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PRODUCTION OF BIOSURFACTANT BY *PSEUDOMONAS AERUGINOSA* CH23

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ABSTRACT:

Petroleum Hydrocarbons are the most obvious pollutants in the both terrestrial & aquatic realm**.** Its Damage in agriculture field and normal water system are well documented. Biosurfactant are produced during Hydrocarbon degradation by Bacteria, Which help them to absorb, emulsify, and solubilize the water- immiscible Hydrocarbon. These agents reduce surface tension and viscosity of Hydrocarbon mixtures. Biosurfactant vary in their chemical properties and molecular size. Low molecular weight surfactants are glycolipid like Rahmnolipid. A Rahmnolipid producing bacterium *Pseudomonas aeruginosa* CH23 was previously isolated from the crude oil contaminated soil. After morphological, biochemical and physiological identification, the strain was confirmed by partial DNA sequencing. Biosurfactant was produced by the bacteria by growing it on medium containing glycerol as additional carbon source. The crude extract was obtained and its presence was confirmed by hemolysis test, emulsification test drop collapse test and measurement of cell- surface hydrophobicity. The present study indicates that renewable and relatively inexpensive resource can be efficiently used for biodegradation of oil-spills.

KEY WORDS: *Biosurfactant, Rahmnolipid, Hydrocarbon, Bioremediation.*

INTRODUCTION:

Organic compounds with limited water solubility like petroleum hydrocarbons are biodegraded very slowly because of their low availability to microbial cells. The availability of slightly soluble organic compounds can be enhanced by microbially produced surfactants which increase aqueous dispersion by many orders of magnitude (1). In many cases, Biosurfactants also stimulate the biodegradation of organic compounds. For example, alkane degradation is stimulated by Rhamnolipid (1), sophorose lipids (2) and phospholipids

Chemically-synthesized surfactants have been used in the oil industry to aid clean up of oil spills, as well as to enhance oil recovery from oil reservoirs. These compounds are not biodegradable and can be toxic to environment. Biosurfactant have special ad-vantage over their commercially manufactured counterparts because of their lower toxicity, biodegradable nature, and effectiveness at extreme temperature, pH, salinity and ease of synthesis. They are potential candidate for much

commercial application in the pharmaceutical and food processing and oil recovery industries (3,4).

Pseudomonads are the best-known bacteria capable of utilizing hydrocarbons as carbon and energy sources and producing biosurfactants which enhance the uptake of such immiscible hydrophobic compounds (5,6,7,8). Although the potential for biosurfactant production is dictated by the genetic traits of microorganisms, environmental conditions and nutrients can significantly influence the level of expression as well as the chemical characteristics. Rhamnolipid compounds are frequently the main biosurfactants produced by *Pseudomonas aeruginosa* as a mixture of mono- and di-rhamnolipids which have quite different physico-chemical properties (9). Rhamnolipid Biosurfactant specifically produced by *Pseudomonas aeruginosa* in particular offer special advantage because of their potent emulsifying activity and low critical micelle concentration. This particular bacteria produces two types of glycolipids both containing Rhamnose as the carbohydrate moiety. These glycolipids are produced after attaining the stationary phase when nitrogen is depleted in the medium

The objective of this paper is to evaluate the biodegradation of crude oil by *Pseudomonas aeruginosa* in laboratory. *Pseudomonas aeruginosa* is a typical strain for Rhamnolipid production and can utilize vegetable oil or glycerol as the sole carbon source. We investigated the feasibility of crude oil degradation by microbial process, focusing mainly on the effect of Rhamnolipid on biodegradation. In the present investigation, the Biosurfactant was extracted in crude form by growing the pseudomonas cells in specific medium containing different carbon source in addition to crude oil. The Biosurfactant activity was further assessed by various analytical methods

MATERIALS AND METHODS:

Bacterial culture: Isolation of crude oil degrading bacteria was done by the method mentioned elsewhere. Out of 35 isolates, the most efficient strain was found to be *Pseudomonas spp.* which was indigenously isolated from the vicinity of oil drilling well. After initial characterization, and partial DNA sequencing, the bacteria was identified as *Pseudomonas aeruginosa* CH23 selected for the further study for Biosurfactant production.

Media and Biosurfactant production using different carbon sources: The medium used for growth, screening and enrichment of Pseudomonas spp was Bushnell and Haas broth medium (Hi media). For production of Biosurfactant, the medium was supplemented with 3% glycerol, 3% glucose and 2% crude oil in 3 different 250 ml shake flasks (labeled as sample I, II and III) and incubated on shaker at 35^0C for 5 days.

Extraction of Crude Biosurfactant:

Many *Pseudomonas* strains are known to produce Biosurfactants on dextrose, glycerol, or mannitol etc (11,12). For production of Biosurfactant, the medium was supplemented with 3%

glycerol, 3% glucose and 2% crude oil in 3 different 250 ml shake flasks and incubated on shaker at 35^{0}C for 5 days. After fermentation, the culture medium was centrifuged at 350 g for 20 min and then the isolated supernatant was adjusted to pH of 2.0 by adding 5 mol/l H_2SO_4 for the Rhamnolipid precipitation. The precipitates were extracted with two volumes of diethyl ether/methanol (1:1, *V/V*) mixture. Evaporation of the solvent yielded crude Rhamnolipid.

Biosurfactant activity Assays:

1. Haemolytic activity: Isolated strains was screened on blood agar plates containing 5% (v/v) human blood and incubated at room temperature for 24 h. Hemolytic activity was detected as the occurrence of a defined clear zone around a colony (13).

2. Blue agar plates: containing cetyl trimethyl ammonium bromide (CTAB) (0.2 g/l; and methylene blue (0.005 g/l) were used to detect extracellular glycolipid production (14).Biosurfactant production was observed by the formation of dark blue halos around the colonies.

3. Drop collapsing test : Two µl of hydrocarbon sources like paraffin oil, Diesel, crude oil and kerosene were added to each well of a 96-well micro titer plate lid The lid was equilibrated for 1 h at room temperature, and then 5 µl of the cultural supernatant from all three samples 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 '-' (15).

4. Emulsification activity E_{24}: Emulsification activity was measured according to the method of Cooper and Goldenberg (1987) with a slight modification. To 4 ml of culture supernatant from all three samples including crude extract (0.5%, w/v), 4 ml of *n*-hexadecane or crude oil were added and vortexed at high speed for 2 min. The mixture was allowed to stand for 10 min prior to measurement. The emulsification activity is defined as the height of the emulsion layer divided by the total height and expressed as percentage.

5. Cell surface hydrophobicity

The bacterial adhesion to hydrocarbons (BATH) assay was used to determine changes in cell surface hydrophobicity during growth on minimal salt medium containing three carbon sources (16). Bacteria were harvested from growth cultures by centrifugation at 8000 rpm for 10 min at 4 ^{0}C, washed twice, and suspended in PUM buffer (22.2 g K_2HPO_4.4H2O; 7.26 g KH_2PO_4; 1.8 g urea and 0.2 g $MgSO_4$..$7H_2O$ in 1 l distilled water, pH 7.2) to an initial absorbance at 400 nm to 1.0. Hexadecane (0.5 ml) and cell suspensions (2.0 ml) were vortexed in a test tube for 2 min and equilibrated for 15 min. The bottom aqueous phase was carefully removed with a Pasteur pipette and the A_{400} was measured. The adherence was expressed as the percentage decrease in optical

absorbance of the lower aqueous phase following the mixing procedure, compared with that of the cell suspension prior mixing.

RESULTS:

The indigenously isolated bacteria *Pseudomonas aeruginosa CH23* was selected for the study of enhanced crude oil degradation by virtue of Rhamnolipid production as indicated by the various screening tests. The bacteria was grown in three different media. One containing BHM + 2% crude oil, second containing BHM+ 3% glycerol and third containing BHM+ crude oil . Crude extract was obtained from both the culture flasks. Glycerol containing medium showed more production of Biosurfactant as indicated by various assay methods as showed in Table 1.

Biosurfactant activity Assays:

1. Hemolytic activity:

Hemolytic activity has been used for the isolation of lipopeptide Biosurfactants (17) and Rhamnolipid (18). The hydrophilic part of Biosurfactant -the cationic part- is proposed to initiate electrostatic interaction with the negatively charged components of the membrane of microbes; the hydrophobic portion is supposed to permit the peptides to insert into and permeate the membrane (19). Biosurfactant producing capacity in liquid medium was found to be associated with hemolytic activity (20, 21). Hemolytic activity therefore appears to be a good screening criterion for surfactant-producing strains (20).The strain *Pseudomonas aeruginosa CH23* is an efficient degrader of oil having the efficiency to degrade crude oil up to 55 %. The crude oil degrading efficiency can be either because of the oil hydrolyzing enzyme Lipase or Biosurfactant Rhamnolipid. The presence of Rhamnolipid was confirmed by occurrence of a clear halo around the bacterial colony on blood agar plate. Fig 1

Fig 1: Occurrence of clear zone hemolysis on blood agar plates

Fig 2: Occurence of dark blue halos around the of β-colony

2.Blue agar plates:

Formation of dark blue halos on blue agar plates, detect the production of extracellular glycolipids by *Pseudomonas spp*. (14). As shown in the fig the strain formed dark blue halos around the bacterial colony thereby confirming the production of extracellular glycolipid. Fig 2

3. Drop collapsing test:

The Biosurfactant decreases the surface tension of the drop when it comes in contact of the lipid containing hydrocarbon. As a result of this, the drop collapses after one minute when the crude extract of the Biosurfactant is mixed with four different hydrocarbon sources –Paraffin oil, crude oil, Kerosene and Diesel as shown in the Table 1. In all the cases, The test was positive except in case of sample III where the collapse was delayed.

Table 1: Results of drop collapse test for all three samples

S.NO.	Type of hydrocarbon	Sample I	Sample II	Sample III
1.	Paraffin oil	++	++	+
2.	Crude oil	++	++	+
3.	Kerosene	+++	+++	++
4.	Diesel	++	++	++

4.Emulsification Index:

Emulsification activity is one of the criteria to support the selection of potential Biosurfactant producers. Emulsifying activity E24 determine the productivity of bioemulsifier The three samples I,II and III containing BHM and three different carbon source, the sample containing glycerol showed maximum emulsification as shown in the Table 2.

5. Cell surface hydrophobicity (Biosur 2):

It has been suggested previously that cell surface hydrophobicity is an important factor in predicting adhesion to surfaces. Thus, cell hydrophobicity was used as a measure of potential cell affinity for hydrophobic substrates and was determined by bacterial adherence to hydrocarbon (BATH) assay. At the beginning of stationary phase hydrophobicity of *Ps. aeruginosa CH 23* grown on glucose was slightly higher than when grown on glucose as the carbon source and there was not any important change in its values during growth. This suggests that Biosurfactant production does not contribute for decreasing or increasing cell surface hydrophobicity. While in case of crude oil, the cell surface hydrophobicity was found to be maximum because of the high insolubility of crude oil in aqueous phase. Table 2.

Table 2: Various tests for Biosurfactant production by *Ps. aeruginosa CH 23* using three different carbon sources.

S.NO.	TEST	SAMPLE I	SAMPLE II	SAMPLE III
1.	Drop collapse test	positive	positive	positive
2.	Emulsification Index	68%	62%	53%
3.	Cell surface hydrophobi	58%	50%	48%:

DISCUSSION:

Biosurfactants play a key role in emulsifying hydrocarbons Biosurfactant producing bacteria are found in higher concentrations in hydrocarbon contaminated areas (22). These strains represent a valuable source of new compounds with surface-active properties, and potential application for

bioremediation. After hemolysis test, stabilization of an oil and water emulsion is commonly used as a surface activity indicator. Several studies focused on high emulsifying abilities (23,24,25). Various Biosurfactant activity assays performed confirm the presence of an emulsifying agent which not only solublizes the crude oil but also makes it available to be utilized as a carbon source.

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