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A Study on Production of Biosurfactant By Bacteria Isolated From Oil Contaminated Soil and Its Optimization

KEYWORDS: Biosurfactants, petrochemical, sucrose, olive oil, FTIR.

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ABSTRACT *Biosurfactants are the structurally diverse group of surface-active molecules synthesized by diverse microbes. Hence, there could probably be a potential chance of producing biosurfactant using locally isolated bacteria originated from petrochemical wastes. Furthermore, their role as anti-adhesive agents against several pathogens indicates their utility as appropriate anti-adhesive coating agents for medical insertional materials prime to reduce a large number of hospital infections without the use of synthetic drugs and chemicals. In the proposed study, Pseudomonas aeruginosa was isolated from petrol contaminated soils at Chennai. The isolated strain was screened for the production of biosurfactant. The various factors affecting production of biosurfactant was assayed, which include temperature, pH, different carbon sources, and different oil substrates. In addition to that, sucrose was found to be better carbon source and olive oil showed better production among the oils used. Finally it was confirmed as glycolipids by FTIR analysis.*

INTRODUCTION

Biosurfactant are a heterogeneous group of surface active molecules synthesized by microorganisms, that are either adhere to cell surface or excreted extracellulary in the growth medium. These molecules reduce surface tension and Critical Micelle Dilution (CMD) in both aqueous solutions and hydrocarbon mixtures. The majority of known biosurfactants are synthesized by microorganisms grown on water immiscible hydrocarbons, but some have been produced on such water- soluble substrates as glucose, glycerol and ethanol. These microbial products are engrossed more as substitutes to chemical surfactants because of their capability of reducing surface, interfacial tension with low toxicity, high specificity and biodegradability. They are mainly formed by hydrocarbon utilizing microorganisms. They occur in nature as a diverse group of molecules comprising of glycolipids, lipoprotein and lipopeptides, fatty acids, neutral lipids, phospholipids, polymeric and particulate biosurfactants. The interest in bio surfactant has been steadily increasing in recent years due to the possibility of their production through fermentation, their potential applications in various extents and its environmental protection.

The present study was intended to isolate biosurfactant producing bacteria from petrochemical oil contaminated soil. The bacterial isolates were screened for the potential bio surfactant producer(s) by various screening methods and also the production level in terms of yield by utilizing different agro substrates was optimized. Finally characterization of the produced bio surfactant was studied.

MATERIALS AND METHODS

Sample collection: 9 soil samples were collected from oil contaminated sites such as loading area at Chennai. Samples were subjected to pretreatment and serially diluted up.

Screening for the bio surfactant production

Hemolytic activity

The fresh single bacterial colony from nutrient agar was then streaked on the freshly prepared on Blood Agar and incubated at 37°C for 48-72 hours. Result was recorded based on the type of clear zone observed i.e. α-hemolysis.

Blue agar plate method

Mineral salts agar medium supplemented with carbon sources (2%) and cetyltrimethylammonium bromide (CTAB: 0.5 mg/ml) and methylene blue (MB: 0.2 mg/ml) were prepared and bacterial colony was inoculated. A dark blue halo zone around the culture was observed as a positive.

Drop collapsing test

Crude oil was used in this test, 2 µl of oil was applied to the well regions delimited on the covers of 96-well micro plates and were left to equilibrate for 24 h. The flatted drops indicate positive and the rounded drops were scored as negative, i.e. lack of bio surfactant production.

Emulsification test (E_{24})

The bacterial culture were suspended in 2 ml of Mineral salt medium after 48 hrs of incubation , 2ml hydrocarbon (oil) was added to each tube and mixture vortexes at high speed for 1 min, they were allowed to stand for 24hrs.

Biosurfactant production

Mineral Salts medium was used as a production medium. The positive isolates of bio surfactant producing bacterial colonies were inoculated in production medium and incubated at 37°C for 48 hours.

Estimation of biosurfactant

The spectrometric chemical assay for the determination of biosurfactant from the sample was estimated using Orcinol assay.

Optimisation studies: The effect of various pH, different temperature, and different time courses, carbon sources and oils on the overall production level of biosurfactant were investigated. A volume of 200µl of acidified culture supernatant was extracted with 1 ml of diethyl ether, and then the fractions were pooled, dried, and resuspended in 1 ml of 0.05 M sodium bicarbonate, which were utilized for further studies.

Thin layer chromatography

A 40µl volume of the partially purified biosurfactant in

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the methanol was spotted onto the TLC plates (silica gel 60 A°, 20 by 20 cm, 250 -μm thickness; whatman, Clifton ,NJ) and developed in chloroform - methanol- 5 M NH_4OH (80:25:4,vol/vol). RF values were calculated.

Fourier Transform-Infrared Spectroscopy:
48 h cells were harvested and biosurfactant was extracted using chloroform and cold methanol, dried and subjected to FT-IR spectroscopy. The partially purified samples were prepared in KBr pellet and FT-IR (Bruker) absorption spectrum was measured in room temperature of range 4000–600cm 1.

Molecular studies
Bacterial genomic DNA isolation using CTAB method and Electrophoresis was performed in a horizontal sub-marine apparatus. The PCR amplification of 16S ribosomal RNA gene was carried out in Thermocycler.

Forward: 5' AACGGCTCACCAAGGCGACG 3'

Reverse: 5' GTACCGTCAAGGTGCCGCCC 3'

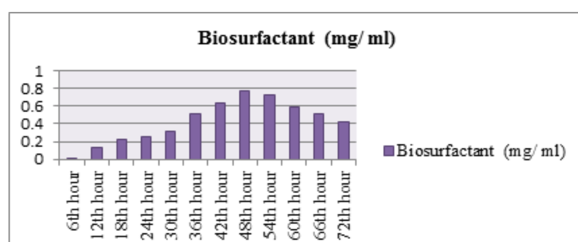
The gene sequencing was carried out using Beckman Coulter CEQ 8000 auto analyzer. BLAST – Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/blast/) provides a method for rapid searching of nucleotide and protein databases. Phylogenetic tree analysis was performed to predict the divergent evolution from a common ancestor or the evolution.

RESULTS AND DISCUSSION
In our study, 21 bacterial colonies were isolated from petrochemical contaminated soil samples collected in Chennai. Results of screening for biosurfactant producing bacteria were presented in Table 1.

Table 1 : Screening of biosurfactant producing bacteria

Tests	Haemolytic assay	Blue agar Plate	Drop Collapsing Test	Emulsification Index (> 60 %)
Sample No.	BS1, BS6, BS9, BS14, BS20	BS9	BS1, BS9, BS14, BS20	BS1, BS9, BS14, BS20

Based on the screening methods, BS9 isolate showed positive result for all the tests and it was considered for further studies. The environmental parameters like pH, temperature showed great influence on the growth of the organisms and the production of biosurfactant. Graph 1 indicates the effect of growth rate at different time intervals.



Graph 1. Effect of incubation period on Biosurfactant
Based on the results analyzed at different time intervals, it was determined that the maximum production was obtained at 48th hour.

Table 1: Effect of Temperature on Biosurfactant production

S.No	Temperature (°C)	Biosurfactant (mg/ ml)
1	25	0.53±0.01
2	30	0.66±0.00
3	35	**0.82**±0.05
4	40	0.62±0.11
5	45	0.5±0.03

The production of biosurfactant was claimed to be sensitive to the changes of incubation temperature. It has also been reported that temperature could significantly affected the yield of biosurfactant, as well as to alter the composition of biosurfactant produced (Desai and Desai, 1993). Temperature may cause alteration in the composition of the biosurfactant produced by *Pseudomonas* sp. DSM-2874 (Syldatk *et al.*, 1987; Abu-Ruwaida *et al.*, 1991) It was observed that the selected strain in our study have a temperature optima at 35°C (Table 1).

Table 2: Effect of pH on Biosurfactant Production

S.No	pH	Biosurfactant (mg/ ml)
1	6.5	0.58±0.11
2	7	0.64±0.03
3	7.5	**0.92**±0.00
4	8	0.65±0.01
5	8.5	0.52±0.05
6	9	0.42±0.00

Based on the readings it was observed that the selected strain showed maximum biosurfactant production when it was maintained at pH 7.5 (Table 2). Kim *et al.*, 2000 and Akram Tabatabaee *et al.*, 2005 obtained the maximum yield at pH range from 6.2-7.2.

Table 3 : Effect of Different Carbon Sources on Biosurfactant Production

S.No.	Carbon source	Biosurfactant (mg/ ml)
1	Glucose	0.83±0.01
2	Fructose	0.66±0.14
3	Sucrose	**0.98**±0.05
4	Lactose	0.56±0.01
5	Mannitol	0.62±0.00
6	Xylose	0.42±0.60

Carbon source is very important in the production of biosurfactant. In general, optimal yield are obtained with hydrocarbon or carbohydrate and lipids. The genus *Pseudomonas* is capable of using different substrates, such as glycerol, mannitol, fructose, glucose, n-paraffins and vegetable oils, to produce rhamnolipid-type biosurfactants (Koch *et al.*, 1991).

Mulligan and Gibbs, 1993 observed that *B. subtilis* and *P. aeruginosa* showed maximum production in presence of glucose. Water-soluble carbon sources, such as mannitol, glycerol and ethanol could be used for rhamnolipid production in *Pseudomonas* sp., (Desai and Banat, 1997).

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Different carbon sources were screened for maximum production for the selected isolate. As it was seen from Table 3, mannitol and sucrose offered satisfactory production of biosurfactant**.**

The most economical and valuable bioproducts are produced from the natural sources and industrial wastes. One of the limiting factors in the commercial success of biosurfactant production schemes is the cost of the sugar substrate used for biosurfactant formation. In this study, various left over oil products such as coconut oil, sunflower oil, groundnut oil, palm oil and olive oil were used (2% concentration) as substrates for effective biosurfactant production. Olive oil was the best carbon source for surfactant synthesis (Table 4). Similar results were found with *P. aeroginusa* 44T1 by Robert *et al.*, 1989 and Abouseoud *et al.*, 2007.

Table 4: Production of Biosurfactant from Agro products

S.No	Oil sources	Biosurfactant (mg/ ml)
	Coconut oil	0.44±0.07
	Sunflower oil	0.33±0.00
	Groundnut oil	0.5±0.06
	Palm oil	0.38±0.80
	Olive oil	0.67±0.01

Thin layer chromatography& Fourier transform-infrared spectroscopy

The calculated Rf value 0.68 confirmed the presence of the rhamnolipid as a red spot i.e. a glycolipid biosurfactant. In our study similar to the findings of rhamnolipid produced from

P. aeruginosa in TLC plate (Rashedi *et al.*,2005; Kannahi and Sherley, 2012). The FTIR studies shows C-H stretching bands of -CH2- and -CH3 groups were observed in the region 3000–2700 cm^{-1}. The carbonyl stretching peak was observed at 1635 cm^{-1}, shows the presence of ester compounds (Plate 1). The FTIR studies shows C-H stretching bands of -CH2- and -CH3 groups were observed in the region 3000–2700 cm^{-1}. The carbonyl stretching peak was observed at 1635 cm^{-1}, shows the presence of ester compounds.

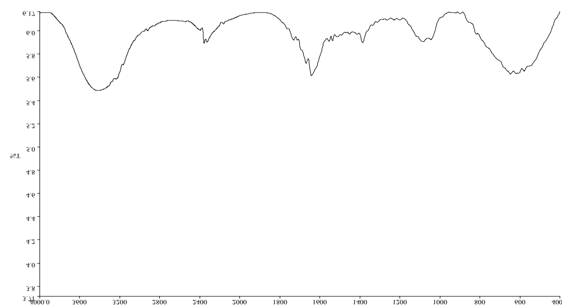

Plate 1. Fourier transform-infrared spectroscopy

Several C-H stretching bands of -CH2- and -CH3 groups were observed in the region 3000–2700 cm^{-1}. The carbonyl stretching peak was observed at 1635 cm^{-1}, which is characteristic of ester compounds. The ester carbonyl group was also confirmed from the peak at 1013 cm^{-1}, which corresponds to C-O stretching vibration as described by Tuelva *et al.,* 2002.

Molecular identification of *Pseudomonas aeruginosa*

The potential biosurfactant producing bacteria, BS9 *Pseudomonas aeruginosa* was ascertained its systematic position based on 16S rRNA sequence analysis and with the aid of BLAST homology analysis. The isolate BS9 (1343 bps) showed the maximum homology (99%) with other *Pseudomonas aeruginosa.*

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