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Isolation, characterization and identification of bacterial biosurfactant

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ABSTRACT

Biosurfactants are extracellular, amphiphilic compounds produced by microorganisms as secondary metabolites which has a wide variety of application in food, pharmaceutical and cosmetics as well as in bioremediation purpose. In this study, two potential biosurfactant producing bacterial strains were isolated from diesel oil contaminated soil sites in Dindigul ,Tamilnadu, India and were identified as Pseudomonas sp and Bacillus sp. The biosurfactant production from the two bacterial strains were determined by standard qualitative methods. Both the bacterial strains has better emulsifying activity and surface tension reduction. Based on the chemical and FT-IR spectra analysis the biosurfactant compound produced by the Pseudomonas sp and Bacillus sp were identified as glycoprotein and lipoprotein respectively.

Keywords: Bacteria, Biosurfactants, bioremediation, glycoprotein, lipoprotein

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INTRODUCTION

Biosurfactants are surface active agents can be produced by yeasts, bacteria and fungi (1). These biosurfactants are amphiphilic molecules consisting of hydrophilic and hydrophobic domain. Due to their amphiphilic nature, biosurfactants can reduce surface tension, Critical Miscelle Concentration (CMC), in both aqueous solution and hydrocarbon mixture (2). When the carbon source is an insoluble substrate like a hydrocarbon, microorganisms facilitate their diffusion into cell by producing biosurfactant. Biosurfactants have several advantages including low toxicity, environmental friendly, high biodegradability, low irritancy, higher foaming, high selectivity and specific activity at extreme temperature, pH and salinity (3) and compatibility with human skin. They possess a wide range of industrial application such, health care, oil & food processing industries, pharmaceuticals and a potential place in bioremediation. In cosmetic industry surface active substances are found in shampoo and many skin care products (4). Hence this present study is focussed on the isolation of biosurfactant producing bacterial strains from hydrocarbon contaminated soil.

MATERIALS AND METHODS

Isolation and enrichment of biosurfactant producing bacteria

Two biosurfactant producing bacterial strains were isolated and enriched from diesel oil contaminated soil in Dindigul (10.3540^0N, 77.9850^0E), Tamil Nadu, India using the standard methods (5).

Preliminary test for biosurfactant production

Hemolytic activity (HA)

The two bacterial isolates were inoculated on blood agar medium (5% of fresh human blood) and incubated at 28^0 C for 48-72 hours. Hemolytic activity were asssesed based on α, β and γ type hemolysis to ensure preliminary conformation on biosurfactant activity and results were recorded **(**6) .

Screening for microbial biosurfactant

The selected two bacterial isolates were grown in mineral salt medium supplemented with diesel (1%) as carbon source at 37^0C in shaking incubator with 150 rpm for 24hrs.The supernatant of each bacterial isolates were collected using centrifugation at 10000 rpm for 15 min cooling centrifuge (Version Remi (24). The biosurfactant property of bacterial culture supernatant was assesed by standard techniques as follows:

(i) Drop collapse test

The supernatant from the two different bacterial cultures (24 hrs old) were taken in a clean glass slide individually and added with 0.1ml of diesel oil. The flatening property of diesel oil was observed for a period of 1 minute and the result were recorded (7).

(ii) Oil spreading test

40 µl of distilled water was added to a Petri dish (15 cm diameter) followed by the addition of 20 µl of diesel oil to the surface of water then 10 µl of culture extract from the two bacterial isolates were dropped on to the oil surface. The diameter of clear zones on the oil surface was measured and the results were recorded (8).

(iii) Emulsification index *(%EI_{24})*

Equal volume of culture supernatant of two bacterial isolates with diesel oil (1:1) were mixed in a glass tube (125mm x 15mm). Then the mixture was vortexed for 2 minutes and left to stand for 24 hours. The emulsification index was calculated by the dividing height of the emulsion layer by total Height × 100 (9).

(iv) Blue agar plate method

Bushnell- Hass agar medium (BHA) supplemented with glucose as a carbon source (2%) and cetyltrimethyl ammonium bromide (CTAB-0.5 mg/ml) and methylene blue (0.2 mg/ml) were used for determination of anionic biosurfactants. Different wells (4mm dia) with equal distance were prepared in blue agar plate using cork borer. 30 µl of culture extract were loaded into respective labelled wells and incubated at 37^0 C for 48-72 hours. A dark blue halo zone was observed and the results were recorded (10)..

(v) Surface tension measurement

The plate and glassware were cleaned with chromic acid, Milli-Q water, and acetone. They were then flamed with a Bunsen burner. The instrument was calibrated with pure water and all the measurements were taken in triplicate. The surface tension property was studied by taking a 40 ml sample of the 72 hrs old culture broth of two bacterial isolates and centrifuging at 10000 rpm for 20 min. The cell pellet was discarded, and the surface tension of the supernatant was measured using a surface tensiomat model 21 tensiometer (Fisher Scientific) by the Du Noüy Ring method. The criterion used for selecting biosurfactant-producing isolates was the emulsification and reduction of the surface tension of the medium to below 40 dynes/cm (11).

Production, Extraction and identification of bacterial biosurfactants

a) Production of biosurfactant

The inoculum was prepared using Luria Bertani (LB) broth and incubated at 37◦C for over night with 100 rpm agitation.The fresh overnight culture were used as an inoculum for the production of biosurfactant.To the 100 ml of production medium with 2% diesel oil ,1% inoculum was transferred aseptically and incubated at 37^0C for 48 hrs at 120 rpm in shaking incubator (12).

b) Extraction of biosurfactant

The culture supernatant of the two bacterial isolates were obtained by centrifugation at 10000 rpm for 15 minutes and then filtered through Millipore membrane system. The biosurfactant was recovered from the cell free culture supernatant by cold acetone precipitation in which three volumes of chilled acetone was added and allowed to stand for 10 h at 4°C. The precipitate was collected by centrifugation and evaporated to dryness to remove residual

acetone after which it was re-dissolved in sterile water.The resulting product was considered as the crude biosurfactant for further analysis (13).

c) Identification of biosurfactant using biochemical and FT-IR analysis

The crude biosurfactant thus collected from the bacterial isolates were subjected to a series of tests viz., ninhydrin test for aminoacid, iodine test for carbohydrates, solubility and saponification test for lipids and acrolein test for glycerol by the standard methods. Further the product was confirmed by Fourier Transform Infrared Spectrometer (Model spectra 100 series, Perkin-Elmer Corporation, Norwalk, CT, USA) (14).

Statistical analysis

All the experiments were carried out with three independent replicates.Inorder to verify significant difference,results were evaluated statistically,at 95% confidence level ($p<0.05$) using Graphpad Prism 5.

RESULTS AND DISCUSSION

The oil spreading technique is very sensitive in detecting low level of biosurfactant producers (15). The quantitative analysis including emulsification index ($\%EI_{24}$) (16) and surface tension measurement test was found to be more reliable method for the quantification of the soluble biosurfactant producer if it is reduced, the surface tension below 40 dynes/cm (17) and or maintained atleast 50% of the original emulsion volume in 24 hrs after the formation of emulsification which determines the productivity of bioemulsifiers (18). In this study, the biosurfactant producing ability of the two bacterial isolates such as *Pseudomonas* sp. and *Bacillus sp* were screened and the result showed positive for hemolytic activity, drop collapse test, oil displacement test, emulsification index ($\%EI_{24}$) and surface tension measurement (Table1). Similiar results were obtained in the biodegradation of automobile oil (19).

Among the two bacterial strains, *Pseudomonas* sp comparatively showed higher reduction in surface tension (51.9±0.07 dynes/cm) and a higher percentage of emulsification at 24 hrs (78.5±0.00) (Table2). The statistical analysis showed a positive correlation between emulsification data and the day interval and in Pearson r correlation, the values are significant ($p<0.05$) , r^2 =0.98 and 0.93 *Pseudomonas* sp. and *Bacillus* sp. for respectively. In linear regression 95% of confidence interval was analysed (20).

The biosurfactant produced by the two bacterial strains were futher analyzed chemically for the presence of amino acids, carbohydrates, lipid and glycerol. The results of biochemical characteristics of the two biosurfactant were recorded in table 3. The *Bacillus* sp. showed positive results for Ninhydrin test indicates the presence of amino acid by the formation of violet blue colour and iodine test indicates the presence of carbohydrate by reddish brown complex formation. No colour formation in *Pseudomonas sp.* reveal absence of both the amino acid and carbohydrate in their biosurfactant. In Bial's test for pentose sugar blue green coloured complex observed in *Pseudomonas sp.* indicates the presence of sugar but is absent in the *Bacillus* sp. In solubility test for lipids biosurfactant from both the strains were insoluble in water whereas soluble in alcohol and choloform which determines the presence of lipid. In acrolin test for glycerol, the tested two biosurfactant doesnot produce pungent smell that reveals the absence of glycerol. Similar results on chemical analysis of biosurfactant was reported (21) and (22).

Biochemical composition of the biosurfactant produced by *Bacillus* sp revealed that, it is a mixture of lipid and protein. Futher,by FT-IR analysis, strong bands at 3431 cm-1, indicating the presence of a peptide component resulting of the N-H stretching mode and at 1656cm-1 resulting of the stretching mode of CO-N bond (Fig.1). C–H stretching bands of -CH3 and -CH2 groups were observed in the region 3000 cm-1 to 2000 cm-1 . Deformed vibration of a CH2 group adjoining a carboxyl ester was also observed at 1350 cm-1. Infrared spectrum strongly suggest that the extracted biosurfactant from *Bacillus* sp belongs to lipopeptide type.Similar studies reported that three different strains of *Bacillus substilis* produce biosurfactant belongs to lipopeptide type (23).

Whereas,biochemical composition of the biosurfactant produced by *Pseudomonas* sp revealed that, it is a mixture of lipids and sugar. Futher with FT-IR Spectrometry, a broad absorption valley at 3400 cm-1 indicating the presence of OH groups in the molecules. Strong absorption valleys observed in the range from 2720 to 2810 cm-1 demonstrated typical CH stretching variation in the alkaline chain. Absorption valleys at 1606 cm-1 indicated stretching vibration of C-O and C = O bonds in carboxyl esters. Scissoring vibration of a CH2 group adjoining a carboxyl ester was also observed at 1352 cm-1 .The peak in the region of 1105 cm−1 indicates C–O–C stretching in the rhamnose (Fig.2).

These results confirm that the extracted biosurfactant from *Pseudomonas* sp belonged to rhamnolipid type. Studies on the biosurfactant produced by *Pseudomonas aeroginosa* LBM10 was belonged to rhamnolipid type (24),(25) and (26)

Table 1: Qualitative characteristics of biosurfactant produced by *Pseudomonas* sp. And *Bacillus* sp

S. No	Name of test	Qualitative results of two bacterial strains	
		***Bacillus* sp**	***Pseudomonas* sp**
1.	Drop collapse test	+	++
2.	Oil spreading test	+	+
3.	$\%EI_{24}$	++	+
4.	Blue Agar plate	+	++

+ = efficient, ++ = very efficient

Table 2: Biochemical characterics of biosurfactant compound produced by *Pseudomonas* sp (1) and Bacillus sp (2)

S .No	Test	Colour change	Result1	Result2
1	**Amino acid:** Ninhydrin test	No color change	-ve	+ve
2	**Carbohydrate:** Anthrone test	Bluish green	+ve	-ve
	Iodine test	No color change	-ve	-ve
	Barfoed's test	Formation of red precipitate	+ve	-ve
	Bial's test	No color change	-ve	-ve
3.	**Lipid test:** Solubility test	Insoluble in water but soluble in organic solvents	+ve	+ve
	Saponification	Formation of soap bubbles	+ve	+ve
4.	**Glycerol test:** Acrolein	No color change	-ve	-ve

Table 3: Surface Tension and E24 index of biosurfactants produced by *Pseudomonas* sp and *Bacillus* sp

S.No.	Organism	Surface Tension (dynes/cm)	E24 index
1.	*Bacillus* sp	57.6±0.07	64.68±0.03
2.	*Pseudomonas* sp	51.9±0.05	78.5±0.03

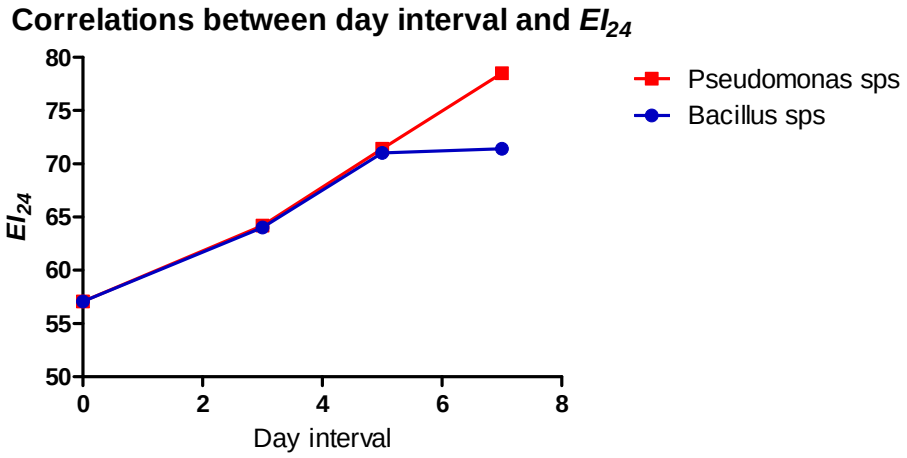

Fig 1: A graph on correlation between day interval and EI_{24} by the two microbial biosurfactants
r^2 =0.98 and 0.93

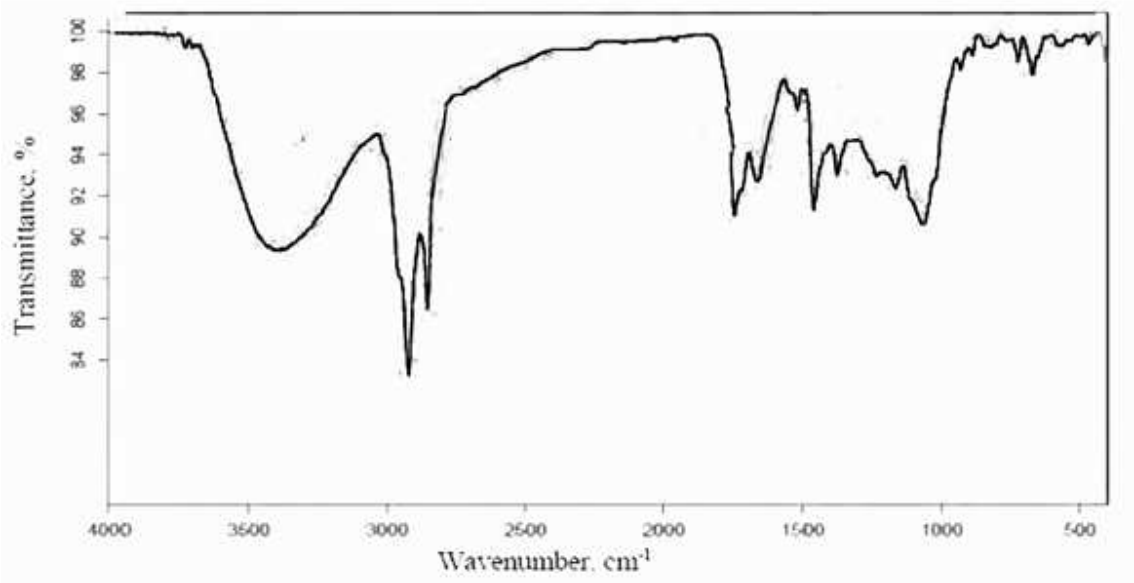

Fig 2: (a).FTIR spectra of biosurfactant produced by *Bacillus* sp.

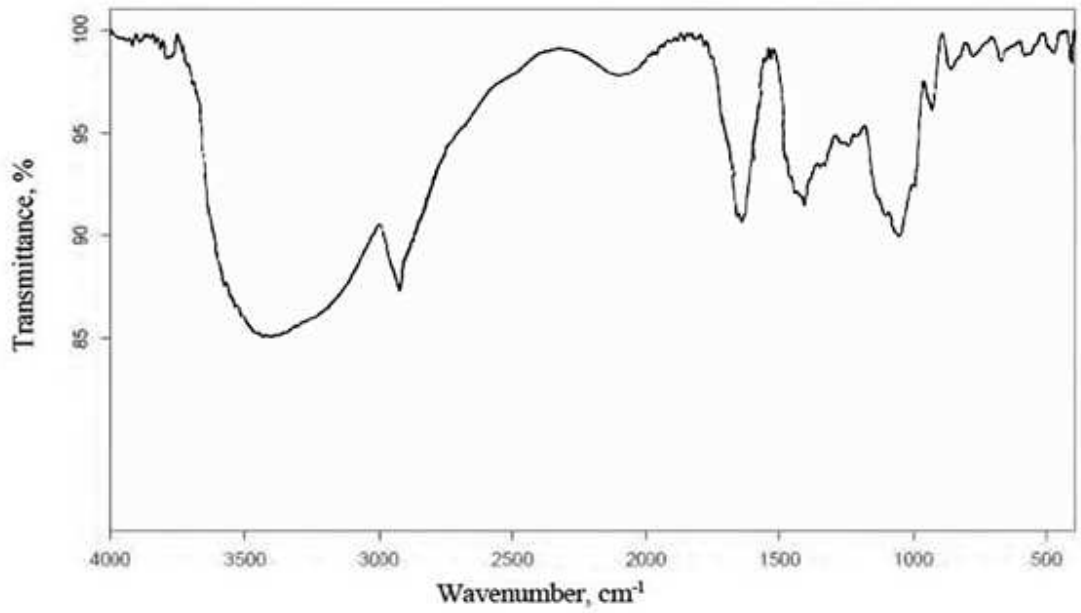

Fig 2: (b) FTIR spectra of biosurfactant produced by *Pseudomonas* sp.

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