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Biosurfactant production by pseudomonas putida and Aspergillus niger from oil contaminated site

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ABSTRACT

Biosurfactant is a structurally diverse group of surface – active molecule, synthesized by microorganisms. In the study, the soil sample was collected from the oil contaminated site at Vellakudi, Thiruvarur (Dt), Tamilnadu. From that sample, organisms were isolated by serial dilution agar plating method. The colonies were identified as *Pseudomonas putida* and *Aspergillus niger* by morphological and biochemical characteristics. Biosurfactant producing organisms were screened by using oil spreading technique and blood haemolysis test. Organisms were inoculated into the Mineral salt broth with crude oil, olive oil, groundnut oil and coconut oil as a carbon source for biosurfactant production. Biosurfactant was analyzed by using different pH, temperature, carbon and nitrogen sources. Emulsification activity of biosurfactant was analyzed. The extracted biosurfactant was characterized by using Thin Layer Chromatography. This study concluded that *Pseudomonas putida* produces higher amount of biosurfactant compared to *Aspergillus niger*.

Key words: Biosurfactant, *Pseudomonas putida*, *Aspergillus niger*, Emulsification, Thin layer chromatography.

1. INTRODUCTION

Biosurfactant are surface active organic compound synthesized by many micro organism during their growth that cells for the utilization of hydrocarbon compounds[1].The term "biosurfactant" refers to any compound obtained from microorganism which has striking influence on interfaces, further it brings down the interfacial tension between the two liquids.

Naturally occurring surface – active compounds derived from microorganisms are called biosurfactants. Biosurfactants are amphiphilic biological compounds produced extracellularly or as part of the cell membrane by a variety of yeast, bacteria and filamentous fungi[2]. Among the bacteria, *Pseudomonas spp.* is the best known for degrading hydrocarbon and producing biosurfactants mainly rhamnolipid in nature [3] whereas few reports are available with production and characterization of biosurfactants by yeast.

Surface active agents are needed for a large number of diverse applications. Emulsion stabilization is a very common requirement for food products cosmetics and other products. Surfactants are also useful as soap and detergents both for cleaning applications and for petrochemical purposes such as enhanced oil recovery and oil spill cleanup. Surface wetting and solid dispersal are important for preparations of coal slurries for pipelining. Colloid preparations are necessary for paints and related products penetration rates of inks and dyes are important for the pulp paper and textile industries. Foam stabilization is also necessary for five extinguishers and food industry[4] .

Other applications are for lubrication, corrosion inhibition and static inhibition. Biosurfactants appear potentially applicable to enhance oil recovery as alternative to, or in formulation with synthetic surfactants. Biosurfactants can also be used to reduce heavy crude oil viscosity either insitu, to effect oil displacement from rock or exsitu, for the pipelining and transport of produced oil [5]. The aim of the study is biosurfactant production by *Pseudomonas putida* and *Aspergillus niger* from oil contaminated site.

2. MATERIAL AND METHODS

2.1. Soil sample collection

Soil sample was collected from the oil contaminated site at Vellakudi, Thiruvarur (Dt), Tamilnadu. The isolated colonies were identified by cultural, morphological and biochemical characteristics.

2.2. Screening for biosurfactant production

The isolated colonies were tested for the biosurfactant production by two methods [6]

i) Oil spreading technique

ii) Blood haemolysis test

2.3. Oil spreading technique

30ml of distilled water was taken in the petriplate and 1ml of crude oil, olive oil, groundnut oil and coconut oil was added to the center of the plate. 20µl of the culture supernatant was added to the centre of the plate. The biosurfactant producing organisms can displace the oil and spread in the water[7].

2.4. Blood haemolysis test

The fresh single colony from the isolated culture was taken and inoculated into blood agar plates. The plates were incubated for 48-72 hrs at 37°C. Then the plates were observed for the presence of clear zone around the colonies. The clear zone indicates the presence of biosurfactant producing organisms [8].

2.5. Biosurfactant production

Pseudomonas putida and *Aspergillus niger* culture were inoculated on the mineral salt broth containing 2% of crude oil, olive oil, groundnut oil and coconut oil and it was incubated at an optimized condition for 24 to 48 hrs in a shaker operating at 120 rpm/min. After incubation the broth was screened for the production of biosurfactant [9].

2.5. Extraction of Biosurfactant

Biosurfactant produced by *Pseudomonas putida* and *Aspergillus niger* were extracted by acid precipitate methods . After incubation, the bacterial cells were removed by centrifugation at 5000 rpm, 4°C for 20 minutes. The surfactant was taken and pH of the supernatant was adjusted by using Hcl. Equal volume of choloroform, methanol 2:1 was added and mixed well. It was left overnight for evaporation. White colored sediment was obtained as a result of the production of biosurfactant [10].

2.7. Dry weight of Biosurfactant

Sterile petriplate was taken and the weight of the plate was measured. Now the sediment was poured on the plates.They were placed on the hot air oven for drying at 100°c for 30 minutes. After drying, the plates were weighed [9].The dry weight of the biosurfactant was calculated by the following formula,

Dry weight of biosurfactant = Weight of the plate after drying - Weight of the empty plate

2.8. Physical Parameters

2.8.1. Effect of pH

100 ml of nutrient broth and potato dextrose broth were prepared, 1% of inoculum was added to it. Then the broth was incubated at different pH at 5, 6, 7 and 8 for 48-72 hrs.

2.8.2. Effect of temperature

100ml of nutrient broth and potato dextrose broth were prepared, 1% of inoculum was added to it. Then the broth was incubated at different temperature at 24°C, 37°C, 45°C and 50°c, for 48-72 hrs.

2.9. Chemical parameters

2.9.1. Effect of carbon sources

100 ml of nutrient broth and potato dextrose broth was prepared and separated into different conical flasks. 1% of inoculum was added. Carbon sources like glucose, starch and galactose was added. Then the broth was incubated for 24-72 hrs at 37°C .

2.9.2. Effect of Nitrogen Sources

100 ml of nutrient broth and potato dextrose broth was prepared and separated into different conical flasks. 1% of inoculum was added. Nitrogen sources like urea, NaCl and $NaNo_3$ was added. Then the broth was incubated for 24-72 hrs at 37°C.

2.10. Estimation of Emulsification Activity [11]

Partially purified biosurfactant (5mg) was dissolved in 5 ml of Tris buffer (pH 8.0) in 30ml test tubes. Hydrocarbons like waste crude oil, olive oil, groundnut oil, coconut oil and xylene were tested for emulsification activity. 5mg of hydrocarbon was added to the above biosurfactant solution and shaken well for 20 min and the mixture was allowed to stand for 20 min. The optical density of the emulsified mixture was measured at 610 nm and the results were expressed as D_{610} [11].

2.11. Analytical method

2.11.1.Thin layer chromatography

Preliminary characterization of the biosurfactant was done by TLC method. A portion of the crude biosurfactant was separated on a silica gel plate using chloroform: Methanol water (10:10:0.5 v/v/v) as developing solvent system with different color developing reagent. Ninhydrin reagent was sprayed to detect lipopeptide biosurfactant as red spot, produced by *Pseudomonas putida* and *Aspergillus niger* [9].

RF value is calculated by using following formula :-

$$RF = \frac{\text{Distance moved by analyte from origin}}{\text{Distance moved by solvent front from origin}}$$

2.12 Statistical analysis

The results obtained in the present investigation were subjected to statistical analysis like Mean ($\bar{X}$) and Standard Deviation (σ) [12].

3. RESULTS AND DISCUSSION

Biosurfactant or bioemulsifiers play a key role in emulsifying hydrocarbons. Biosurfactant and bioemulsifiers are thought to be very suitable alternatives to chemical surfactants due to their properties like eco friendly, less or no toxicity, biodegradability, high specificity, selectivity at temperature, pH, salinity and synthesis from cheaper renewable substrates. The present study was carried out to determine the production of biosurfactants by using the organism isolated from paper waste contaminated soil.

3.1. Screening of biosurfactant producing microorganisms

The isolated colonies for their biosurfactant production were analyzed by two method.

3.1.1. Oil spreading technique

In oil spreading technique, *P. putida* and *A. niger* showed a zone of displacement in the oil. The biosurfactant producing organisms can only be able to displace the oil. The result of this technique revealed that the maximum zone of displacement was noted in crude oil for *P. putida* (0.54 ± 0.01mm) and *A. niger* (0.49 ± 0.01mm)respectively, when compared with other oils (Table – 1)

Table – 1: Zone Displacement of Oil Spreading Technique by *Pseudomonas putida* and *Aspergillus niger*

Sample	*P. putida* (mm)	*A. niger* (mm)
Crude oil	0.54 ± 0.04	0.49 ± 0.01
Olive oil	0.48 ± 0.01	0.45 ± 0.02
Groundnut oil	0.43 ± 0.01	0.46 ± 0.01
Coconut oil	0.45 ± 0.02	0.40 ± 0.01

Values are expressed by Mean ± Standard deviation

3.1.2. Blood hemolysis test

On blood agar plates, *Pseudomonas putida* showed the beta – hemolytic activity.

3.2. Production of Biosurfactants

Biosurfactant production in mineral salt broth with crude oil, olive oil, groundnut oil and coconut oil as carbon sources showed a colloidal thin white layer formation on the surface of the broth.

3.3. Extraction of biosurfactants

The biosurfactants was extracted by acid precipitation method with chloroform, methanol and solvent. White sediment was retained when the mixture was placed in the rotor.

3.4. Dry weight of biosurfactants

The dry weight of the biosurfactants was measured and estimated. The result revealed that the maximum dry weight was in crude oil for *P. putida* and *A. niger* respectively, when compared with other oils (Table - 2).

3.5. Physical Parameters

3.5.1. Effect of pH

Biosurfactant was analyzed in various pH ranges such as 5, 6, 7 and 8 (Table - 3).

3.5.2. Effect of temperature

Biosurfactant was analyzed in various temperature ranges such as 24°C, 37°C, 45°C and 50°C (Table - 3).

3.6. Chemical parameters

3.6.1. Effect of carbon sources

Biosurfactant was analyzed in various carbon sources such as glucose, starch and galactose (Table - 4).

3.6.2. Effect of nitrogen sources

Biosurfactant was analyzed in various nitrogen sources such as urea, NaCl and $NaNo_3$ (Table - 4).

3.7. Estimation of Emulsification activity

Emulsification activity of biosurfactant was analyzed by using various hydrocarbon such as crude oil, olive oil, groundnut oil and coconut oil (Table - 5).

3.8. Characterization of biosurfactants

Lipopeptide were obtained as rhamnolipid i.e. a glycolipid while sprayed at ninhydrine reagent on the TLC plate. In our study similar to the findings of rhamnolipid produced from *P. aeruginosa* in TLC plate [13].

The biosurfactant production was characterized by using TLC plate. On TLC plate, the biosurfactant production by *P. putida* and *A. niger* were detected as red spot. Larger red spot on TLC plate was observed for crude oil. The RF values for the biosurfactants production for *P. putida* in crude oil was (0.61 ± 0.05mm) which

Table – 2: Dry weight (g) of biosurfactants for *P. putida* and *A. niger*

Sources	*Pseudomonas putida*			*Aspergillus niger*		
	Plate Weight	After Dry	Dry Weight	Plate Weight	After Dry	Dry Weight
Crude oil	48.2 ± 1.68	47.4 ± 3.04	1.4 ± 0.69	45.5 ± 1.57	44.3 ± 1.43	1.31 ± 0.96
Olive oil	46.0 ± 2.50	45.1 ± 1.54	0.38 ± 0.36	44.2 ± 1.60	43.0 ± 1.23	0.71 ± 0.23
Groundnutoil	44.1 ± 3.64	43.2 ± 1.50	0.21 ± 0.32	43.1 ± 1.50	42.1 ± 1.23	0.62 ± 0.34
Coconut oil	44.2 ± 1.30	43.0 ± 1.05	0.25 ± 0.24	42.0 ± 1.54	41.0 ± 0.25	0.30 ± 0.14

Values are expressed by Mean ± Standard deviation

Table – 3: Effect of pH and temperature on biosurfactant production

pH	*P. putida*		*A. niger*		Temperature	*P. putida*		*A. niger*	
	OD Value	g/100 ml	OD Value	g/100 ml	°C	OD Value	g/100 ml	OD Value	g/100 ml
5	0.43 ± 0.02	0.36 ± 0.01	0.43 ± 0.03	0.32 ± 0.02	24	0.36 ± 0.01	0.24 ± 0.01	0.26 ± 0.01	0.14 ± 0.01
6	0.55 ± 0.04	0.49 ± 0.03	0.40 ± 0.02	0.30 ± 0.01	37	0.45 ± 0.02	0.36 ± 0.02	0.32 ± 0.02	0.23 ± 0.02
7	0.42 ± 0.02	0.31 ± 0.01	0.46 ± 0.04	0.35 ± 0.02	45	0.36 ± 0.01	0.24 ± 0.01	0.31 ± 0.02	0.21 ± 0.01
8	0.51 ± 0.03	0.26 ± 0.01	0.36 ± 0.01	0.34 ± 0.02	50	0.21 ± 0.01	0.16 ± 0.01	0.21 ± 0.01	0.11 ± 0.01

Values are expressed by Mean ± Standard deviation

Table – 4: Effect of carbon and nitrogen sources on biosurfactant production

Carbon sources	*P. putida*		*A. niger*		Nitrogen sources	*P. putida*		*A. niger*	
	OD Value	g/100 ml	OD Value	g/100 ml		OD Value	g/100 ml	OD Value	g/100 ml
Glucose	0.56 ± 0.03	0.47 ± 0.02	0.31 ± 0.01	0.27 ± 0.01	Urea	0.36 ± 0.02	0.24 ± 0.01	0.56 ± 0.04	0.43 ± 0.02
Starch	0.31 ± 0.01	0.25 ±0.01	0.45 ± 0.02	0.31 ± 0.02	NaCl	0.40 ± 0.03	0.31 ± 0.01	0.35 ± 0.01	0.21 ± 0.01
Galactose	0.43 ± 0.02	0.36 ± 0.01	0.33 ± 0.01	0.24 ± 0.01	$NaNo_3$	0.45 ± 0.03	0.36 ± 0.02	0.41 ± 0.02	0.30 ± 0.01

Values are expressed by Mean ± Standard deviation

Table – 5: Emulsification activity on biosurfactant production

Sources	*P. putida*		*A. niger*	
	OD Value	g/100 ml	OD Value	g/100 ml
Crude oil	0.9 ± 0.04	0.87 ± 0.05	0.6 ± 0.01	0.52 ± 0.05
Olive oil	0.6 ± 0.02	0.54 ± 0.04	0.4 ± 0.02	0.39 ± 0.03
Groundnut oil	0.3 ± 0.01	0.38 ± 0.03	0.2 ± 0.01	0.28 ± 0.01
Coconut oil	0.6 ± 0.02	0.54 ± 0.04	0.3 ± 0.01	0.38 ± 0.02

Values are expressed by Mean ± Standard deviation

was higher than the olive oil (0.47 ± 0.03 mm), groundnut oil (0.50 ± 0.03 mm) and coconut oil (0.53 ± 0.04 mm) (Table – 6).

Table – 6: Analysis of Biosurfactants Using Thin Layer Chromatography

Sources	RF Value (mm)	
	P. putida	*A. niger*
Crude oil	0.61 ± 0.05	0.58 ± 0.04
Olive oil	0.57 ± 0.02	0.47 ± 0.03
Groundnut oil	0.48 ± 0.02	0.50 ± 0.03
Coconut oil	0.59 ± 0.03	0.53 ± 0.04

Values are expressed by Mean ± Standard deviation

From this study, *Pseudomonas putida* is able to produce the biosurfactant by using pH (6), temperature (37°C), carbon source (glucose) and nitrogen source ($NaNo_3$). *A. niger* is able to produce the biosurfactant by using pH (7), temperature (45°C), carbon source (starch) and nitrogen source (urea).

In our study correlated with the biosurfactant isolated from *P. aeruginosa* and Triton X – 100 showed maximum emulsification activity against waste motor lubricant oil [9]. In our study reports similar to the biosurfactant producing organisms showed beta – haemolytic activity on blood agar plate [8] .

Biosurfactant used in this study was higher than the emulsification activity recorded with Triton X – 100 against waste motor lubricant oil, crude oil and peanut oil. Compare to Triton X – 100 emulsification activity of the biosurfactant was low against kerosene, diesel, xylene, naphthalene and anthracene. However, while considering the advantages of biosurfactant over chemically synthesized surfactants, such as lower toxicity, biodegradability and ecological acceptability, the possibility of replacing the chemical surfactant in oil pollution[14] .

Biosurfactant produced by *P. aeruginosa* inferred that, biosurfactant produced with one carbon source like waste motor oil or peanut oil cake could be used to remediate different hydrocarbon pollution. In the present study, the attempt made on biodegradation of crude oil in a laboratory scale experimental setup revealed that maximum biodegradation rate was found with biosurfactant and fertilizer addition. Above information obtained in this study may be useful for the bioremediation of hydrocarbon polluted environments[15] .

4. CONCLUSION

Thus from the above study, it is concluded that *P. putida* produces higher amount of biosurfactant compared to *A. niger*. The optimum pH (6), temperature (37°C), carbon source (glucose) and nitrogen source ($NaNo_3$) for the production of biosurfactant was analysed. This study concludes that biosurfactants are easily biodegradable and safe to environment and low toxicity and low cost than synthetic surfactants. Hydrocarbons in oil contaminated soil are not easily degraded by normal flora of soil. So, they cause pollution to the environment but biosurfactants from microorganisms are able to easily degrade hydrocarbons in oil contaminated soils when compared to synthetic surfactants. Biosurfactants are likely to gain wide acceptance since they are readily biodegradable and have lower toxicity as compared to their chemically synthesized counterpart.These biosurfactants are easily degradable and well suited for the environmental applications such as bioremediation and oil spills.

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