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RESEARCH ARTICLE

PRODUCTION OF BIOSURFACTANT FROM POTENT STRAIN ISOLATED FROM OIL SPILL SOIL IN DEOLI REGION, WARDHA DISTRICT

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ABSTRACT

Biosurfactant is a structurally diverse group of surface-active molecule, Synthesized by microorganisms. In present study isolation and screening of microorganisms from oil spill soil with biosurfactant producing ability was investigated by drop collapse test, oil spreading test, measurement of surface tension, hemolytic assay, emulsification index, emulfication activity and methylene blue agar plate method. Five soil samples from oil contaminated soil of various automobile workshops, petrol pumps and saw mills were collected. Total 25 bacterial strains were isolated, out of which 8 potential bacterial strains with ability to produce biosurfactant were selected for further study. On the basis of cultural, morphological and biochemical characteristics these 8 bacterial strains were tentatively identified as belongs to *Pseudomonas, Citrobacter* and *Klebsiella species(spp). Pseudomona spp.* from petrol pump near Deoli region of Wardha district was found to be the potent biosurfactant producer. It is evident from the results that biosurfactants can be used in place of synthetic surfactants due to their efficiency and ecofriendly nature.

Key words: Biosurfactant, surface tension, emulsification index.

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INTRODUCTION

Chronic release of oil in the soil and water from numerous natural and anthropogenic sources poses a continuous-serious threat for the environment. Oil spills have become a global problem particularly in industrialized and developing countries. 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 (http://www.itof.com/stats.html). 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). The percentage of oil remaining in a reservoir varies from field to field. However, in a recent study, it was found that 65% of the original oil in place (OOIP) was left behind after primary and secondary-oil recovery processes (Pillai, 1999). In general residual oil can be recovered by enhanced oil recovery (EOR) technologies which are of three types i.e. thermal, miscible and chemical enhanced oil recoveries (CEOR). Among these, CEOR is one of the most common methods of EOR. This method involves the recovery of residual oil by using different types of chemical formulations which are capable enough in reducing mobility ratio and/or increasing capillary number when injected in the reservoir as displacing fluid.

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Use of chemical surfactant for this purpose is one of the common methods(Alvarado, 2010). Chemically-synthesized surfactants have been used in the oil industry to aid cleanup of oil spills, as well as to enhance oil recovery from oil reservoirs. But, chemical surfactants are hazardous, toxic, non-biodegradable and hence having many disadvantages in environmental as well as application point of view (Tabatabaee, 2005). From literature it was found that biosurfactants produced from various microorganisms have potential for many applications in industries. Biosurfactant are group of secondary metabolites, amphiphilic compounds produced on living surface mostly by microbial cells and contain hydrophobic and hydrophilic moieties that decrease surface tension between individual molecules at the surface and interface respectively (Priya, 2009).Surfactants are key ingredients used in detergents, shampoos, toothpaste and a number of other industrial products. The biosurfactants are complex molecules covering a wide range of chemical types including peptides, fatty acids, phospholipids, glycolipids, antibiotics, lipopeptides etc. (Anandaraj, 2010). Biosurfactants have several advantages over the chemical surfactants, such as lower toxicity, higher biodegradability (Zajic, 1997), better environmental compatibility (Georgiou, 1992), higher foaming (Razafindralambo, 1996), higher selectivity and specific activity at extreme temperatures, pH and salinity (Velikonja, 1993) and the ability to be synthesized from renewable feedstock (Desai, 1997). The biosurfactant could be used as an emulsifier to form emulsion between water and hydrocarbon such as palm oil, benzene, toluene with various stability. For this purpose, an effort has been made to replace chemical surfactant with the eco-friendly biosurfactant which is

produced from an isolated bacterial strains from oil spill soil of Wardha region.

MATERIALS AND METHODS

Sampling Sites and Sample Collection: The sample collection was aimed for isolation of biosurfactant producing organism. For this purpose five oil contaminated soil samples from various automobile workshops, Petrol Pumps and Saw mills were collected during November 2018 in sterile zip lock bag from Deoli region of Wardha district- Maharashtra and transported to the laboratory and were stored at 4^0C till analysis.

Enrichment and Screening of Biosurfactant Producing Bacteria: 1 g soil was inoculated in 250 ml Erlenmeyer flask containing 100 ml Minimal Salt Medium having composition (g/l):$NaNO_3$ 2.5g; KCl 0.1g; $KH2PO_4$ 3.0g; $K2HPO_4$ 7.0g; $CaCl_2$ 0.01g; $MgSO_4.7H_2O$ 0.5g and 5ml of trace element solution contains: $FeSO_4.7H_2O$ 0.116g/l; $H3BO_3$ 0.232g/l; $CoCl_2.6H_2O$ 0.41g/l; $CuSO_4.5H_2O$ 0.008g/l; $MnSO_4.H_2O$ 0.008g/l; $[NH_4]6Mo7O_{24}$ 0.022g/l; $ZnSO_4$ 0.174g/l with 2% kerosene oil as a sole source of carbon and were incubated at 30^0C at 200rpm on rotary shaker for 7days and same procedure was successively repeated for 3 time for enrichment of bacterial culture (Namir, 2008).

Isolation, biochemical characterization and Identification of bacteria: After enrichment the samples were inoculated on solid nutrient agar plate and well grown, isolated and morphologically distinct microbial colonies were selected and transferred on agar slants as a stock culture at 4^0C for further study. All these isolates were further identified on the basis of cultural, morphological and biochemical characterization according to Bergey's manual of systematic bacteriology (9th edition).

Preliminary screening for biosurfactant production

Surface tension measurement: Reduction in surface tension of cell-free culture was measured according to the Drop counting method using stalagmometer. For this purpose, five mL of inoculums of the bacterial culture were added to 250 mL Erlenmeyer flask containing 100 mL Nutrient broth with 2% kerosene oil as a carbon source. The experimental flasks were incubated at 30^0C on rotary shaker at 200 rpm. After 5 days of incubation, broth was centrifuged at 8000 rpm for 20 min for cell removal and cell free supernatant was collected in sterile flask. The reduction in surface tension of cell free broth was determined by using stalagmometer by drop counting method. The stalagmometer was calibrated before each measurement using distilled water (Morikawa, 1993).

Oil displacement assay: The oil spreading assay was developed by Morikawa. For this assay, 20 µl of kerosene oil was added to the surface of 50 ml of distilled water in a petri dish to form a thin oil layer. Then10 µl of culture supernatant was gently placed on the center of the oil layer. If biosurfactant is present in the supernatant, the oil is displaced and a clearing zone is formed. The diameter of this clearing zone on the oil surface correlates to surfactant activity, also called oil displacement activity (Rahman, 2002).

Blue agar plate method: Minimal salt agar medium supplemented with glucose as carbon source (2%) and cetyl trimethyl ammonium bromide (CTAB: 0.5 mg/mL) and methylene blue (MB: 0.2 mg/mL) were used for the detection of anionic biosurfactant. 30 µl of cell free supernatant was loaded into the each well prepared in methylene blue agar plate using cork borer (4 mm) and these plates were incubated at 30^0C for 3 days. If anionic surfactants are secreted by the microbes growing on the plate, they form a dark blue, insoluble ion pair with cetyl trimethyl ammonium bromide and methylene blue. Thus, productive colonies are surrounded by dark blue halos. A dark blue halo zone around the culture was considered positive for anionic biosurfactant production (Satpute, 2008).

Blood agar hemolysis: Blood agar hemolysis method is used to screen biosurfactant producing strain. This method is based on the fact that biosufactants are able to haemolyse the red blood cell present in blood. Pure culture of bacterial isolates were streaked on the freshly prepared blood agar and incubated at 30°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. This zone of hemolysis indicates production of biosurfactant (Krepsky, 2004).

Foaming activity: Isolated strains were grown separately in 250 mL Erlenmeyer flasks, each containing 100 mL of nutrient broth medium. The flasks were incubated at 30°C on a shaker incubator (200 rpm) for 72 hrs. Foam activity was detected as duration of foam stability, foam height and foam shape in the graduated cylinder.

Emulsification assay: Emulsification activity of culture supernatant was calculated by emulsification index known as E24. Emulsification assay was carried by adding 2 ml kerosene oil in 1ml cell free supernatant which was obtained after the centrifugation, and then it was vortexed for 5 minutes confirming regular mixing of both the liquids. The emulsification activity was observed after 24 hrs(Cooper, 1984).

It was calculated by using the formula:

E24 = Total height of the emulsion layer/height of the aqueous layer * 100

Confirmatory method

Phenol:Sulfuric acid method: Biosurfactant producing strains selected from above screening methods were inoculated in Minimal salt medium broth & incubated at 30^0C on rotary shaker for 4-5 days. After incubation, broth was centrifuged at 10,000 rpm for 15 min and supernatant was collected while pellet was discarded. 1ml collected supernatant was mixed with 1ml of 5% phenol then 5ml of concentrated H_2SO_4 was added in drop wise manner. Presence of biosurfactant in supernatant produces orange color from yellow color.

Production of biosurfactant: Biosurfactant was produced by using minimal salt medium as formulated by Makkar and Cameotra (Makkar, 1998). Biosurfactant production was carried out in 250mL conical flasks containing 50 mL of a minimal salt medium containing 2% kerosene oil as the sole source of carbon.

The temperature of the medium was maintained at 30°C with shaking at 150 rpm. Culture medium samples were drawn for estimation of biosurfactant production once every 24 hours for five days. Biosurfactant concentration in the culture broth was estimated after its crude extraction and concentration. A conical flask without the kerosene oil was maintained as control. All the experiments were performed in triplicate.

Table 1. Morphological and biochemical characteristics of isolates.

	NS1	NS2	NS3	NS4	NS5	NS6	NS7	NS8
Gram reaction	Gram Positive	Gram Negative	Gram Positive	Gram Positive	Gram Positive	Gram Positive	Gram Positive	Gram Positive
Motility	Non Motile	Non Motile	Non Motile	Non Motile	Non Motile	Non Motile	Non Motile	Non Motile
Indole	+	+	-	+	+	+	+	+
MR	-	+	+	+	+	+	-	+
VP	+	-	+	-	-	-	-	-
Citrate utilization	+	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	-	+	+	+	+
H_2S production	+	-	-	+	-	-	-	-
Catalase	+	+	+	-	+	+	+	+
Oxidase	+	-	-	+	-	-	+	+
Glucose	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+
Blood Haemolysis Test	β Heamolysis	β Heamolysis	β Heamolysis	β Heamolysis	β Heamolysis	β Heamolysis	β Haemolysis	β Heamolysis
CTAB	Haloes around colony appeared	Haloes around colony appeared	Haloes around colony appeared	Haloes around colony appeared	Haloes around colony appeared	Haloes around colony appeared	Haloes around colony appeared	Haloes around colony appeared
Fluorescence Test	Appeared	Appeared	Appeared	Appeared	Appeared	Appeared	Appeared	Appeared
Foaming Activity	Appeared	Appeared	Appeared	Appeared	Appeared	Appeared	Appeared	Appeared

Table 2. Surface tension, Biomass and Biosurfactant yield for the isolates

Isolates	Oil displacement zone	Emulsification index (E24%)	Biomass at 600nm (gL-1)	Biosurfactant yield at 500nm (gL-1)
NS1	1.0 cm	64.70	0.601	2.256
NS2	0.5 cm	64.51	0.554	-1.091
NS3	1.4 cm	63.33	0.438	0.970
NS4	2.0 cm	63.33	0.397	0.397
NS5	0.7 cm	60.00	0.540	3.603
NS6	1.5 cm	63.63	0.369	0.364
NS7	1.0 cm	63.63	0.615	2.721
NS8	1.5 cm	62.5	0.000	-0.620

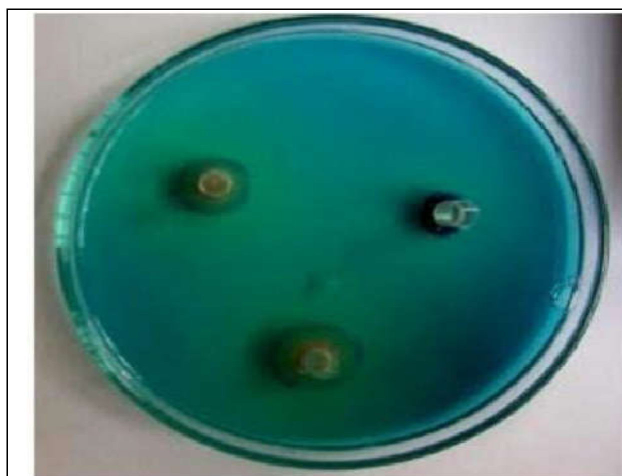

Fig 1:CTAB Methylene Blue Agar Test

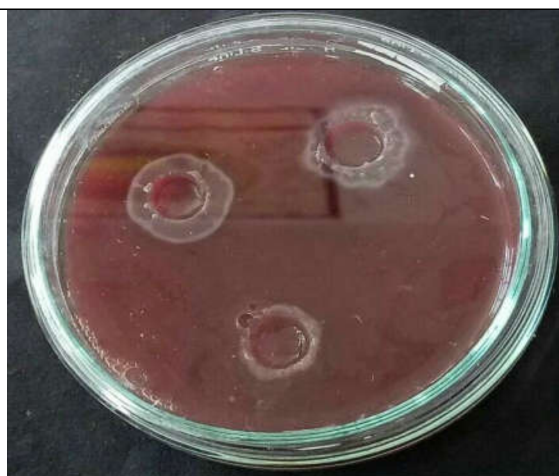

Fig 2: Blood Haemolysis Test

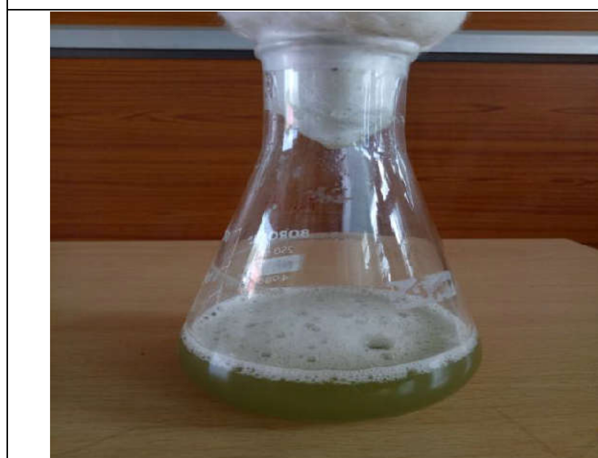

Fig 3: Foaming Activity

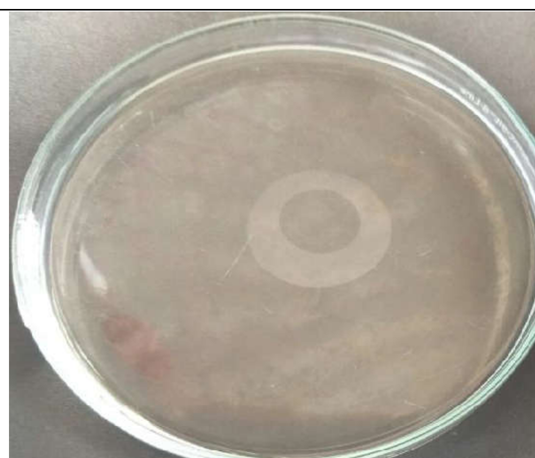

Fig 4: Oil Spreading Test

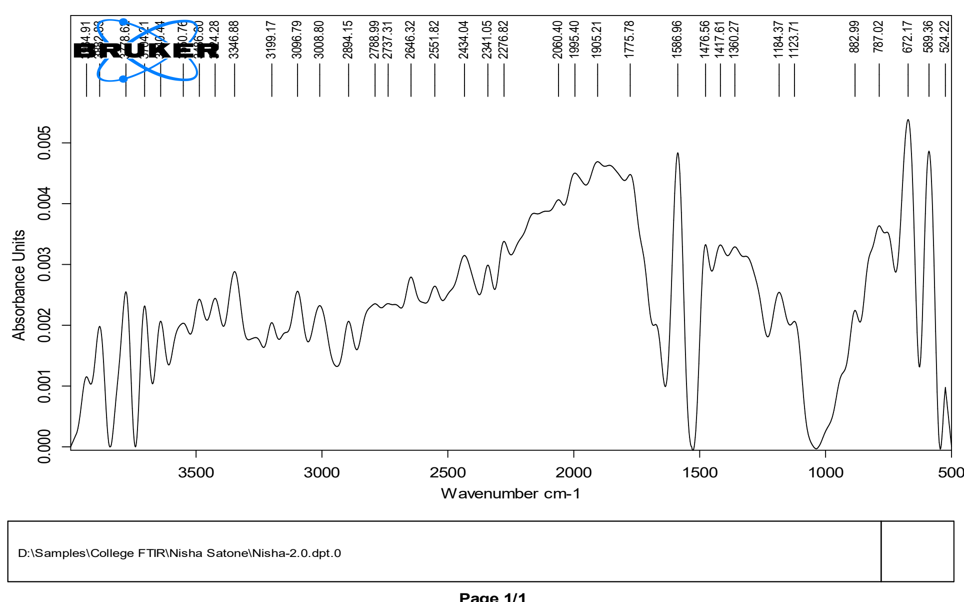

Fig 5. FTIR analysis of biosurfactant

Extraction of biosurfactant: The culture broth was centrifuged at 5000 rpm, 4^0 C for 20 minutes to obtain a cell-free supernatant. The pH of the supernatant was adjusted to 2 using 6 N HCl and was subjected to acid precipitation by placing it at 4^0C overnight. The off-white precipitate was separated by centrifugation at 10000 rpm for 30 min at 4^0C. The precipitate was extracted thrice with a 2: 1 chloroform-ethanol mixture. The organic phase was removed, and the biosurfactant was concentrated using a rotary evaporator at 40^0C. The solvents were evaporated leaving behind relatively pure biosurfactant as a viscous light brown matter.

Measurement of biosurfactant concentration: The biosurfactant concentration was measured indirectly by measuring the absorbance of biosurfactant-methylene blue complex at wavelength of 500nm. Methylene blue with a concentration of 0.015mg/ml was prepared and its optical density was measured using spectrophotometer. The solution was used as standard. 2 ml of the broth culture was pipetted into a test tube containing 2 ml of the standard, and 1 ml of chloroform was added to the mixture. The mixture was vortexed for two minutes and allowed to stand. The biosurfactant-methylene blue complex was siphoned using pasteur pipette into a cuvette and the absorbance was measured using spectrophotometer (Rahman, 2002).

Biuret method: It detects the presence of lipopeptide biosurfactants. 2ml of crude Extract solution was first heated at 70^0C then mixed with 1M NaOH solution. Drops of 1% $CuSO_4$ were slowly added to observe any colour change (Violet or Pink ring).

Fourier Transform Infrared Spectroscopy: To understand the overall chemical nature of the extracted biosurfactant, Fourier transform infrared spectroscopy (FTIR) [Bruker (Alpha)] was employed. The technique helps to explore the functional groups and the chemical bonds present in the crude extract. Samples were prepared by homogeneous dispersal of 1mg of the biosurfactant sample in pellets of potassium bromide. IR absorption spectra were obtained using a built-in plotter. IR spectra were collected over the range of 450–4500cm^{-1} with a resolution of 4cm^{-1}. The spectral data were the average of 50 scans over the entire range covered by the instrument. The spectrum was studied to interpret the chemical nature of the biosurfactant fraction.

RESULTS AND DISCUSSION

Total 25 bacterial strains were isolated from five samples collected from soil of various automobile workshops, petrol pumps and saw mills. Out of 25 isolates, 8 potential bacterial strains (NS1, NS2, NS3, NS4, NS5, NS6, NS7 and NS8) with ability to produce biosurfactant were selected for further study. The study comprises of biomass yield, biosurfactant yield and reduction in surface tension of the culture media. The isolates were screened by oil displacement method, blue agar plate method, blood agar heamolysis, foaming activity etc. characterized on the basis of cultural, morphological, and biochemical tests. All the strains were unable to ferment galactose, fructose and trehalose. All the strains showed β-hemolysis on blood agar. On the basis of cultural, morphological and biochemical characterization, isolates NS1, NS2, NS3, NS4, NS5, NS6 ,NS7, NS8 were tentatively identified as *Pseudomonas sp., Citrobacter sp., Klebsiella sp.* The isolates were further characterized for their ability to produce biosurfactant by using 2% kerosene oil as a sole source of carbon. All the eight isolates showed oil displacement and drop collapse test positive. The isolates showed oil displacement zone of 1.0cm, 0.5cm, 1.4cm, 2.0cm, 0.7cm, 1.5cm, 1.0cm and 1.5cm respectively. Further test showed emulsification index of 64.70%, 64.51%, 63.33%, 63.33%, 60.00%, 63.63%, 63.63% and 62.50%respectively. Kerosene oil as a carbon source favored extracellular production of surface active product by *Pseudomonas, Citrobacter, Klebsiella sp.* which was indicated by the reduction in surface tension of the broth. *Pseudomonas* sp. was found to reduce the surface tension of broth from 65N/m to 39N/m. while *Citrobacter and Klebsiella sp.* were found to reduce the surface tension up to 41.5 and 42 N/m respectively. Whereas the surface tension of control sample was found to be 48.89 N/m. FTIR spectra of biosurfactant produced was

performed to identify the types of functional groups present in the compound. Characteristic absorption bands corresponding to functional groups typically forming parts of rhamnolipids could be observed (Fig 5). The characteristic peak at 3429 cm^{-1} represents the O-H stretching (free hydroxyl of phenolic groups).Presence of N-H stretch was observed at 3450 $cm^{-1.}$ Presence of CH_2 and CH_3 at 1476.56 cm^{-1} was observed. Absorption around 1775.78 cm^{-1}represents ester (C=O) and carbonyl (COO^-) groups.

Conclusion

The present study is an attempt to isolate and characterize biosurfactant producing bacteria from oil spill soil. *Pseudomonas* strain screened from the environment gave maximum biosurfactant production (0.20 g/L-^{1}) with kerosene oil as sole source of carbon and could reduce the surface tension of broth from 65mN/m to 39 mN/m. It is evident from the results that biosurfactants can be used in place of synthetic surfactants due to their efficiency and ecofriendly nature.

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