Biosurfactants from marine bacterial isolates

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Biosurfactants are extracellular surface active compounds produced by bacteria, fungi and yeast. Most microbial surfactants are complex molecules, comprising different structures that include lipopeptides, glycolipids, polysaccharide protein complexes, fatty acids and phospholipids. In the past two decades, biosurfactants have gained increasing attention due to their useful properties such as biodegradability, low toxicity, ecological acceptability and ability to be produced from renewable and cheaper substrates. The range of industrial applications of biosurfactants includes enhanced oil recovery, crude oil drilling, lubricants, and bioremediation of environmental pollutants, health care, food processing, medical applications as adjuvants and as antimicrobial biocontrol agents. In this chapter we report on an investigation to explore biosurfactant producing marine bacteria. The main criteria used for the isolation of biosurfactants producers were hemolytic assay, bacterial adherence to hydrocarbons (BATH), emulsification of crude oil and the drop-collapse test. Bacterial strains were isolated and subjected to screening tests for biosurfactants production. 3 bacterial strains were found as potential biosurfactant producers and identified as *Bacillus megaterium, Corynebacterium kutscheri* and *Pseudomonas aeruginosa*. Crude oil was used as a carbon source for biosurfactant production in shake flask fermentation experiments to optimize the culture conditions. Fermentor production of biosurfactant was carried out with economically cheap or sustainable carbon sources such as waste motor lubricant oil and peanut oil cake. Preliminary characterization of biosurfactant products for isolated *B. megaterium, C. kutscheri* and *P. aeruginosa* were glycolipid, glycolipopetide and lipopeptide respectively.

Key Words: Biosurfactant, glycolipid, glycolipopetide, lipopeptide

1. Introduction

Biosurfactants are amphiphilic compounds produced by bacteria, fungi and yeast. They belong to various classes including glycolipids, lipopeptides, fatty acids, phospholipids, neutral lipids and lipopolysaccharides [1]. The properties/applications of biosurfactants includes excellent detergency, emulsification, foaming, dispersing traits, wetting, penetrating, thickening, microbial growth enhancement, metal sequestering and resource recovering (oil) which allows biosurfactants an ability to replace some of the most versatile process chemicals. In addition biosurfactants are promising natural surfactants that offer several advantages over chemically synthesized surfactants, such as lower toxicity, biodegradability and ecological acceptability.

Although biosurfactants exhibit such important advantages, they have not been yet employed extensively in industry because of relatively high production costs. One possible strategy for reducing costs is the utilization of alternative substrates such as agro-industrial wastes [2]. The main problem related to use of alternative substrates as culture medium is to find a waste with the right balance of nutrients that permits cell growth and product accumulation [3]. Molasses [3], peat hydrolysate [4] and potato processing effluents [5] are examples of alternative substrates that have been suggested for biosurfactant production. The establishment of waste-based medium for biosurfactant production also faces another problem, in relation to the properties of final product which are dependent on the composition of the culture medium [6]. Hence in the present study an attempt was made to screen new biosurfactant producing bacteria capable of growth on cheap carbon sources. The objectives of the present study includes: i. screening of marine bacteria for biosurfactant production, ii. Characterization of biosurfactants produced and iii. Evaluation of cheaper carbon sources for large scale production of biosurfactants. In this study peanut oil cake and waste motor lubricant oil were tested as cheap carbon sources for biosurfactant production. Peanut oil cake is a carbohydrate, protein and lipid rich residue generated in large amounts during the production of peanut oil and the cost of this cake is very low when compared to other carbon sources such as glucose, fructose, crude oil and other hydrocarbons. Waste motor lubricant oil is waste oil drained from geared motor vehicles after a long run, which contains weathered hydrocarbon fractions and may be a suitable substrate for biosurfactant production.

2. Materials and methods

2.1. Screening for biosurfactant production

2.1.1 Microorganism and Hemolytic activity

B. megaterium, C. kutscheri and *P. aeruginosa* were isolated from water sample collected at Tuticorin harbor (08°45'N; 78°13'E) South East coast of India using Bushnell–Haas agar with 0.1% of crude oil and identified to the species level by Thavasi and Jayalakshmi [7] following Bergey's Manual of determinative bacteriology [8].

Hemolytic assay was performed in blood agar plates [9]. 50µl broth culture was spot-inoculated on to blood agar plates and incubated for 48h at 37°C. The plates were visually inspected for zone of clearance (hemolysis) around the colony. The diameter of the zone of clearance is a qualitative method used as an indicator of biosurfactant production [9,10].

2.1.2 Bacterial adhesion to hydrocarbons (BATH)

Cell hydrophobicity was measured by BATH assay according to a method similar to that described by Rosenberg et al.[11]. Bacterial cells were washed twice and suspended in a buffer salt solution (g/l 16.9 K_2HPO_4, 7.3 KH_2PO_4) to give an optical density (OD) at 600 nm of ~ 0.5. The cell suspension (2ml) with 100µl crude oil added was vortex-shaken for 3 min in test tubes (10x100mm). After shaking, crude oil and aqueous phase were allowed to separate for 1h. OD of the aqueous phase was then measured at 600nm in a spectrophotometer. Hydrophobicity is expressed as the percentage of cell adherence to crude oil calculated as follows: 100 x (1-OD of the aqueous phase/OD of the initial cell suspension). For a given sample, three independent determinations were made and the mean value was accounted.

2.1.3 Visualization of bacteria in oil droplets

A few drops of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) solution was added to the BATH assay culture broth and observed under the microscope. The INT turned red if it was reduced inside the cells, indicating the viability and adherence of cells with crude oil droplets [12].

2.1.4 Drop-collapse test

All three bacterial strains were cultured in mineral salts medium with 0.1% crude oil for 48 h. Screening of biosurfactant production was performed using the qualitative drop-collapse test described by Jain et al.[13] as modified by Bodour and Miller-Maier [14]. Crude oil was used in this test, 2µl of oil was applied to the well regions delimited on the covers of 96-well microplates and these were left to equilibrate for 24h. 5µl of the 48h culture, after centrifugation at 12,000g for 5 min to remove cells, was transferred to the oil-coated well regions and drop size was observed 1 min later with the aid of a magnifying glass. A result was considered positive for biosurfactant production when the drop diameter was at least 1mm larger than that produced by deionized water (negative control).

2.1.5 Emulsification assay

Partially purified biosurfactant (5mg) was dissolved in 5ml of Tris buffer (pH 8.0) in 30ml test tubes. Hydrocarbons like waste motor lubricant oil, crude oil, peanut oil, diesel, kerosene, naphthalene, anthracene and xylene were tested for emulsification activity. 5mg of hydrocarbon was added to the above solution and shaken well for 20 min the mixture was allowed to stand for 20 min. The optical density of the emulsified mixture was measured at 610nm and the results were expressed as D_{610} [15]. Emulsification activity of the biosurfactant was compared with Triton X-100 (1mg/ml), concentration and conditions for emulsification study were maintained similar to that of biosurfactant.

2.2. Cell growth and Biosurfactant production

2.2.1 Biosurfactant production in fermentor

Laboratory scale biosurfactant production was performed in a 3L fermentor (Scigenics, India Pvt. Ltd. Chennai) with a 2.1L working volume. Optimization of culture conditions was carried out and reported elsewhere [16]. The culture conditions are as follows - pH 8.0, temperature 38°C, salinity 30‰ (w/v) and 2.0% substrate concentration and 8.0 mg/l of dissolved oxygen (DO). Substrates used were crude oil, peanut oil cake and waste motor lubricant oil.

2.2.2 Estimation of growth

Two milliliters of culture broth was collected at 12h intervals for a period of 168h and the biomass was estimated gravimetrically. For gravimetric estimation of biomass 1ml of broth culture was taken and allowed to stand for 20 min. When the oil phase separated, the bottom phase with cells was siphoned out and filtered through a 0.45µm sized Millipore filter paper. The filter paper with cells was dried at 80^{o}C in a hot air oven for a period of 24h and weighed; a control was maintained to exclude the weight of crude oil adhered to the filter. Biomass was quoted in terms of mg/ml (dry weight).

2.2.3 Purification of biosurfactant

Culture broth was centrifuged at 12,000g for 20min and extracted twice with chloroform and methanol (2:1v/v). The solvents were removed by rotary evaporation and the residue was purified in a silica gel (60–120 mesh) column and the elution were made with chloroform and methanol ranging from 20:1 to 2:1v/v in a gradient manner and 10 fractions were obtained. The fractions were pooled and the solvents were evaporated, the resulting residue was dialysed against distilled water and lyophilized [17]. Weight of the biosurfactant was expressed in terms of mg/ml (dry weight).

2.3 Characterization of biosurfactant

2.3.1 Biochemical composition of biosurfactant

Chemical composition of the biosurfactant was analyzed following standard methods. Carbohydrate content of the biosurfactant was determined by the phenol–sulfuric acid method [18] using D-glucose as a standard. Protein content was determined by the Lowry et al.[19] method using bovine serum albumin as a standard and lipid content was estimated adopting the procedure of Folch et al.[20].

2.3.2 Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) is most useful for identifying types of chemical bonds (functional groups), therefore can be used to elucidate some components of an unknown mixture. One milligram of freeze-dried partially purified biosurfactant was ground with 100mg of KBr and pressed with 7500kg for 30 seconds to obtain translucent pellets. Infrared absorption spectra were recorded on a Thermo Niocolet, AVATAR 330 FTIR system with a spectral resolution and wave number accuracy of 4 and 0.01cm^{-1}, respectively. All measurements consisted of 500 scans, and a KBr pellet was used as background reference.

2.3.3 Mass spectrometric analysis of biosurfactant

Biosurfactant was dissolved in methanol and mixed thoroughly. The mass spectrometric analysis of the biosurfactant was carried out in LCQ^{TM} quadrupole iontrap mass spectrometer (Finnigan MAT, San Jose, California, USA) utilizing electrospray ionization (ESI). Standard solutions and samples under investigation were infused into the mass spectrometer at a flow rate of 10µl/min. In the ESI, nitrogen and auxiliary gas flows were maintained at 50 and 5ml/min respectively and refer to arbitrary values set by the software. The heated capillary temperature was 250^{o}C and the spray voltage was set to 5kV. Negative ion mode was used and scanning was done at 50–2,000 *m/z* range.

3. Results and Discussion

3.1 Screening for biosurfactant production

The haemolytic activity results revealed that maximum activity was found in *P. aeruginosa* (3cm) followed by *B. megaterium* (2cm), and *C. kutscheri* (1.8cm). Hemolysis was included in this study since it is widely used to screen biosurfactant production and in some cases, it is the sole method used [21,22,]. Mulligan et al. [9] recommended blood agar lysis as a preliminary screening method for biosurfactant production. The hemolytic activity of biosurfactants was first discovered when Bernheimer and Avigad [23] reported that the biosurfactant produced by *B. subtilis*, surfactin, lysed red blood cells. Blood agar lysis has been used to quantify surfactin [24] and rhamnolipids [25] and has been used to screen biosurfactant production by new isolates [21,26]. Carrillo et al. [26] found an association between hemolytic activity and surfactant production, and they recommended the use of blood agar lysis as a primary method to screen biosurfactant production. However, in some cases hemolytic assay excluded many good biosurfactant producers [27]; hence in the present investigation BATH assay and drop collapse test with crude oil were included to confirm biosurfactant production.

Table 1. Emulsification of hydrocarbons by biosurfactant isolated from [a]*B. megaterium*, [b]*C. kutscheri*, [c]*P.aeruginosa*

Hydrocarbons	Emulsification activity (D_{610})			
	a	b	c	Triton X 100
Waste motor lubricant oil	1.86	1.72	2.01	1.93
Crude oil	1.72	1.69	1.97	1.85
Peanut oil	1.42	1.31	1.95	1.56
Kerosene	1.01	0.95	1.12	1.12
Diesel	0.85	0.81	0.90	0.94
Xylene	0.53	0.51	0.61	0.78
Naphthalene	0.46	0.41	0.51	0.63
Anthracene	0.42	0.41	0.45	0.58

BATH assay results revealed that *P. aeruginosa* showed high affinity (95.3%) with crude oil followed by *C. kutscheri* (49.7%) and *B. megaterium* (40.2%). Visualization of bacterial cells adhered with crude oil using INT staining also confirmed the BATH results. All the strains showed a positive drop collapse activity with crude oil. Both the drop-collapse and visualization of cells adhered to crude oil have several advantages in requiring a small volume of samples, are rapid and easy to carry out, and do not require specialized equipment.

Emulsification activity of the isolated biosurfactant from all three strains were in the order of waste motor lubricant oil > crude oil > peanut oil > kerosene> diesel > xylene > naphthalene > anthracene and emulsification activity results for each strain for different hydrocarbons were listed in Table (1). These emulsification results showed that, biosurfactant produced from a substrate can emulsify different hydrocarbons to a greater extent which confirmed its applicability against different hydrocarbon pollution.

3.2 Cell growth and Biosurfactant production

Three carbon substrates were used in this study for biosurfactant production namely crude oil, waste motor lubricant oil and peanut oil cake. Among the three substrates used, maximum growth and biosurfactant production was found with peanut oil cake. *P. aeruginosa* seems to be the maximum producer of biosurfactant for all the substrates used followed by *B. megaterium* and *C. kutscheri* (Fig. 1). Waste motor lubricant oil and peanut oil cake used in the present study were economically more viable than crude oil. Youssef et al. [28] reported a maximum biosurfactant production of 90mg/l by *B. subtilis* using glucose as carbon source. Cooper and Goldenberg [29] reported 1.6g/l of biosurfactant production by *B. cereus* using sucrose as a carbon source. Where as in the present study we have used waste motor oil and peanut oil cake as carbon sources, biosurfactant production also found higher than their study (7.8mg/ml). The possibility of biosurfactant production using cheaper carbon sources was already reported by earlier workers; olive oil mill effluent and animal fat [30], frying oil [31], molasses [32], and starch-rich wastes [33] supporting the present study on use of renewable carbon sources for biosurfactant production. Biosurfactant produced in the present study using waste motor lubricant oil and peanut oil cake showed good emulsification activity against seven different hydrocarbons and peanut oil. Further it encouraged the aim of the present study to produce biosurfactants from cheaper carbon sources with emulsification property against seven different hydrocarbons.

Parallel increases in biomass and biosurfactant production were found from 12h to 120h, but maximum biosurfactant concentration was found at 132h (Fig.1). Higher concentrations of the biosurfactant even after the offset of growth may be because of the release of cell-bound biosurfactant at the early stationary phase (132h), which leads to an increase in extracellular biosurfactant concentration in the medium [34].

3.3 Characterization of biosurfactant

The biosurfactant produced by *B. megaterium* was classified as a glycolipid with carbohydrate and lipid combination of 28:70%. The FTIR analysis of the biosurfactant revealed that, the most important bands were located at 2929cm^{-1} (for the CH aliphatic stretching), 1700cm^{-1} (for the C=O ester bond), 1066cm-1 (PII band: polysaccharides) and 764, 699cm^{-1} (for the CH_2 group) and 3342cm^{-1} (for O–H bonds) confirming the presence of glycolipid moieties [10]. In addition, the mass spectrometric analysis of the biosurfactant also confirmed the above results with peaks observed at m/z = 326.5, 413.3, 429.3 for lipids and at 663.4 for carbohydrate moieties [35].

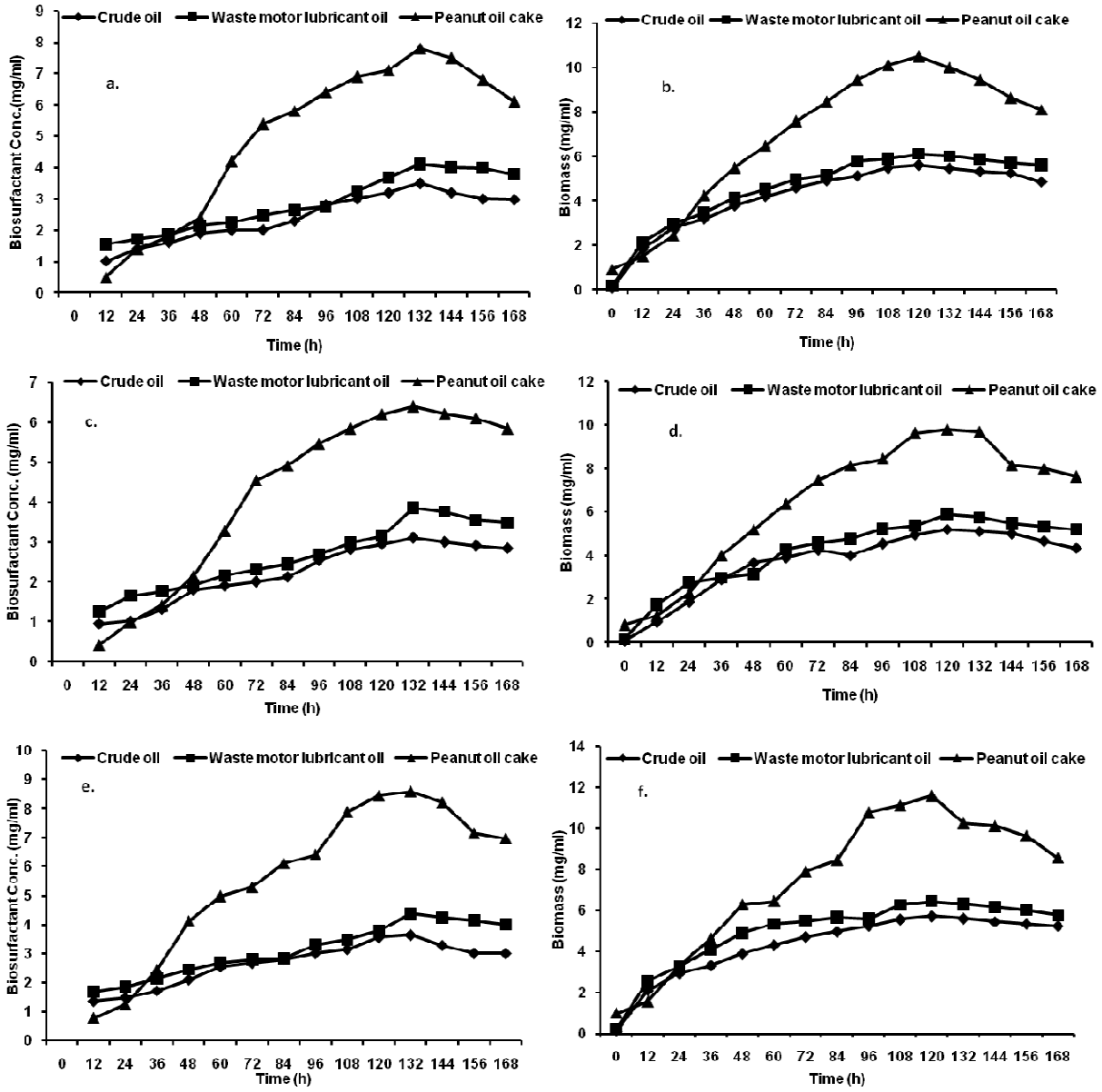

Fig. 1. Biosurfactant production and growth by *B.megaterium* (a,b), *C.kutscheri* (c,d) and *P.aeruginosa* (e,f).

Biosurfactant isolated from *C. kutscheri* is a mixture of carbohydrate, lipid and protein with a combination of 40:27:29% respectively. FTIR spectral analysis of the biosurfactant inferred that, wave number 3513cm^{-1} indicated the presence of carboxylic acids and 3444cm^{-1} for N-H/C-H bonds of protein. CH_2/C-H asymmetric vibrations were found at 2918 and 2858cm^{-1} which confirmed the (C-H) presence of alkanes. CH and CH_2 deformation was found at 880 and 856cm^{-1}. Presence of C-O bond was found at 1113, 1022, 797, and 711cm^{-1} The above information from the respective wave numbers confirmed the glycolipopeptide nature of the biosurfactant. The mass spectrometric analysis of the biosurfactant also complements the biochemical and FTIR results that, the peaks observed at *m/z* = 326, 413, 663 and 1075 were corresponding to carbohydrate, lipid and protein moieties. Glycolipopeptide nature of the biosurfactant produced in the present study was similar to the results obtained by earlier workers in *Corynebacterium* spp. [36].

Chemical composition of the biosurfactant isolated from *P.aeruginosa* is a mixture of lipid and protein with a combination of 49.8:50.2% respectively. FTIR analysis of the biosurfactant showed that, wave numbers 3422 and 3246cm^{-1} for N-H bonds indicated the presence of amine groups. C-H bonds of the CH_3, CH_2 and CH groups observed at wave numbers 2962, 2923, 2863, 1481 and 1425cm^{-1} which confirmed the presence of alkanes. The wave number 1650cm^{-1} (amide I bond) indicated the presence of peptide groups. The wave number 1066 cm^{-1} indicated the presence of C-O bonds. The above information from the respective wave numbers confirmed the lipopeptide nature of the biosurfactant. The mass spectrometric analysis of the biosurfactant also complements the biochemical and FTIR results that, the peaks observed at *m/z* = 1076.2, 1347.3, 1348.4 and 326.5, 413.3, 29.3 indicated the presence of proteins and

lipid moieties. Analysis of lipopeptide biosurfactant produced by *P. putida* using mass spectrometry was reported by Kupier et al. [37] and their results were correlated with peaks observed in this study.

This work aims to contribute to biosurfactant production using economically cheaper carbon sources. Peanut oil cake proved to be a suitable substrate for biosurfactant biosynthesis, providing not only bacterial growth and product accumulation but also a surfactant that has interesting and useful properties with potential of many industrial applications. Further research on structural characterization, gene regulation of biosurfactant production and cost of production are in progress.

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