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ORIGINAL ARTICLE **OPEN ACCESS**

BIOSURFACTANT PRODUCTION USING *PSEUDOMONAS* SPP. ISOLATED FROM OIL CONTAMINATED SOILS

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ABSTRACT

Biosurfactants are diverse group of surface-active molecules / chemical compounds synthesized by microorganisms. These are amphipathic molecules with both hydrophilic and hydrophobic domains. Biosurfactants are environment friendly and have potential industrial and environmental applications. The oil contaminated soil samples were collected from various sources such as petrol pumps and automobile garages from Karad city. The strains of Pseudomonas species were isolated from the soil samples using enrichment culture technique using minimal salt medium followed by isolation on solid nutrient medium (centrimide agar). The isolate was gram negative motile short rods oxidase test positive and were taken as Pseudomonas spp. In all six different isolates were obtained from which four were selected out during screening processes. They were designated as PS1, PS2, PS3 and PS4. The strain designated PS3 was found to be the best as it showed Emulsification index as 50 %(Drop collapse test). Optimization studies of biosurfactant using oil supplemented medium were carried out using isolate PS3. The optimum pH and temperature for biosurfactant production were found to be 7 and 37°C respectively.

Keywords*: Biosurfactant, Pseudomonas species, Centrimide agar, Drop collapse test.*

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INTRODUCTION

Biosurfactants reduce surface tension and critical micelle dilution (CMD) in both aqueous solution and hydrocarbon mixtures.These properties create micro-emulsions in which micelle formations occur in which hydrocarbons can solubilize in water or water in hydrocarbons. Microorganisms have been reported to produce several classes of biosurfactants, such as glycolipids, lipopeptides, phospholipids, neutral lipids or fatty acids and polymeric biosurfactants. [1] These compounds are metabolic products produced during the growth of microorganisms on water-soluble and water immiscible substrates. The most commonly isolated and the best studied groups of biosurfactants are mainly glycolipids and phospholipids in nature. Nevertheless, rhamnolipids are glycolipid compounds produced by *Pseudomonas spp*. that can reduce water surface tension and emulsify oil. Biosurfactants are environmentally friendly and have potential industrial and environmental applications. [2] Present study was aimed at isolation of *Pseudomonas* species from oil contaminated soil and their screening for biosurfactant producing ability. Optimization of biosurfactant production was studied using the best isolate obtained from screening of the isolated *Pseudomonas* species.

MATERIAL AND METHODS

Enrichment and isolation of organisms:

Oil contaminated soil samples were collected from the petroleum pump and automobile shops in Malkapur, karadIndia and were enriched by inoculating each soil sample into 50 mL of minimal salt medium containing (g/L); 15 g $NaNO_3$, 1.1 g KCl , 1.1 g NaCl , 0.00028 g $FeSO_4.7H_2O$, 3.4g KH_2PO_4, 4.4 g K_2HPO4, 0.5 g $MgSO_4.7\ H_2O$, 0.5 g yeast extract and incubating at 37°C in shaker incubator (100 rpm). Aftera week of incubation, the samples were serially diluted using sterile saline (0.85% NaCl) and plated on nutrient agar supplemented with 1% oil different bacterial isolated were selected based on the organism grown in enrichment technique, cenrimide agar and morphology (gram negative rods) motile, nonspore and oxidase positive were taken as Pseudomonas isolates. The selected isolates were screened for the production of biosurfactants using screening methods as below:

Screening for Biosurfactant production

Hemolytic activity: [3]

Pure cultures of bacterial isolates were streaked on the freshly prepared blood agar and incubated at 37°C for 48-72h. 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.

Drop collapsing test: [4]

Drop collapsing was performed in order to screen the best surfactant producing *Pseudomonas*. A twoμL of motor oil was spotted onto a piece of parafilm sheet while five μL cell free culture broth was added and drop size was observed 5 min later. Positive results were considered for biosurfactant production when the drop diameter was atleast 1mm larger than that produced by deionize water (negative control).

Oil spreading test: [4]

30 mL distilled water was put into petri dishes.50 μLdisel drop was dropped on to the water surface to form oil film, in the midst of which 5 μLof fermentation broth (diluted by 10 times with water) was dropped. The diameter of the oil expelling circles formed was measured. Distilled water (without surfactant) was used as negative control and triton –X100 was used as positive control. The increased in the diameter of oil circles was taken as indication and activity of biosurfactant.

Emulsification test (E_{24}): [4]

Colonies of pure culture were suspended in test tube containing 2 mL of mineral salt medium and 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.

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

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 tentatively identified by its microscopic appearance and biochemical tests based on Bergey's Manual of Determinative Bacteriology volume 1(1986).

Biosurfactants production in shake flasks: [5]

For preparation of inoculum one loopful of PS3 culture (10^8CFU/mL) was inoculated in 20 mL of seed media(nutrient broth)in a 100 ml flask and incubated on rotary shaker at 180 rpm at 30º C for 11h. 5% of inoculum was transferred to each production medium (100mL) and flasks were incubated on rotary shaker at 180 rpm at 30º C in an incubator shaker at 180rpm for 3 days. The growth of the isolates was carried out using fermentation medium composition : (g/l) NaNo3 2.5,K_2HPO_4 4.0, KH_2PO_4 4.0, $CaCl_2$ 0.1, $MgSO_4$ 0.3, NaCl 1.0, KCL1.0, yeast extract 1.0and 1% palm oil(as a sole source of carbon). Flask without carbon source was used as control. The pH of the medium was adjusted to 7.0 using 1N NaOH and autoclaved by 121^0C for 20 min. In the time course samples of culture medium were drawn in appropriate time intervals and monitred for cell growth and biosufactant production in terms of emulsification index test and biosurfactant weights.

Extraction and partial purification of biosurfactant : [6]

The biosurfactant was extracted from culture medium from each flask of separate isolate after cells removal by centrifugation at 12,500 rpm for 30 min. The pH of the supernatant was adjusted to 2.0 by 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 .

Characterization of biosurfactant by phenol-sulfuric acid method: [7]

Glycolipid type of biosurfactant was confirmed by using phenol sulfuric acid method. In this test 20 mg of crude biosurfactant obtained by acid precipitation was dissolved in 5 mL of distilled water to that 5% phenol was added. Further 5 mL of H_2SO_4 was added drop by drop. Yellow orange color indicated the presence of glycolipid.

Optimization of environmental conditions on biosurfactant stability:[7]

4 mLcell free culture broth and 6 mL of soyabean oil was taken in different test tubes. The mixture was vortex at 3000 rpm for 2 min on cyclomixer. The tubes allow to stand for 10 min in temperature 20^0,25^0,37^0,55^0 and 100^0C. After that tubes were cooled under running tap water and O.D. was taken at 500 nm.

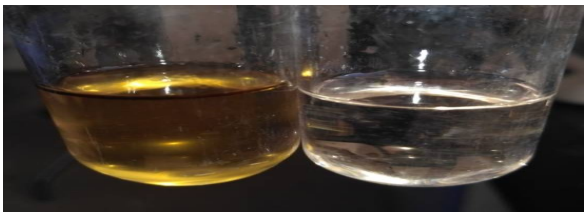
Effect of pH on biosurfactant activity

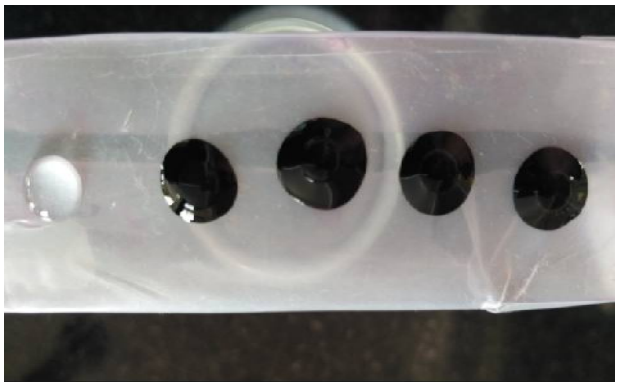
4 mLcell free culture broth and 6 mL of soyabean oil was taken in different test tubes. In each respective tubes added 1ml respective buffer solution of pH 5, 6,7,8,9 and 10.The mixture was vortex at 3000 rpm

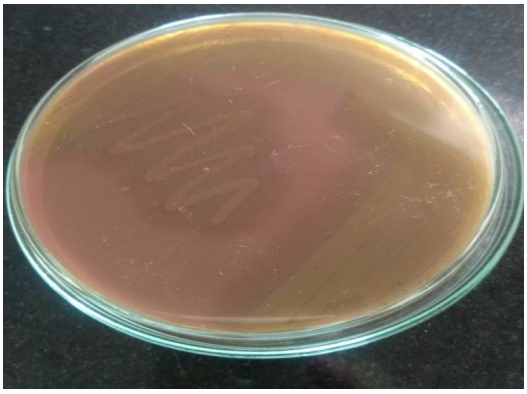
for 2 min on cyclomixer. The tubes were allowed to stand for 15 min. after that take O.D. at 500 nm. plot graph of O.D. vs pH.

RESULTS AND DISCUSSION

The assay was 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 4 isolates which showed positive results for all the 4 screening methods viz., hemolytic test, oil spreading test, drop collapsing and emulsification index. The 4 isolates were further tested for maximum biosurfactant production by inoculating into the Mineral salt medium (MSM). Among the selected isolates, PS3 showed maximum biosurfactant producing ability. The best isolate PS3 was identified as *Pseudomonas aeruginosa* based on microscopic and biochemical analysis.







Photograph 1: Photograph showing Drop collapsing,

Photograph 2: Photograph showing Haemolytic test done on paramfilm from left to right: distilled water activity shown by biosurfactant (rhamnolipid) (negative control),PS1,PS2,PS3 AND PS4.produced by *Pseudomonas aeruginosa* (PS3).

Photograph 3: Photograph showing Detection of glycolipid biosurfactant produced by *Pseudomonas aeruginosa*by phenol-sulphuric acid method. Yellow orange color indicating presence of glycolipid.

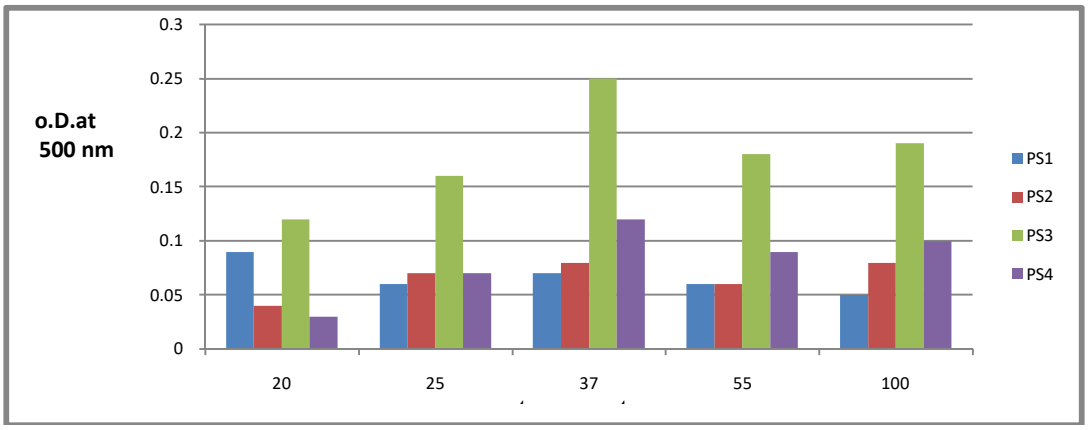


Fig 1 : Graph of effect of temperature on bioemulsifier activity

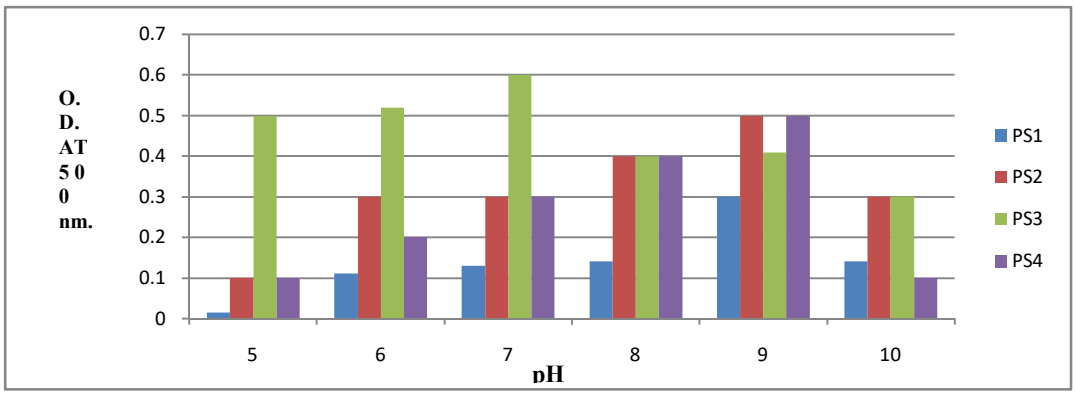


Table 1 Results of primary screening for biosurfactant activity

Test isolates	Haemolytic assay	Drop collapsing test	Oil spreading test	Emulsification index
PS1	+	+	+	47.5%
PS2	+	+	+	45%
PS3	+	+	+	50%
PS4	+	+	+	40%
PS5	-	-	-	27.2%
PS6	-	+	-	30%

Positive = +, Negative= -

Table 2 : Effect of temperature on bioemulsifier activity/stabilty:

Temperature	*O.D.at 500nm PS1*	*O.D.at 500nm PS2*	*O.D.at 500nm PS3*	*O.D.at 500nm PS4*
20	0.09	0.04	0.12	0.03
25	0.06	0.07	0.16	0.07
37	0.07	0.08	0.25	0.12
55	0.06	0.06	0.18	0.09
100	0.08	0.08	0.19	0.10

Table 3: Effect of pH on bioemulsifier activity/stability:

PH	*O.D.at 500nm PS1*	*O.D.at 500nm PS2*	*O.D.at 500nm PS3*	*O.D.at 500nm PS4*
5	0.014	0.1	0.5	0.1
6	0.11	0.3	0.52	0.2
7	0.13	0.3	0.6	0.3
8	0.14	0.4	0.4	0.4
9	0.3	0.5	0.41	0.5
10	0.14	0.3	0.3	0.1

Conclusions

The isolates strains obtained from oil contaminated soils showed biosurfactant producing ability. Maximum production of biosurfactant was found with PS-3 isolate identified as Pseudomonas aeruginosa. Further study should be carried out to explore the biosurfactant potential of the isolated *Pseudomonas* species for commercial application.

Conflict of interest: No conflict of interest is there amongst authors

Ethical clarification: No any type of study was done on humans or animals.

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