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ORIGINAL ARTICLE

Biosurfactant Production by *Pseudomonas aeruginosa* from Renewable Resources

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Abstract This study deals with production and characterization of biosurfactant from renewable resources by *Pseudomonas aeruginosa.* Biosurfactant production was carried out in 3L fermentor using waste motor lubricant oil and peanut oil cake**.** Maximum biomass (11.6 mg/ml) and biosurfactant production (8.6 mg/ml) occurred with peanut oil cake at 120 and 132 h respectively. Characterization of the biosurfactant revealed that, it is a lipopeptide with chemical composition of protein (50.2%) and lipid (49.8%). The biosurfactant (1 mg/ml) was able to emulsify waste motor lubricant oil, crude oil, peanut oil, kerosene, diesel, xylene, naphthalene and anthracene, comparatively the emulsification activity was higher than the activity found with Triton X-100 (1 mg/ml). Results obtained in the present study showed the possibility of biosurfactant production using renewable, relatively inexpensive and easily available resources. Emulsification activity found with the biosurfactant against different hydrocarbons showed its possible application in bioremediation of environments polluted with various hydrocarbons.

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Introduction

Many microorganisms produce extracellular or membrane associated surface-active compounds (biosurfactants). Biosurfactants are organic compounds belonging 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 make surfactants replace some of the most versatile process chemicals [1]. 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 cost. One possible strategy for reducing cost 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 [4], peat hydrolysate [5] and potato process effluents [6] are examples of alternative substrates that have been suggested for biosurfactant production by *Bacillus megaterium.* The establishment of waste-based medium for biosurfactant production also faces another problem; once the kind and the properties of final product are depend on the composition of culture media [7].

Although biosurfactants have many interesting properties, their industrial importance depends on the ease of production [8]. Low yield of biosurfactant is a major limitation influencing its commercialization. Hence, in the present study peanut oil cake and waste motor lubricant oil were tried as cheaper carbon sources as compared to glucose and other petroleum based substrates for biosurfactant production. Peanut oil cake is a carbohydrate, protein and lipid rich residue generated at large amounts during the production of peanut oil, the cost of this cake is also very low when compared to other carbon sources like 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 useful for the biosurfactant production. By considering the importance of biosurfactants and their role in biodegradation of hydrocarbons and other industrial applications, the present investigation was conducted with the following objectives: production of biosurfactant by *P. aeruginosa* using waste motor lubricant oil and peanut oil cake, estimation of emulsification activity of the biosurfactant against hydrocarbons and influence of biosurfactant addition on biodegradation of crude oil.

Materials and Methods

Organism

Pseudomonas aeruginosa was isolated from sea water sample collected at Tuticorin harbor (08°45′ N; 78°13′ E) using Bushnell Haas agar supplemented with 0.1% (v/v) of crude oil and identified to species level by following Bergey's Manual of Determinative Bacteriology [9].

Media and Growth Conditions

Biosurfactant production was carried out in a 3 L laboratory fermentor with working volume of 2.1 L (Scigenics India Pvt. Ltd., Chennai). The strain was cultured in mineral medium (g/l, 1.0 K_2HPO_4, 0.2 $MgSO_4{\cdot}7H_2O$, 0.05 $FeSO_4{\cdot}7H_2O$, 0.1 $CaCl_2{\cdot}2H_2O$, 0.001 $Na_2MoO_4{\cdot}2H_2O$, 30 NaCl). Optimization of culture conditions was carried out elsewhere [10]. The culture conditions are as follows—pH 8.0, temperature 38°C, salinity 30‰ (w/v) and 2.0% substrate concentration (waste motor lubricant oil/peanut oil cake) and 8.0 mg/l of dissolved oxygen (DO).

Estimation of Growth and Biosurfactant Production

Five ml samples of culture broth were collected at 12 h intervals for a period of 168 h. Biomass was estimated gravimetrically, broth culture was filtered through a Millipore filter paper (0.45 μm) and dried at 80°C in hot air oven and weighed. Biomass was quoted in terms of mg/ml (dry weight).

Biosurfactant concentration in the culture broth was estimated according to the procedure described by Li et al. [11] and the biosurfactant concentration was expressed as mg/ml. The culture broth was centrifuged at 6,000 rpm for 20 min at 4°C and extracted twice with chloroform and methanol (2:1 v/v). The solvents were removed by rotary evaporation and the residue was partially purified in silica gel (60–120 mesh) column eluted with chloroform and methanol ranging from 20:1 to 2:1 (v/v) in a gradient manner. The fractions were pooled and solvents were evaporated, resulting residue was dialyzed against distilled water and lyophilized.

Estimation of Emulsification Activity

Partially purified biosurfactant (5 mg) was dissolved in 5 ml of Tris buffer (pH 8.0) in 30 ml test tubes. Hydrocarbons like waste motor lubricant oil, crude oil, peanut oil, diesel, kerosene, naphthalene, anthracene and xylene were tested for emulsification activity. 5 mg 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} [12]. Emulsification activity of the biosurfactant was compared with Triton X-100 (1 mg/ml) (Hi-Media, Laboratories, Mumbai, India), concentration and conditions for emulsification study were maintained similar to that of biosurfactant. Stability of the emulsion was measured by measuring the height of the emulsion layer and monitored for 24 h.

Laboratory Scale Experiment on Biodegradation of Crude Oil with Biosurfactant

This experiment was conducted to study the impact of the biosurfactant isolated from *P. aeruginosa* on biodegradation of crude oil in natural sea water. Crude oil used in this study was obtained from Chennai Refineries Limited, Chennai, India. Its specific gravity was reported by them as 0.844 at 25°C. Seventy-five liter plastic tanks were filled with 50 L of filtered and UV treated sea water with 30‰ salinity, and pH 8.0. The experiment was conducted with four different sets—(i) bacterial cells alone, (ii) with fertilizer (Urea and Dipotassium Phosphate 0.1% w/v) and cells, (iii) with cells and biosurfactants (0.1% w/v) and (iv) with cells, fertilizer and biosurfactant. 2.0% (w/v) of crude oil was added to the filtered sea water, inoculation was done with 24 h old culture (10^3–10^4 CFU/ml) at the rate of

1% (v/v) concentration. Continuous aeration was provided at the rate 1.5 L min^{-1} with an oil free aerator and the set up was maintained at room temperature for a period of 168 h. Biodegradation of crude oil was estimated fluorometrically as described in Intergovernmental Oceanographic Commission Manuals and Guide No. 11 [13]. An uninoculated control was maintained to assess the natural weathering of crude oil.

Characterization of Biosurfactant

Biochemical Composition of Biosurfactant

Carbohydrate content of the biosurfactant was determined by the phenol–sulfuric acid method [14] using D-glucose as a standard. Protein content was determined by the method of Lowry et al. [15] using bovine serum albumin as a standard and lipid content was estimated by following the procedure of Folch et al. [16].

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. Freeze-dried crude biosurfactant (10 mg) was ground with 100 mg of KBr and pressed with 7,500 kg for 30 s 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 0.01 cm^{-1}, respectively. All measurements consisted of 500 scans, and a KBr pellet was used as background reference.

Mass Spectrometric Analysis of Biosurfactant

Biosurfactant was dissolved in methanol and mixed thoroughly. The mass spectrometric analysis of the biosurfactant was carried out in a LCQ^{TM} Quadrupole ion-trap mass spectrometer (Finnigan MAT, San Jose, California, USA) utilising electrospray ionisation (ESI). Standard solutions and samples under investigation were infused into the mass spectrometer at a flow rate of 10 µl min^{-1}. In the ESI, nitrogen and auxiliary gas flows were maintained at 50 and 5 ml/min respectively and refer to arbitrary values set by the software. The heated capillary temperature was 250°C and the spray voltage was set to 5 kV. Negative ion mode was used and scanning was done at 50–2,000 *m/z* range.

Results

Estimation of Growth and Biosurfactant Production

Physiological and biochemical characteristics of *P. aeruginosa* are illustrated in Table 1. Biosurfactant production was studied using 2.0% of waste motor oil and peanut oil cake. Figure 1 show the time-course of biosurfactant production by *P. aeruginosa* with waste motor oil as the substrate. Maximum biosurfactant concentration of 4.37 mg/ml occurred at 132 h of incubation, when the cells reached their early stationary phase. Maximum biomass was observed at 120 h (6.45 mg/ml). Biosurfactant production with peanut oil cake also showed similar trend, but with higher biosurfactant production (8.6 mg/ml) than waste motor lubricant oil (Fig. 1).

Estimation of Emulsification Activity

Biosurfactant isolated from *P. aeruginosa* and Triton X-100 showed maximum emulsification activity against waste motor lubricant oil. Emulsification of different hydrocarbons by the biosurfactant was in the order of waste motor lubricant oil > crude oil > peanut oil > kerosene > diesel > xylene > naphthalene > anthracene (Table 2). The emulsion formed by the biosurfactant against each hydrocarbon was stable up to 48 h. Emulsification of eight different hydrocarbons by the biosurfactant reflects the

Table 1 Characteristics of *P. aeruginosa*

Name of the test	Result
Gram reaction	−
Shape of the cell	Rod
Utilization of carbohydrates	
Adonitol	−
Glucose	+
Lactose	−
Trehalose	+
Xylose	+
Hydrolysis	
Starch	+
Gelatin	+
Fat	+
Catalase	+
Oxidase	+
Nitrate reduction	+
Litmus reaction	P
Decarboxylation	
Arginine	+

+ Positive, − negative, *P* peptonization

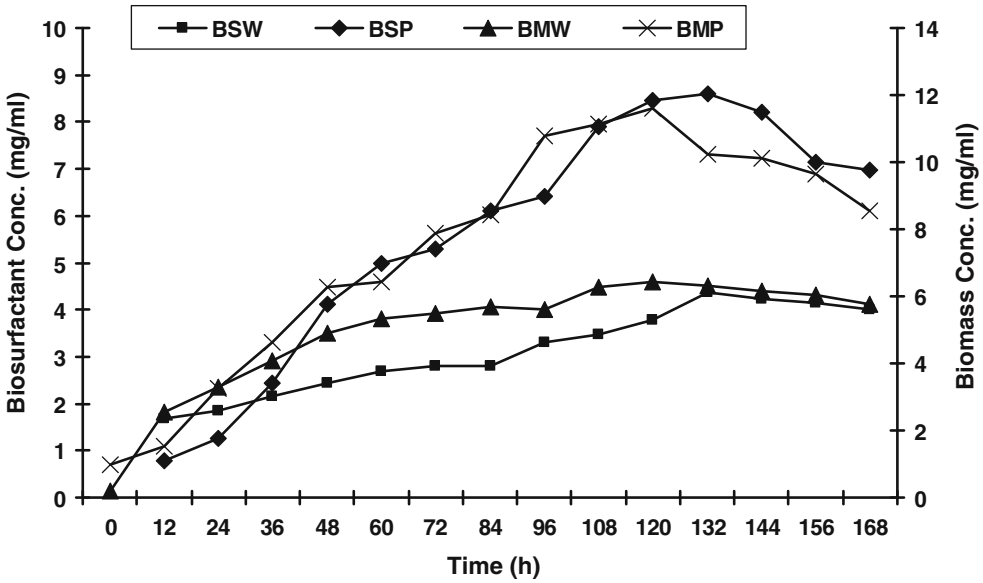

Fig. 1 Growth and biosurfactant production by *P. aeruginosa* in fermentor. *BSW* biosurfactant production using waste motor lubricant oil, *BSP* biosurfactant production using peanut oil cake, *BMW* biomass concentration with waste motor lubricant oil, *BMP* biomass concentration with peanut oil cake

possibility of its application against different hydrocarbon pollution.

Laboratory Scale Experiment on Biodegradation of Crude Oil with Biosurfactant

Biodegradation of crude oil in the laboratory scale experiment inferred that, maximum biodegradation was found with biosurfactant and fertilizer added set (88.2%) followed by biosurfactant (85%), fertilizer (71%) and 50.5% in normal setup.

Characterization of Biosurfactant

Biochemical composition of the biosurfactant revealed that, it 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 3,422 and 3,246 cm^{-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 1425 cm^{-1} confirmed the presence of alkanes. The wave number 1,650 cm^{-1} (amide I bond) indicated the presence of peptide groups. The wave number 1,066 cm^{-1} indicated the presence of C–O bonds (Fig. 2). 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 protein and lipid moieties (Fig. 3).

Table 2 Emulsification of hydrocarbons by biosurfactant isolated from *P. aeruginosa* and Triton X-100

	Emulsification activity (D_{610})	
	Biosurfactant	Triton X-100
Waste motor lubricant oil	2.01	1.93
Crude oil	1.97	1.85
Peanut oil	1.95	1.56
Kerosene	1.12	1.12
Diesel	0.90	0.94
Xylene	0.61	0.78
Naphthalene	0.51	0.63
Anthracene	0.45	0.58

*Experiment was carried out in duplicates and the mean values expressed as D_{610}. Biosurfactant used in this assay was produced from peanut oil cake

Discussion

This study revealed the possibility of biosurfactant production using cheaper carbon sources like waste motor lubricant oil and peanut oil cake. The possibility of biosurfactant production using cheaper carbon sources was already reported by earlier workers; Mercade et al. [17] used olive oil mill effluent, animal fat and frying oil by Deshpande and Daniels [18], molasses by Benincasa et al. [19], and starch-rich wastes by Nitschke and Pastore [20] supporting the present study on use of renewable carbon sources for biosurfactant production. Haba et al. [21] reported that, biosurfactant produced from firing oil have low emulsifying properties, where as biosurfactant produced in the present study using waste motor lubricant oil and peanut oil cake showed good emulsification activity against eight different hydrocarbons. Further, it encouraged the aim of the present study to produce biosurfactants from

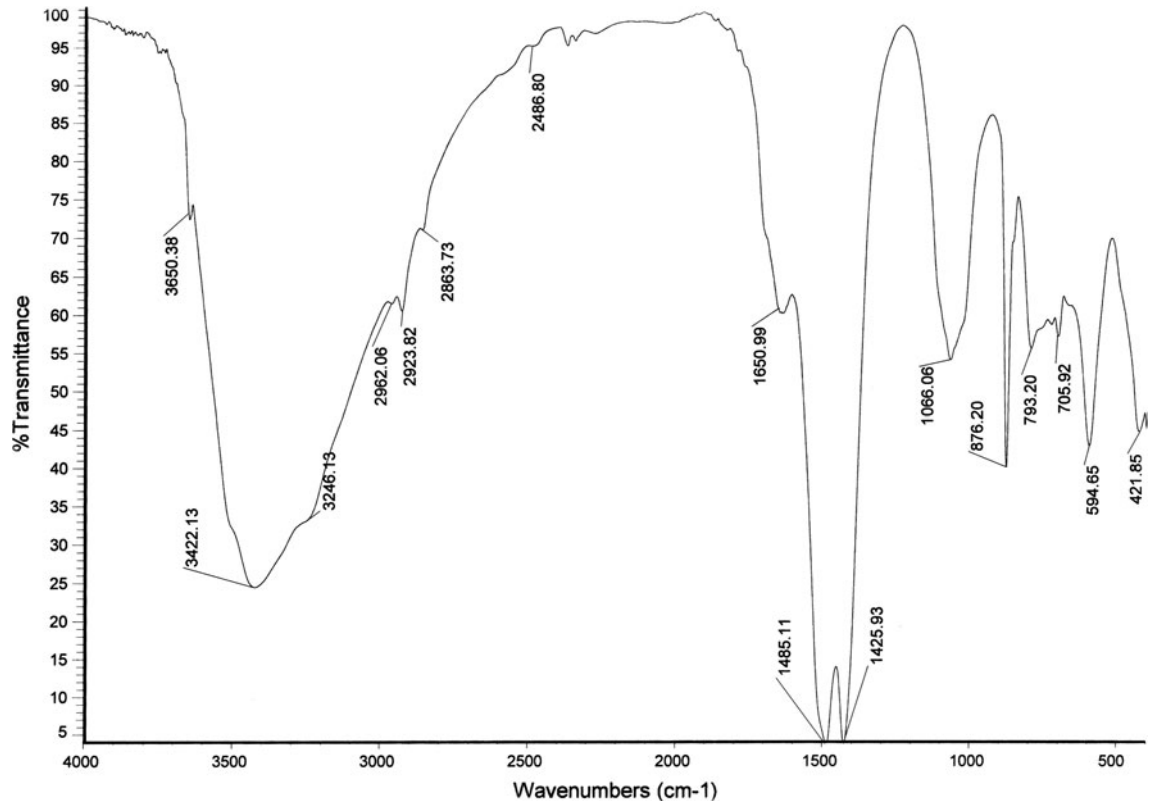

Fig. 2 FTIR spectrum of biosurfactant produced by *P. aeruginosa*

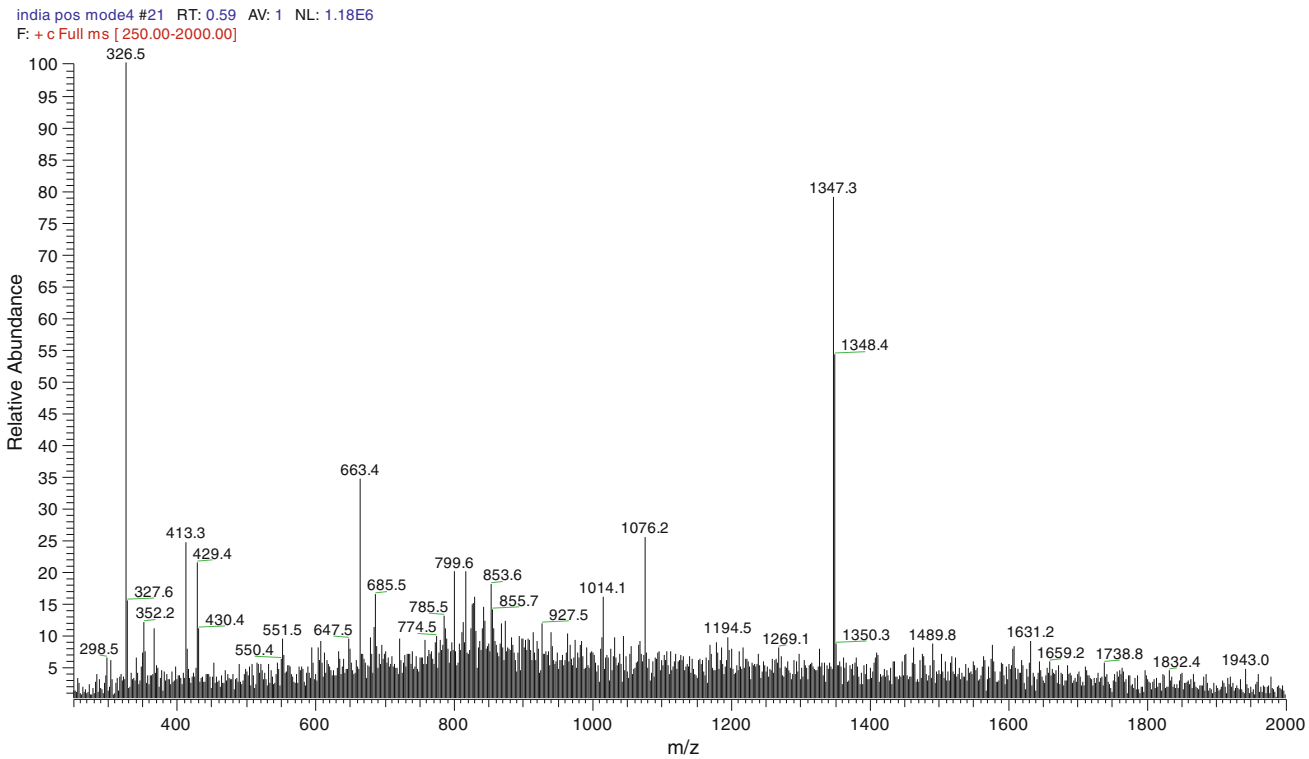

Fig. 3 Mass spectrum of biosurfactant produced by *P. aeruginosa*

cheaper carbon sources with high emulsification property. Parallel increase in biomass and biosurfactant were found from 12 to 120 h, but maximum biosurfactant concentration was found at 132 h. Higher concentration of biosurfactant even after the off set of biomass may be due to the release of cell bound biosurfactant at the early stationary phase (132 h), which leads to rise in extracellular biosurfactant concentration in the medium [22].

The emulsification activity of 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 with biosurfactant is sought and need further research with different kind of experiments. Emulsification activity found with biosurfactant produced by *P. aeruginosa* in this study inferred that, biosurfactant produced with one carbon source like waste motor oil or peanut oil cake could be used to remediate different hydrocarbon pollution.

Microbiological studies in laboratories, experimental field trials and cleanup operations following actual marine oil spill incidents, have demonstrated that bioremediation strategy based on the enhancement of oil biodegradation rates through nutrient addition was effective [23–27]. An understanding of the microbial hydrocarbon degradation process makes it possible to develop models for predicting the fate of hydrocarbon pollutants and to find strategies for incorporating microbial utilization of petroleum hydrocarbons in contaminated ecosystems [28]. 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.

Analysis of lipopeptide biosurfactant produced by *P. putida* using mass spectrometry was reported by Kuiper et al. [29] and their results were correlated with peaks observed the present study. Kalinovskaya et al. [30] also analyzed surfactin, a lipopeptide biosurfactant produced by *Bacillus pumilus* and got similar results. Chemically, biosurfactants produced with waste motor lubricant oil and peanut oil cake are similar.

In conclusion, the present study is an attempt to find economically cheaper carbon sources for the large scale production of microbial biosurfactants. Results obtained in biosurfactant production with waste motor lubricant oil and peanut oil cake suggested the possibility of industrial production of biosurfactants using economically cheaper carbon sources. Satisfactory emulsification activity of the biosurfactant against eight different hydrocarbons indicated its diverse applicability against different hydrocarbon pollution. Further purification, structural characterization of biosurfactant and genetic regulation of biosurfactant production are in progress.

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