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Full Length Research Paper

Improved Microbial oil revitalization using effective biosurfactant synthesized by *Pseudomonas* sp. from Arabian Sea, Mumbai

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Increase in oil pollution due to accidental leakages during various ship operations and human activities make these hydrocarbons the most common global environmental pollutants. Current understanding in degradation of these harmful oils involves the isolation of biosurfactants from various isolated microbial strains from contaminated sites of Marine Sea. Biosurfactants are the surface active molecules synthesized by microorganisms. Due to various side effects of chemical surfactants the demand for biosurfactant production and its synthesis has been steadily increasing and may eventually replace their chemically synthesized counterparts. In this study, isolation and identification of biosurfactant producing bacteria were assessed from oil-spilled area of Arabian Sea. To confirm the ability of isolates in biosurfactant production, various biosurfactant activity assay tests were performed. Marine biosurfactants produced by some marine microorganisms have been paid more attention, particularly for the bioremediation of the sea polluted by crude oil. Among all of the isolated strains *Pseudomonas* sp. showed the highest biosurfactant activity. The biosurfactant component was detected as glycolipid or other anionic surfactants in analysis of a phenotypic assay test using CTAB. The isolated culture filtrate was found to be highly effective in microbial enhanced oil recovery (MEOR) using sand pack method.

Key words: Biosurfactant, *Pseudomonas* sp., microbial enhanced oil recovery, oil spilled area.

INTRODUCTION

Petroleum products are the major source of energy for industry, transportation as well as day today life; it also poses major concern over hydrocarbon release during its production, transportation and accidental leakages. On 7th of August, 2010, 800 tonnes of oil being spilled into the Arabian Sea, due to the collision of Panamanian vessels MSC Chitra and MV Khalijia III had severe impact around 1,273 ha of mangroves. The oil spills from marine water are treated using bioremediation methods using biosurfactant as it is one of the promising technologies in future. Microbial compounds, which exhibit pronounced surface activity are classified as biosurfactants. Biosurfactants or surface-active compounds are a heterogenous group of surface active molecules produced by microorganisms, which either adhere to cell surface or are excreted extracellularly in the growth medium (Fietcher, 1992; Zajic and Stiffens, 1994; Makker and Cameotra, 1998). Biosurfactants have unique amphiphatic properties due to hydrophilic and hydrophobic portion in it. As a result, they can partition

preferentially at the interfaces (Desai and Banat, 1997) and are environmentally compatible (Georgiou et al., 1990). Biosurfactants have various advantages, such as high biodegradability, low toxicity, environmental compatibility, high selectivity, and specific activity at extreme temperatures, pH, and salinity (Desai and Banat, 1997; Lang and Wullbrandt, 1999). The use of biosurfactants to protect the marine environment seems possible since a number of marine bacterial strains can produce biosurfactants during growth on hydrocarbons (Bertrand et al., 1993). Microbial enhanced oil recovery (MEOR) is a good alternative in improving the recovery of crude oil from reservoir rocks by using microorganisms and their metabolic by-products. Recently many investigations on MEOR have used whole cells and their biosurfactants to improve the efficiency of oil recovery (Joshi et al., 2008; Toledo et al., 2008; Jinfeng et al., 2005; Rashedi et al., 2005; Mei et al., 2003). There are three mechanisms by which microorganisms can contribute to increased oil production: i) microorganisms can produce biosurfactants and biopolymers on the cell surface, ii) microorganisms produce gases and acids to recover trapped oil and iii) microorganisms can selectively plug high permeability channels into the reservoir (Bryant, 1987). The main objective of this paper is to isolate biosurfactant producing bacteria from oil spilled area of Marine Sea for use in MEOR.

MATERIALS AND METHODS

Isolation and enrichment of biosurfactant producing microorganisms

Soil samples were collected from different oil spilled areas of Marine sediment. Each sample was loaded into sterile 250 ml flasks. The sediments were collected through scuba diving to the depth of 1 to 100 m. The samples were collected in plastic bags and immediately transported to the Microbiology lab, IIS University, Jaipur, India, and stored in a refrigerator at 4°C up to further processing. 1 g of soil sample was taken and serially diluted in 0.85% sterile saline water. All dilutions were performed in triplicates and then the samples were spread on nutrient agar plates and incubated at room temperature for 1 to 2 days. After incubation, plates were enumerated and morphologically different bacteria were selected for biosurfactant screening (approximately 5 to 6 isolates per plate) and purified by re-streaking twice. Isolated colonies were inoculated into 100 ml of Marine Broth 2216 (Difco) containing 2 to 3 drops of petrol+kerosene+diesel (P+K+D) in 1:1:1 ratio, and incubated with continuous shaking (200 rpm) for 24 to 48 h at room temperature using a shaker. Colonies possessing biosurfactant-producing activity, as evidenced by emulsification of oil, were chosen for further experimentation. In addition, the cell suspensions of isolated strains were tested for presence of surfactant by using haemolytic activity, the qualitative drop collapsing test, quantitative oil displacement test and emulsification activity.

Screening for biosurfactant producers

Haemolytic activity

Biosurfactant producing capacity was found to be associated with haemolytic activity. Haemolytic activity therefore appears to be a good screening criterion for surfactant-producing strains. Isolated strains were screened on blood agar plates containing 5% (v/v) goat blood and incubated at room temperature for 24 h. Haemolytic activity was detected as the occurrence of a define clear zone around a colony (Carrillo et al., 1996).

Drop collapsing test

Two micro-liter of mineral oil was added to each well of a 96-well micro-liter plate lid. The lid was equilibrated for 1 h at room temperature, and then 5 µl of the cultural supernatant was added to the surface of oil. The shape of the drop on the oil surface was inspected after 1 min. Biosurfactant-producing cultures giving flat drops were scored as positive '+'. Those cultures that gave rounded drops were scored as negative '-', indicative of the lack of biosurfactant production (Youssef et al., 2004).

Oil activity assay test

The isolates present in the clearing zone on Haemolytic activity test were re-screened by hydrolyzing oil activity assay on oil agar plates (Morikawa et al., 1993). Potential biosurfactant-producing strains were inoculated into the Minimal salt medium (MSM) (Himedia), and the cultured filter was further analyzed by improved degreasing effect assay test and emulsification activity measurement. Improved degreasing effect assay was carried out following Bi et al. (2009) work. The emulsification activity was determined by adding 5 ml of kerosene and an equal volume of cell-free supernatant to a 20 ml tube. The sample was homogenized in a vortex at high speed for 2 min and allowed to settle for 24 h. The emulsification index was then calculated by given formula.

Emulsification Activity = Height of emulsion layer/Total height

Identification of potential strain

The potential strain that was showing good biosurfactant activity was identified using Bergey's manual of determinative bacteriology (Buchanan and Gibbons, 1974).

Production, isolation and identification of rhamnolipids

The identified strain was initially maintained on *Pseudomonas* isolation agar at 30°C for 24 h. Random single colonies were transferred into the MSM with the addition of 200 g/ml cetyltrimethylammonium bromide (CTAB, Sigma), 5 g/ml methylene blue, and 1.5% (w/v) agar, as described by Siegmund and Wagner (1991). A colony showing a dark blue halo was selected and grown in Kay's minimal medium (0.3% $NH_4H_2PO_4$, 0.2% K_2HPO_4, 0.2% glucose, 0.05 mg% $FeSO_4$, 0.1% $MgSO_4$) at 30°C for 5 days with shaking at 250 rpm. The culture filtrate was centrifuged at 8,000 × g for 10 min at 4°C to remove the cells and the debris. The pH of the supernatant obtained was adjusted to 2.0 using 12 M HCl, and then stored overnight at 4°C. The precipitates were collected by centrifugation at 8,000 × g for 20 min, and then extracted three times with a chloroform–methanol (2:1, v/v) mixture. The mixture was evaporated, leaving behind an oil-like appearance as the crude biosurfactant (Liu et al., 2011).

Microbial enhanced oil recovery (MEOR)

The MEOR process was carried out by the sand pack method described by Abu-Ruwaida et al. (1991). Hydrocarbon saturated

Table 1. Oil displacement activity, emulsification activity and drop collapsing test of cultural supernatant from different-strains.

Strain	Emulsification activity (%)	Oil displacement test (cm^3)	Drop collapsing test	Haemolytic activity	Degreasing effect assay test
B1	65.2 ± 0.26	2.25 ± 0.02	+	+	+
B2	40.0 ± 0.18	1.11 ± 0.02	+	+	+
B3	60.0 ± 0.30	2.30 ± 0.03	+	+	+
B4	50.0 ± 0.30	1.14 ± 0.02	+	+	+
B5	25.0 ± 0.13	1.05 ± 0.01	+	+	+
B6	50.0 ± 0.27	1.13 ± 0.03	+	+	+
B7	28.0 ± 0.15	1.09 ± 0.02	+	+	+

Table 2. Characterization of bacterial strains.

Bacterial isolates	Gram's stains	Cell shape	Spore	Motility	Penicillin sensitivity	Oxidase	Catalase	Urease	Gelatinase	Flurescent pigment
B1 (*Pseudomonas* Sp.)	-	Rods	-	+	-	+	+	-	+	+

sand pack column was treated with the culture filtrate and cell-free supernatant at 30°C. Distilled water served as the control. The oil displacement rate is calculated by given formula.

Oil displacement (%) = $(M_2/M_1) \times 100$

M_1: Oil content in the sand (g)
M_2: Wash out oil content (g).

The assay was repeated three times with three replications for each treatment.

RESULTS

This study revealed that B1 strains detected as *Pseudomonas* sp. out of 15 isolated strains had shown the higher biosurfactant activity. Among the 15 isolates, 7 formed a hydrolyzing oil spot on the oil agar plate, and were considered potential biosurfactant-producing isolates. Moreover, as determined by improved degreasing effect assay, B1 strain showed the highest emulsification activity (65.2 ± 0.26) and oil displacement test (2.25 ± 0.02) as shown in Table 1. This isolate was characterized as Gram negative having a slender rod with rounded ends. The morphology of the colonies, as well as the physiological and biochemical characteristics of the strain, is shown in Table 2. It was therefore found as *Pseudomonas* sp*.* Bacterium can be screened for rhamnolipid production using CTAB–methylene blue indicator plates. The results of the current work showed a dark blue halo after 48 h of incubation at 30°C, whereas the other strains did not show a positive reaction, demonstrating that the isolate can produce biosurfactant rhamnolipids. The oil displacement rates caused by the fermentation broth and culture filtrate (except for the cell) at 30°C were 68% and 60.2% respectively, whereas those caused by bacterial cell suspensions and distilled water were 11 and 10%, respectively. The results showed that both the fermentation broth and culture filtrate (except for the cell) produced by *Pseudomonas* sp*.* was highly effective in recovering crude oil from the sand pack column.

DISCUSSION

Present study revealed that biosurfactant produced by various microbes can reduce pollution of Sea that occurred due to various human activities. Chemical treatment has several disadvantages. Thus biological treatment may be preferred due to their eco-friendly nature, low toxicity, biodegradable and biocompatible and selective (Desai and Banat, 1997). In present study the initial isolation of suspected biosurfactant producers was done on blood agar plates, utilizing the ability of many biosurfactants to lyse erythrocytes, which results in a band of beta hemolysis surrounding biosurfactant-producing bacterial colonies (Bernheimer and Avigad, 1970; Banat, 1995a, b; Lin, 1996). Single screening method is unsuitable for identifying all types of biosurfactants, and recommended that more than one screening method should be included during primary screening to identify potential biosurfactant producers (Kiran et al., 2010). Therefore, drop collapsing test, oil activity assay test and emulsification activity measurement were used to screen the biosurfactant producer. Strain *Pseudomonas* sp*.* showed positive results in all the screening methods used. Thus, we confirm that this bacterium can produce biosurfactants with positive responses. *Pseudomonas* spp*.* are known to produce different types of rhamnolipids. For example, production of polymeric biosurfactant by *Pseudomonas nautica* was achieved (Husain et al., 1997). The major

constituents were proteins, carbohydrates and lipid at the ratio of 36:63:2, respectivey. A simple method using CTAB-methylene blue indicator plates can be used to screen rhamnolipids produced by a wide range of *Pseudomonas* species, as well as other types of bacteria. We conclude that *Pseudomonas* sp. B1 may produce rhamnolipids, and first assayed the strain by this method. The main advantages of microbiological method of bioremediation of hydrocarbon polluted sites are use of biosurfactant producing bacteria without necessarily characterization of the chemical structure of the surface active compounds. The cell free culture broth containing the biosurfactants can be applied directly or by diluting it appropriately to the contaminated site. The other benefit of this approach is that the biosurfactants are very stable and effective in the culture medium that was used for their synthesis (Płociniczak et al., 2011).

Conclusion

Biosurfactants have been proven as one of the promising agent for controlling the oil pollution by hydrocarbons, particularly oil polluted in marine environment. Synthetic detergents used to clean up these spillages have often led to more destruction of the environment. From the environmental view point it is important that all substances released into the environment are degradable, firstly to assess their potential for causing environmental damage and secondly to safeguard against the possibility of future harm due to build up in the environment. Therefore, potent biosurfactant producing microorganisms should be intensively isolated and screened for bioremediation without causing adverse effects to the environment.

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