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Pseudomonas aeruginosa WJ-1, A Potent Biosurfactant Producer: Exploring Enhanced Oil Recovery Under Laboratory Conditions

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ABSTRACT

Due to energy scarcity the search for new and renewable energy sources has gained a lot of momentum. Chemical surfactants have been used in the oil industry for enhanced oil recovery from oil reservoirs. Though these compounds seem useful but are not biodegradable and eco-friendly. Potential use of bacteria in enhanced oil recovery therefore becomes the useful tertiary treatment to recover oil from a reservoir which cannot be otherwise removed by primary and secondary treatments.

In this study, attempts are being made in order to improve the efficiency of recovering crude oil using enhanced oil recovery (EOR) techniques by biosurfactant. Various oil contaminated soil and marine samples were screened to isolate potent biosurfactant producing bacteria and then characterized. Out of the two bacteria, isolate producing high level of biosurfactant *P. aeruginosa* WJ-1 was found to be the most effective. Partially purified preparation of biosurfactant derived from *P. aeruginosa* WJ-1 lowered the surface tension of the Mineral Salt Medium (MSM) from 63 to 29 dyne/cm after 48 hrs. Reduction in interfacial tension of the MSM and oil was noted to be 21 dyne/cm to 3dynes/cm with 100% EI-24 for diesel, petrol and sunflower oil. Further, usefulness of biosurfactant in the recovery of oil from sand was tested. It was found that more than 67% of low viscosity oil was recovered from an oil saturated pumice stone as compared to 10% removed from the control. At the ambient temperature 26% of crude oil was recovered using cell free broth. Moreover, the percentage recovery obtained by partially purified biosurfactant was 32.86% in contrast with 3.4% removal in control from a sand column saturated with oil indicating potential of biosurfactant in oil recovery .

Keywords: EOR, Biosurfacatnt, Surface Tension, Interfacial tension, Crude oil

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INTRODUCTION

Microbial Enhanced Oil Recovery (MEOR) is the use of microorganisms to retrieve additional oil from existing wells, thereby enhancing the petroleum production of an oil reservoir. In this technique, selected natural microorganisms are introduced into oil wells to produce harmless by-products, such as slippery natural substances or gases, all of which help to propel oil out of the well. Because these processes help to mobilize the oil and facilitate oil flow, they allow a greater amount of oil to be recovered from the well [1].

Surfactants are amphiphilic substances containing both hydrophilic and hydrophobic moiety that concentrate at the interface between water and a polar fluid to form interfacial films. They reduce the surface tension of liquids, critical micelle concentration and also the interfacial tension between two liquids or a liquid and a solid and therefore are able to show a variety of surface activity [1]. These properties make them highly useful in various industries like petroleum, textiles, detergents and soaps, medicine, agriculture, food and solubilization of hydrophobic compounds. The petroleum industry has traditionally been the major user of surfactants for recovery of oils as chemically synthesized surfactants used in oil industry are not biodegradable and toxic to the environment and have issues related to safe disposal. The major application of biosurfactant is therefore the safe, efficient and enhanced recovery of oils that are difficult to extract.

Numerous microorganisms have been identified from soil, fresh and marine water as biosurfactant producers. These microbial biosurfactant could be promising due to the striking advantages over chemically synthesized surface-active compounds, viz. non-toxicity or reduced toxicity, biodegradability and broad range of characteristics for different applications. The use of biosurfactant therefore may be a better alternative to overcome the toxicity of synthetic compounds. Biosurfactant can be manufactured on a large scale at low operating costs and they possess the unique attributes such as high temperature, pH and salt stability [2, 3]

The properties of the various biosurfactants have been extensively reviewed [4]. Most microbial surfactants are complex molecules comprising a wide variety of chemical

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structures such as glycolipids, lipopeptides, fatty acids, polysaccharide-protein complexes, peptides, phospholipids and neutral lipids. Rhamnolipids produced by *Pseudomonas aeruginosa* have been widely studied [5, 6, 7]. Since microorganisms have diverse synthetic capabilities, they can produce a range of biosurfactants and novel compounds, providing new possibilities for industrial applications [8]. Hence, the present work is directed toward the isolation of microorganisms that have high rates of production of biosurfactant and demonstrate the feasibility of using these strains for cost-effective and large-scale recovery of oils at laboratory level.

OBJECTIVES

The primary aim of the research work was isolation and screening of biosurfactant producing bacteria from hydrocarbon contaminated soil and marine water samples. The next goal was to indentify and characterize potent biosurfactant producing organisms. The main objective was studies on the potential of partially purified biosurfactant in enhanced oil recovery operations.

MATERIAL AND METHODS

(i) Sampling

Crude oil contaminated marine water samples were collected from Sasoon Dock and Jawaharlal Nehru Port Trust (JNPT), Mumbai. Soil samples were collected from Petrolium distribution center and various petroleum contaminated sites in the vicinity of local petrol pump and motor garage areas from Nashik District, Maharashtra, India. Soil Samples were collected from a level of about 4-5 cm below the soil surfaces and marine water contaminated with oil was collected in sterile containers. Moisture level in soil samples was maintained with help of 0.85% saline and all samples were stored at 4^0c for subsequent use.

Isolation of bacterial isolates

For isolation of bacteria, the samples (1 g soil or 1 ml water) were inoculated in 150 ml Mineral Salts Medium in 500ml Erlenmeyer flasks containing 5% of either diesel, petrol or dodecane. The flasks were incubated at room temperature (25°C) on an orbital shaker at 120 rpm. At predetermined intervals of 7, 14, 21 and 28 days, 0.1 ml of the flask contents were withdrawn aseptically and plated on nutrient agar and mineral salts (MS) agar. The plates were incubated at 37^0C and observed after 24 hrs for isolated colonies that appeared on the medium. Subsequently colony characters of each isolates were recorded and then maintained on nutrient agar and MS agar slants at 4°C.

Growth media

Mineral salts medium supplemented with 5% (v/v) diesel was used for screening of potent hydrocarbon degrading and biosurfactant producing bacterial isolates. The MSM used for enrichment contained : glucose- 10 gm; $(NH_4)_2SO_4$- 1 gm; Na_2HPO_4-, 4 gm; yeast extract- 5 gm; KH_2PO_4- 3 gm; NaCl- 2.7 gm; $MgSo_4$- 0.6 gm and 5 ml/L trace element solution containing $FeSo_4$,$7H_2O$- 5mg; $ZnSO_4$ $7H_2O$ - 3.34 mg; $MnSO_4$ $7H_2O$-1.56 mg; $CoCl_2$, $2H_2O$ -2 mg in 1L distilled water. pH was maintained at 6.8 throughout enrichment period [9, 10].

(ii) Screening of potent biosurfactant producing bacteria:

Each individual bacterial isolate was screened by both qualitative and quantitative methods in order to select the cultures that could produce high levels of biosurfactant. All samples were tested in triplicates.

Qualitative tests

(a) Hemolytic activity was carried out as described by Carrillo *et. al* ,1996 [11]. Briefly, the bacterial isolates were plated on blood agar plate containing 5% blood and incubated at 37^0C for 24-48 hrs. Hemolytic activity (zone of clearance around the bacterial colonies) was indicative of RBC lysis due to biosurfactant production by the bacteria. [1, 3, 12].

(b) The drop collapse technique (DCM) was carried out in a 96-well microtiter plate as described by Jain *et. al.,* 1991 [13] and Bodour and Miller Mair, 1998 [3]. For this, 100 µl culture supernatant was added to the wells of a 96-well microtiter plate and 5 µl of crude oil was added on top of the culture supernatant. Biosurfactant producing cultures were identified on the basis of a flat oil drop compared to the rounded drop in case on non-biosurfactant producing cultures.

(c) The oil spread method (OSM) was carried out according to Morikova *et. al.*, 2000 [14] and Youssef *et. al.*, 2004 [15]. For this, 50 ml distilled water was added to a petridish (25 cm diameter) followed by addition of 100 µl of crude oil on the surface of water. After this, 10 µl of culture filtrate was put on the crude oil surface. A clear zone on oil surface indicated biosurfactant activity of the culture and diameter of the clear zone was proportional to the production of biosurfactant by the bacteria.

Quantitative tests

(a) The emulsification index (EI-24) was determined using a method described by Bosch *et. al.*, 1988 [16] and Willumsen and Karlson, 1997 [17]. Briefly, 7 ml of culture

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filtrate was overlaid by 3 ml of diesel, petrol, kerosene and toluene and vortexed for 1 min to obtain an emulsion. The stability of the emulsion was determined after 24 hrs in terms of the Emulsification Index (EI-24) which was calculated by dividing height of emulsion layer by the height of hydrocarbon phase and multiplying the result by 100. An emulsion was defined as stable if the EI-24 was 60% or more [18].

(b) Tensiometer based on du Nouy ring method was used for measuring the ability of bacteria to reduce the surface tension (SFT) of liquids. For this, bacterial isolates were grown in MS medium for a period of up to 96 hrs. Samples were withdrawn from the culture medium after every 24 hr and cells separated by centrifugation at 10,000 rpm for 15 min. Cell-free supernatants were placed in a clean 50 ml glass beaker and surface tension was measured using tensiometer having a platinum ring [3, 8].

(iii) Identification of bacterial isolates: The isolates were identified by routine bacteriological and biochemical tests followed by the API 32 and 16SrRNA techniques.

(iv) Quantification of biosurfactant yield : The yield of crude biosurfactant (glycolipid) was determined by orcinol assay which was used for determination of rhamnose concentration. For this, of culture supernatant (333 µl) was extracted twice with 1 ml diethyl ether. The ether fraction was evaporated to dryness and 0.5 ml of distilled water was added. After heating for 30 min at 80^0C, the sample was cooled at room temperature and $O.D._{425}$ was measured using a spectrophotometer [19]. A standard curve prepared with L-rhamnose was used for determination of rhamnolipid concentration (as rhamnose equivalent, $mg.ml^{-1}$) from the samples. Yield of biosurfactant was also determined by gravimetric analysis using the method described by Yankinov *et. al*. For this, bacterial cells were removed from culture medium by centrifugation (10,000 x g for 15 minutes). To the supernatant, a solution of 6N HCl was added to obtain a final pH value of 2.0 and the mixture was allowed to stand at 4^oC overnight for precipitation of the biosurfactant. The precipitate was subsequently recovered by centrifugation at 10000 rpm for 15 min. The resulting pellet was then dissolved in 0.1M sodium bicarbonate to achieve final pH of 7.0. The biosurfactant was further extracted with ethyl acetate and dried on rotary evaporator in vacuum. Weight of crude biosurfactant was determined in the laboratory using a

precision balance [20,21].

(v) Assesement of biosurfactant activity

(a)Determination of Interfacial tension (IFT):

The interfacial tension between two liquids, viz. oil and culture supernatant was determined by the du Nouy ring method. For this, the heavy phase was first poured into the sample vessel and then the platinum ring was positioned under the surface of heavy phase to wet it completely, and while in this position, the light phase was carefully pipetted onto the surface of heavy phase. The interface was allowed to reach equilibrium and the ring was then slowly pulled out through the light phase. The external force required to pull out the ring was expressed as dyne/cm.

(b) Determination of Critical micelle concentration (CMC):

The activity of partially purified biosurfactant was measured by determining its critical micelle concentration (CMC).CMC of biosurfactant was determined by measuring the surface tension of water supplemented separately with different concentrations of (1 to 10 mg/lit) bacterial surfactant. The CMC was defined as the concentration of biosurfactant in water beyond which there was no further reduction in surface tension. The SFT of non inoculated (control) culture medium was also determined using tensiometer at room temperature (25^0C).

(vi) Cleansing action of biosurfactant and oil recovery

The cleansing of the pumice stones and recovery of oil from oil saturated pumice stones (with a high porosity) was attempted. For this, the pumice stones (2 cm^3) were first washed with acid and then with distilled water to remove surface dust and other impurities. The stones were then dried and weighed using a precision balance. After this, the stones were soaked in oil for about 60 minutes so as to saturate them with the oil. Then, the stones were drained completely, washed with water to remove excess oil, dried and weighed. The stones were then treated with culture supernatant of biosurfactant producing culture (50 ml) for 24 hrs, dried and weighed again. Treatment with sterile MSM medium served as the control. The difference in weight of stones before and after treatment was used to calculate the efficiency of oil recovery from the stones.

(vii) Recovery of oil from sand packed column:

The usefulness of biosurfactant in EOR was evaluated using a sand packed column as per method described by Bordoloi and Konwar

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(2008). A glass column (60 cm x 4 cm) was packed with acid washed sand (250 g). The column was then saturated with (150 ml) crude oil. Culture supernatant (100 ml) of biosurfactant producing organism and partially purified biosurfactant was then applied to the columns separately and allowed to interact with the sand for 24 hr. After this, the eluant was drained out from the bottom of the column and the volume of released oil was measured [1].

RESULTS AND DISCUSSION

(i) Bacterial isolates and screening of potent biosurfactant producers:

A total of 14 bacterial cultures were isolated in pure from hydrocarbon contaminated soil and marine water samples. Using both qualitative and quantitative methods of screening, the isolates that showed maximum potential for biosurfactant production was selected for further studies. Three isolates were identified as potential biosurfactant producers and these were identified by routine bacteriological and biochemical tests. Further, using the API 32 and 16SrRNA techniques, the isolates were identified as *Stenotrophomonas koreensis*, *Pseudomonas aeruginosa*WJ-1 and *Bacillus sp*. It was observed that the bacterial isolates did not only degrade different components of crude oil such as diesel, petrol and dodecane but also produced biosurfactant in hydrocarbon containing medium. **Table 1** gives the results of the screening tests employed for the various bacterial isolates, which shows that *P. aeruginosa* WJ-1 had maximum biosurfactant activity (>50% surface tension reduction). It was observed that *Pseudomonas aeruginosa* WJ-1 was found to be sensitive to most of the antibiotics tested as compared to other two organisms. It could be inferred that the organism does not pose a problem of drug resistance and was selected for further studies. Research data pertaining to experiments on the usefulness of *P. aeruginosa* WJ-1 in MEOR are presented in this paper.

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Table 1. Screening of bacterial isolates for biosurfactant activity

Test	Control	*P. aeruginosa WJ-1*	*S. koreensis*	*Bacillus sp.*	1	2	3	4	5	6	7	8	9	10	11
Blood Hemolysis	-	+++	++	++	+	-	++	-	+	+	-	-	++	+	+
Drop collapse test	-	+++	++	++	-	+	+	-	++	+	+	-	+	-	+
Oil spreading test	-	+++	+	++	-	+	-	-	+	+	+	-	-	-	+
EI-24 (%)	4	100	90	80	4	20	40	8	60	50	25	6	30	40	10
SFT(dyne/cm, 48 h)	63	29.0	31.2	34.2	61	58	60	70	44	38	52	48	42	68	56

Control, sterile growth medium (MSM), EI- Emulsification Index, SFT- Surface Tension +, biosurfactant activity detected (number of + signs indicate degree of activity) -, biosurfactant activity not detected

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(ii) Quantification of biosurfactant yield:

Numerous reports have suggested to involvement of glycolipids in the formation of biosurfactant molecules. Hence, the yield of biosurfactant can be determined in terms of the glycolipid content (especially rhamnolipid). In the present investigations, biosurfactant material was measured spectrophotometrically with L-rhamnose as the standard and it was found that in terms of rhamnolipids, the yield was to the tune of 1.53 mg/ml. Extraction of the biosurfactant material from the broth using ethyl acetate followed by drying yielded biosurfactant to the tune of 4.2 gm/liter. This value is in tune with the yields obtained by other researchers [1].

Efficiency of biosurfactant

Production of biosurfactant by *P. aeruginosa* WJ-1 was assessed for 96 hours with the interval of

24 hours by determining the SFT of MS media supplemented with various oil sources (petrol, diesel and hexadecane) and compared it with control devoid of biosurfactant. SFT of culture medium supplemented with showed maximum reduction to 29 dyne/cm after 48 hours as compared to the control (63 dynes/cm). These results were found to be similar to the findings reported by Bordoloi and Konwar (2008) where *P. aeruginosa* (MTCC7815) lowered SFT up to 29.7 dyne/cm and 31.9

dyne/cm of culture media supplemented with glycerol and glucose respectively.

Properties of biosurfactant

Determination of Interfacial tension: The crude preparation of biosurfactant derived from *P. aeruginosa* WJ-1 could effectively lower the interfacial tension of the mixture of growth media and oil from 21 dyne/cm to 3 dyne/cm.

Determination of CMC value: The CMC value has practical utility since it indicates the concentration of a purified biosurfactant preparation that must be taken for any specified purpose. A lower value of CMC denotes higher effectiveness of the preparation. It was found that the CMC for the partially purified biosurfactant was 6mg/L. Monterio *et.al.,* (2007) reported 13.9mg/L for non purified biosurfactant from *P.aeruginosa* DAUPE, while Wei et.al., (2008) reported CMC value of 5.65mg/L in case of *P. aeruginosa* J16 [21,22]. The biosurfactant reported in the present work is much superior to those mentioned in the above literature [1] .

Cleansing action of biosurfactant: Treatment of low viscosity crude oil saturated porous stone with biosurfactant from *P. aeruginosa* WJ-1 resulted over 67% recovery of oil from the stone. On the other hand, treatment with sterile MSM medium could remove only 10% of the oil. This suggests that the biosurfactant could be potentially useful in recovery of oil.

Recovery of oil from sand packed column:

Treatment of crude oil saturated sand column with biosurfactant containing cell-free supernatant of *P. aeruginosa* WJ-1 resulted in the release of crude oil through the eluant. This was due to the ability of the biosurfactant to reduce surface tension of oil making it more mobile in the column. It was found that the treatment of biosurfactant containing cell free extract allowed the recovery of about 26% crude oil from the saturated sand pack column at ambient conditions. On the other hand, only about 4.3% recovery of oil was possible in the control treated with un inoculated MS medium. At the same time treatment of partially purified biosurfactant resulted in higher recovery of oil to the tune of 32.86% this was sharply in contrast with 3.4% removal in case of control. When Bordoloi and Konwar injected biosurfactant produced by *P. aeruginosa* could release 30-45% more oil from saturated sand packed column as compared to the treatment with the non inoculated culture medium (control). Banat injected biosurfactant produced by *B.subtilis* to crude oil saturated sand packed

columns and reported that 35% release of residual oil, as compared to 21% using nutrient solution[23]. It must be mentioned here that the crude oil sample used in this experiment was highly viscous and almost had a solid-like consistency. Hence, the recovery level of 32.86% was considered encouraging, since the recovery levels for low viscosity oils could be much higher. Similar experiments need to be carried out in larger scale and especially in the *in-situ* conditions in order to establish the utility of this bio-based method for commercial applications.

CONCLUSION:

The present investigations highlight the fact that organisms isolated from a local environment have the potential of crude oil recovery in a safe and efficient manner.

Biosurfactant effectiveness in successful reduction of viscosity of crude oil by decreased SFT and IFT values was verified.

Being biodegradable and eco-friendly , biosurfactant produced in this study promotes its efficacy in EOR. Application of biosurfactant also demonstrated in abatement of heavy metals from the environment[24]. Preliminary findings of the present investigation are quite promising. Further work on the use of purified biosurfactant preparation and scale up of the oil recovery process needs to be carried out in order to bring the potential into fruitful applications

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