonas aeruginosa* based on microscopic and biochemical analysis. The extracted biosurfactants was characterized with Fourier transform infrared spectroscopy (FTIR) spectra.

Keywords: Biosurfactant, hemolytic assay, drop collapse test, emulsification index, methylene blue.

Introduction

Oil pollution and remediation technology has become a global phenomenon of increasing importance. Most of the hydrocarbons are insoluble in water and their degradation using microorganisms have an important role in combating environmental pollution. Hydrocarbon degrading microorganisms produce biosurfactants of different chemical nature and molecular size which are surface active compounds which increases the surface tension of the hydrophobic water-insoluble substrates and thereby enhancing their bioavailability and the rate of bioremediation (Pekdemir *et al.,* 1999). Almost all surfactants currently produced are chemically derived from petroleum. These synthetic surfactants are usually toxic themselves and are hardly degraded by microorganisms. They are, therefore, a potential source of pollution and damage to the environment. These hazards associated with synthetic emulsifiers have, in recent years, drawn much attention to the microbial production of surfactants or biosurfactants (Urum and Pekdemir, 2004).

Biosurfactants derived from living organisms, mainly microorganisms have attracted much attention because of advantageous characteristics such as structural diversity, low toxicity, higher biodegradability, better environmental compatibility, higher substrate selectivity, and lower CMC. These properties have led to several biosurfactant applications in the food, cosmetic and pharmaceutical industries (Xiao-Xia *et al.,* 2003; Thanomsub *et al.,* 2004). The most commonly isolated biosurfactants are glycolipids and lipopeptides. They include rhamnolipids released by *Pseudomonas aeruginosa* (Nitschke *et al.,* 2005), sophorolipids from *Candida* sp. (Daverey *et al.,* 2008), as well as surfactin and iturin produced by *Bacillus subtilis* strains (Ahimou *et al.,* 2000).

Thus, based on their chemical composition, the biosurfactants produced by microorganisms are of many types such as glycolipids, lipopolysaccharides, oligosaccharides, and lipopeptides (Franzetti *et al*., 2010; Banat *et al*., 2010). Biosurfactants has received considerable attention in the field of environmental remediation processes such as bioremediation, soil washing, and soil flushing. Biosurfactants influence these processes because of their efficacy as dispersion and remediation agents and their environmentally friendly characteristics such as low toxicity and high biodegradability. Although biosurfactants exhibit such important advantages, they have not yet been employed extensively in industry because of relatively high production costs (Sivapathasekaran *et al*., 2010; Kiran *et al*., 2010; Satpute *et al*., 2010).

Against these backdrops, this study was aimed at isolating and screening biosurfactants producing microorganisms from oil contaminated soil samples and its characterization by FTIR.

Materials and methods

Reagents and chemicals: Peptone, yeast extract, beef extract, sodium chloride, agar, sodium nitrate, potassium chloride, ferrous sulphate, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, magnesium sulphate, cetyltrimethylammonium bromide, methylene blue, orcinol, sodium sulphate, sodium bicarbonate, hydrocholoric acid and ethyl acetate. All the reagents and media used in this study were purchased from Himedia Laboratories Pvt. Ltd, Mumbai, India, and Sisco Research Laboratories (SRL) Pvt. Ltd. Mumbai, India.

Collection of soil samples and enrichment of microorganisms: Oil contaminated soil samples from garage and petrol bunk were collected at 30 different locations in Chennai and were enriched by inoculating into sterile mineral salt medium (MSM). one gram of each soil sample was inoculated into 50 mL of minimal salt medium (Tahzibi *et al.,* 2004) containing (g/L); 15 g $NaNO_3$, 1.1 g KCl, 1.1 g NaCl, 0.00028 g $FeSO_4.7H_2O$, 3.4 g KH_2PO_4, 4.4 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g yeast extract at 37°C in shaker incubator (100 rpm). After 48 h of incubation, the samples were serially diluted using sterile saline (0.85% NaCl) and different bacterial isolates were selected based on the colony morphology on nutrient agar. The selected isolates were screened for the production of biosurfactants using following screening methods.

Screening for biosurfactant production

Hemolytic activity: Pure culture of bacterial isolates were streaked on the freshly prepared blood agar and incubated at 37°C for 48-72 h. Results were recorded based on the type of clear zone observed i.e. α-hemolysis when the colony was surrounded by greenish zone, β-hemolysis when the colony was surrounded by a clear white zone and γ-hemolysis when there was no change in the medium surrounding the colony (Carrillo *et al.,* 1996).

Drop collapsing test: Screening of biosurfactant production was performed using the qualitative drop-collapse test described by Bodour and Maier (1998). Crude oil was used in this test. Two microlitres of oil was applied to the well regions delimited on the covers of 96-well micro plates and these were left to equilibrate for 24 h. Five micro liters of the 48 h culture, before and after centrifugation at 12,000 g for 5 min to remove cells, was transferred to the oil-coated well regions and drop size was observed after 1 min with the aid of a magnifying glass. The result was considered positive for biosurfactant production when the drop was flat and those cultures that gave rounded drops were scored as negative, indicative of the lack of biosurfactant production (Youssef *et al.,* 2004).

Blue agar plate (Bap) method: Mineral salt agar medium supplemented with glucose as carbon source (2%) and cetyltrimethylammonium bromide (CTAB: 0.5 mg/mL) and methylene blue (MB: 0.2 mg/mL) were used for the detection of anionic biosurfactant (Satpute *et al*., 2008). Thirty microlitre of cell free supernatant was loaded into the each well prepared in methylene blue agar plate using cork borer (4 mm). The plate was then incubated at 37°C for 48-72 h. A dark blue halo zone around the culture was considered positive for anionic biosurfactant production.

Emulsification test (E_{24}): Several colonies of pure culture were suspended in test tubes containing 2 mL of mineral salt medium after 48 h of incubation, 2 mL hydrocarbon (oil) was added to each tube. Then, the mixture was vortexed at high speed for 1 min and allowed to stand for 24 h. The emulsion index (E_{24}) is the height of the emulsion layer (cm) divided by total height (cm), multiplied by 100 (Bodour *et al.,* 2004).

$$\text{Emulsification index } (E_{24}) = \frac{\text{Height of the emulsion layer}}{\text{Total height}} \times 100$$

Based on the screening test results, the positive isolates were inoculated into the mineral salt medium for the biosurfactant production. Based on the quantification of biosurfactant produced, the best strain was selected, and then identified by its microscopic appearance and biochemical tests based on Bergey's manual of determinative bacteriology.

Determination of biosurfactant: The biosurfactant from the sample was estimated using orcinol assay method (Tuelva *et al.,* 2002). The orcinol assay was used for the direct assessment of the amount of glycolipids in the sample. To 100 µL of each sample, 900 µL of a solution containing 0.19% orcinol (in 53% H_2SO_4) was added. After heating for 30 min at 80ºC, the samples were cooled at room temperature and the OD at 421 nm was measured. Control was prepared with distilled water. The rhamnolipid concentrations were calculated from a standard curve prepared with L-rhamnose and expressed as rhamnose equivalents (RE) (mg mL^{-1}).

Purification of biosurfactant: The biosurfactant was extracted from culture medium after cell removal by centrifugation at 12,500 rpm for 30 min. The pH of the supernatant was adjusted to 2.0 mL with 6 M HCl, and an equal volume of ethyl acetate was added in a separation funnel. The mixture was vigorously shaken for several minutes and allowed to set until phase separation. The organic phase was collected by repeating the above procedure 2 to 3 times and using anhydrous sodium sulphate, the water was removed and concentrated using a rotary evaporation. The resulting product was considered as the crude biosurfactant.

For further purification, the crude biosurfactant was dissolved in 0.05 M sodium bicarbonate. After filtration, the pH of this solution was adjusted to 2.0 mL using 6 M HCl, and then the solution was kept at 4 to 8°C for 24 h. The precipitate was finally collected by centrifugation at 12,500 rpm for 15 min, freeze-dried and analyzed by FTIR spectroscopy.

FTIR analysis: Infra Red absorption spectra were obtained with a Perkin– Elmer grating 1430 IR (Norwalk, CT) in a dry atmosphere. Absorption spectra were plotted using a built-in plotter. IR spectra were collected from 400–4000 wavenumbers (cm^{-1}) with resolution of 2 wave numbers per wave number. Samples were prepared by dispersing the solid uniformly in a matrix of potassium bromide.

Table 1. Screening results of ten isolates for the production biosurfactant.

Test isolate	Haemolytic assay	Methylene blue agar plate	Drop collapsing test	Emulsification index (%)	Rhamnolipid (mg/mL)
PB3A	+	+	+	65.5	0.82
PB4E	+	+	+	56.2	0.68
PB6B	+	+	+	50.5	0.56
PB13A	+	+	+	54.8	0.64
PB18D	+	+	+	49.8	0.44
PB23A	+	+	+	51.4	0.59
GG8C	+	+	+	42.5	0.34
GD11C	+	+	+	45.8	0.38
GG18A	+	+	+	52.5	0.59
GG21B	+	+	+	47.2	0.40

Results and discussion

Bacterial isolates about 243 were isolated from oil contaminated soil samples by plate and dilution techniques. They were further screened for biosurfactant activities by hemolytic test, blue agar test, drop collapsing and emulsification index, as reported by Satpute *et al*. (2008) that more than one screening methods should be included in the primary screening as to identify potential biosurfactant producers (Table 1). The results on blood agar media were similar to the work done by Mulligan *et al*. (1984) and Mulligan *et al*. (1989), who have isolated biosurfactant overproducer mutants with blood agar method. The flat drop appearance in micro titer plate confirmed the positive result for drop collapse test as suggested by Jain *et al*. (1991), proving the use of drop collapse method as a sensitive and easy method to test for biosurfactant production. Dark blue halo zone in the methylene blue agar plate supplemented with CTAB confirmed the presence of anionic biosurfactant. An alternative approach previously developed for the detection of extracellular rhamnolipids and other anionic glycolipids (Siegmund and Wagner, 1991) were employed in this study for the screening of rhamnolipid biosurfactants production by *P. aeruginosa* MM1011 and mutants. The assay was developed based on the property that the concentration of anionic surfactants in aqueous solutions can be determined by the formation of insoluble ion pairs with various cationic substances. The formation of insoluble ion pair precipitates in the agar plate containing methylene blue exhibited dark blue color against the light blue background. The diameter of the dark blue region previously has been shown to be semi-quantitatively proportional to the concentration of the rhamnolipid biosurfactants (Siegmund and Wagner, 1991). Only 10 isolates showed positive results for all the 4 screening methods viz., hemolytic test, blue agar test, drop collapsing and emulsification index. The 10 isolates were further tested for maximum biosurfactant production by inoculating into the MSM medium. Among the selected isolates, PB3A showed maximum biosurfactant producing ability (Table 1). The best isolate PB3A was identified as *P. aeruginosa* based on microscopic and biochemical analysis according to Bergey's manual of determinative bacteriology (Fig. 1 and Table 2).

Table 2. Identification and characterization of the test isolate PB3A.

Tests	Results
Gram stain	-
Motility	+
Oxidase	+
Catalase	-
Indole production	-
MR test	-
VP test	-
Citrate	+
H_2S	-
Gelatin liquefaction	+
Glucose fermentation	-
Mannitol fermentation	+
Maltose fermentation	-
Lactose fermentation	-
Xylose fermentation	+
Galactose fermentation	+
Sucrose fermentation	-
Urea hydrolysis	+
Nitrate reduction	-

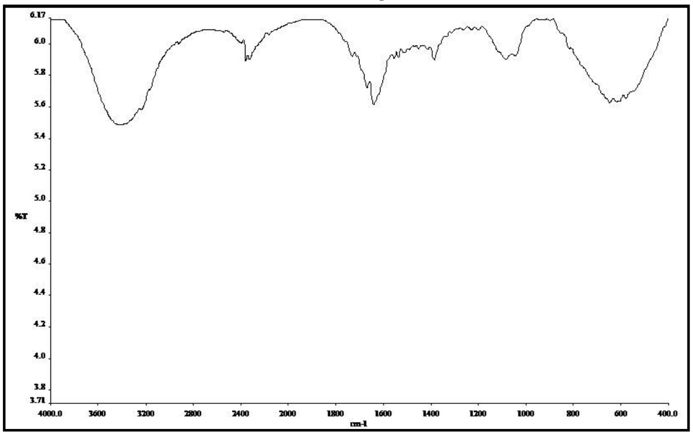
Fig. 1. Colony morphology of *Pseudomonas aeruginosa* PB3A.

The FTIR studies showed C-H stretching bands of $-CH_2$ and $-CH_3$ groups were observed in the region 3000–2700 cm^{-1} (Fig. 2).The deformation vibrations at 1467 and 1379 cm^{-1} also confirmed the presence of alkyl groups. Carbonyl stretching band was found at 1745 cm^{-1} which is the characteristic peak for ester compounds. The ester carbonyl group was also proved from the band at 1250 cm^{-1} which corresponds to C-O deformation vibrations. Scissoring vibration of a CH_2 group adjoining a carboxyl ester was also observed at 1351 cm^{-1}. The peak in the region of 1104 cm^{-1} indicates C–O–C stretching in the rhamnose. Similar results were reported by Thenmozhi *et al*. (2011) and Tuleva *et al*. (2002). Rhamnolipids produced by *Pseudomonas aeruginosa* were the most studied biosurfactants due to their potential applications in a wide variety of industries and the high levels of their production (Toribio *et al*., 2010). Rhamnolipids, in which one or two molecules of rhamnose are linked to one or two molecules of β-hydroxy-decanoic acid, are the best-studied glycolipids. Production of rhamnose-containing glycolipids was first described in *P. aeruginosa* (Jarvis and Johnson, 1949). The findings of this study were in accordance with Rahman *et al.,* (2007) who reported biosurfactant production by *P. aeruginosa* DS10-129 characterized by FTIR technique belonged to rhamnolipid type and Da Rosa *et al*. (2010) reported that the rhamnolipid type biosurfactant was produced by *P. aeruginosa* LBM10 which supported this study.

Conclusion

In conclusion, the study represented surfactant activity of the bacterial strains isolated from oil contaminated soils from the petrol bunk and garage areas. This confirms that environment has an influence on the metabolism of the tested microbes. This study suggests that, *Pseudomonas aeruginosa* isolated from oil contaminated soil showed biosurfactant producing ability. Further study on the utilization of agro industrial wastes as substrates for the large-scale production of biosurfactants is recommended.

Fig. 2. FTIR of partially purified biosurfactant produced by *Pseudomonas aeruginosa* PB3A.



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