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Production and characterization of biosurfactant produced by a novel *Pseudomonas* sp. 2B

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ABSTRACT

Biosurfactant-producing bacteria were isolated from terrestrial samples collected in areas contaminated with petroleum compounds. Isolates were screened for biosurfactant production using Cetyl Tri Ammonium Bromide (CTAB)–Methylene blue agar selection medium and the qualitative drop-collapse test. An efficient bacterial strain was selected based on rapid drop collapse activity and highest biosurfactant production. The biochemical characteristics and partial sequenced 16S rRNA gene of isolate, 2B, identified the bacterium as *Pseudomonas* sp. Five different low cost carbon substrates were evaluated for their effect on biosurfactant production. The maximum biosurfactant synthesis (4.97 g/L) occurred at 96 h when the cells were grown on modified PPGAS medium containing 1% (v/v) molasses at 30 °C and 150 rpm. The cell free broth containing the biosurfactant could reduce the surface tension to 30.14 mN/m. The surface active compound showed emulsifying activity against a variety of hydrocarbons and achieved a maximum emulsion index of 84% for sunflower oil. Compositional analysis of the biosurfactant reveals that the extracted biosurfactant was a glycolipid type, which was composed of high percentages of lipid (~65%, w/w) and carbohydrate (~32%, w/w). Fourier transform infrared (FT-IR) spectrum of extracted biosurfactant indicates the presence of carboxyl, hydroxyl and methoxyl functional groups. The mass spectra (MS) shows that dirhamnolipid (L-rhamnopyranosyl-L-rhamnopyranosyl-3-hydroxydecanoyl-3-hydroxydecanoate, Rha-Rha-C_{10}-C_{10}) was detected in abundance with the predominant congener monorhamnolipid (L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate, Rha-C_{10}-C_{10}). The crude oil recovery studies using the biosurfactant produced by *Pseudomonas* sp. 2B suggested its potential application in microbial enhanced oil recovery and bioremediation.

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1. Introduction

Surfactants are an important class of speciality chemicals widely used in modern industries. Almost all the surfactants commercially available today are chemically synthesized from petroleum. Since the conventional chemical surfactants are derived from non-renewable petroleum products, they are costly and pose potential threats to the environment due to their recalcitrant nature. Microbial systems are the best alternatives in the surfactant production as they offer several advantages over their chemical counterparts such as lower toxicity, higher biodegradability, stability at extremes of temperature, pH, salinity and their ability to be synthesized from renewable feedstock by a wide variety of microorganisms. The robustness of biosurfactants leads to several potential uses in petrochemical, food, cosmetics, and pharmaceutical industries. They also have varied properties such as excellent detergency, emulsification, foaming, dispersing traits, wetting, penetrating, thickening, microbial growth enhancement, metal sequestering and resource recovering (oil). These properties make surfactants replace some of the most versatile process chemicals [1,2].

Biosurfactants are classified according to their molecular structure into mainly glycolipids (e.g., rhamnolipids and sophorolipids), lipopeptides (e.g., surfactin), polymeric biosurfactants (e.g., emulsan and alasan), fatty acids (e.g., 3-(3-hydroxyalkanoyloxy) alkanoic acids) and phospholipids (e.g., phosphatidylethanolamine) [1,3]. Among the different classes of biosurfactants, rhamnolipids, members of the glycolipid group, are the most extensively studied and characterized [4]. Rhamnolipids are promising surfactants owing to several characteristics such as low minimum surface tension (30–32 mN/m), high emulsifying activity, and higher affinity for hydrophobic organic molecules. These properties confer optimal characteristics to rhamnolipids and make them potential carriers of pollutants in soil systems [5]. Rhamnolipids produced by

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Pseudomonas sp. grown with different carbon sources have been traditionally reported as mixtures of the homologs L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Rha-$C_{10}C_{10}$), L-rhamnopyranosyl-β-hydroxydecanoate (Rha-C_{10}), 2-O-L-rhamnopyranosyl-β-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Rha-Rha-$C_{10}C_{10}$), and 2-O-L-rhamnopyranosyl-β-L-rhamnopyranosyl-β-hydroxydecanoate (Rha-Rha-C_{10}). The surfactant properties of rhamnolipids depend on its composition and distribution of homologs that vary according to the bacterial strain, culture conditions, and media composition [6].

Although rhamnolipids are effective surfactants and suitable for the application of bioremediation of oil pollution [7], the main concern for the commercial application and large scale production of rhamnolipid are its low yield and high production cost, which are due to the inefficient bioprocessing methodologies available, poor strain improvement, and the need to use expensive substrates [8]. Due to the increasing demand of microbial biosurfactants, there is a need to isolate efficient biosurfactant producers and develop a low cost-effective process in order to facilitate the large scale production of environmental friendly surfactants. In the present investigation, we report the isolation and characterization of a novel rhamnolipid producing bacterial strain, production of extracellular biosurfactant on low cost carbon sources and characterization of the biosurfactant.

2. Materials and methods

2.1. Materials

All the chemicals used in the present study were of analytical grade and purity.

2.2. Enrichment and isolation of microorganisms

The bacterial strains were isolated using enrichment technique from hydrocarbon contaminated soil samples in Karnataka, India. The soil samples were suspended in Bushnell–Hass medium [9] containing 1% crude oil and incubated at 30 °C, 150 rpm for 7 days.

2.3. Detection of surface active compounds

For the isolation of biosurfactant producing organisms, the enriched media were serially diluted and inoculated on to Cetyl Tri Ammonium Bromide (CTAB)–Methylene blue agar medium. The plates were inspected for the presence of dark blue halos around the bacterial colonies after 48 h of incubation at 37 °C. Colonies showing halos on CTAB–Methylene blue agar medium were isolated and subsequently subjected to two cycles of enrichment at 30 °C for 7 days at 150 rpm. The samples from this culture broth were further screened for the biosurfactant production by drop collapse technique [10]. Among several bacterial strains isolated, an efficient biosurfactant-producing bacterial strain was identified based on positive and rapid drop collapse reaction. The isolated bacterial culture was maintained on nutrient agar slant and stored at 4 °C.

2.4. Identification of the bacterium by biochemical and 16S rRNA sequence analysis

The bacterial strain identification was performed by determining the gene sequence coding for 16S rRNA at Agharkar Research Institute, Pune, India and biochemical reactions according to Bergey's Manual of Determinative Bacteriology. Each PCR reaction mixture contained approximately 10 ng of DNA; 2.5 mM $MgCl_2$; 1× PCR buffer (Bangalore Genei, Bangalore, India); 200 μM each dCTP, dGTP, dTTP and dATP; 2 pmol of each forward and reverse primer; and 1 U of Taq polymerase (Bangalore Genei, Bangalore, India). The PCR was performed using the Eppendorf Gradient Mastercycler system with a cycle of 94 °C for 5 min; 30 cycles of 94 °C, 60 °C and 72 °C for 1 min each; and final extension at 72 °C for 10 min, and the mixture was held at 4 °C. The PCR product was precipitated using polyethylene glycol, washed thrice using 70% ethanol and dissolved in Tris–HCl (10 mM, pH 8).

The ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) was used for the sequencing of the PCR product. A combination of universal primers was chosen to sequence the gene sequence [11,12]. Samples were run on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The sequencing output was analyzed using the DNA sequence analyzer computer software (Applied Biosystems). The sequence was compared with National Center for Biotechnology Information Gen Bank entries by using the BLAST algorithm.

2.5. Nucleotide sequence accession number

The 16S rRNA sequence of the isolated bacterium was submitted in the GenBank database with an accession number JF683582.

2.6. Biosurfactant production from different low-cost carbon sources

In order to economize the biosurfactant production by the bacterial strain, different low-cost substrates such as molasses, whey, glycerol, orange peelings, and coconut oil cake were screened as carbon source for biosurfactant production. The biosurfactant production was carried out in 250 ml Erlenmeyer flasks containing 50 ml of the modified Proteose Peptone Glucose Ammonium Salt (PPGAS) medium composed of: NH_4Cl 0.02 M, KCl 0.12 M, $MgSO_4$ 0.0016 M, peptone 1% and 1% (w/v or v/v) carbon source was added [13]. The initial pH of the medium was set to 7.2. The medium was inoculated with 2% of the 18 h bacterial culture grown on nutrient broth. A control flask without carbon source was also maintained. Incubation was carried out at 30 °C in an incubator shaker at 150 rpm. Time course samples of culture medium were drawn in appropriate time intervals and monitored for biosurfactant production, biomass estimation and surface tension (ST). Bacterial cell growth was monitored by measuring the dry cell weight method [14]. Surface tension was measured with a tensiometer (Surface tension meter, DST 30 series, Surface and Electro Optics Corporation, Korea) accordingly to the ring method [10].

2.7. Extraction of the biosurfactant

Biosurfactant concentration in the culture broth was estimated according to the procedure described by Samadi et al. [15]. The culture broth was centrifuged at 9000 rpm at 4 °C for 30 min. The supernatant was adjusted to pH 2.0 using 1 M sulfuric acid, prior to biosurfactant extraction using equal volume of chloroform–methanol (2:1) mixture. The organic phase was separated and the solvent was evaporated to concentrate the biosurfactant. The biosurfactant was then dried using a rotary evaporator. The dried product was washed with absolute ethanol for three times to complete remove of residual pigments.

2.8. Determination of critical micelle concentration (CMC)

The CMC of the biosurfactant was determined by plotting the surface tension as a function of the biosurfactant concentration [16,17]. The biosurfactant was dissolved in aqueous solutions at concentrations ranging from 0 to 6 g/L. For each concentration, surface tension measurement was carried out until a constant value was reached. The effectiveness and efficiency of the biosurfactant

were measured according to the method described by Parkinson [18].

2.9. Substrate specificity of biosurfactant and determination of emulsifying activity (E24)

The ability of the biosurfactant to emulsify liquid hydrocarbons, such as crude oil, kerosene, hexadecane, n-hexane and benzene was determined. 2 ml of cell free broth containing biosurfactant (0.02 g/L) was added into each test tube containing 2 ml of hydrocarbon. The content of the tubes was vortexed vigorously for 2 min and left undisturbed for 24 h. The emulsion index (E24) was determined as the percentage of height of the emulsion layer divided by the total height [19].

2.10. Studies on the effect of environmental factors on biosurfactant stability

It was based on the determination of temperature, pH, and NaCl effects on the activity of the biosurfactant (0.02 g/L) in the cell free broth. To determine the stability of the biosurfactant at different temperatures, the cell-free broth was maintained at a constant temperature in the range 4–121 °C for 30 min. To determine the effect of pH on the biosurfactant activity, the pH of the cell free broth containing biosurfactant was adjusted in the range 2.0–12.0 with HCl (6 N) and NaOH (6 N) [20]. In each case, surface tension and E24 values were measured. In order to assess the effect of salinity on the emulsification capacity and surface activity of cell free broth containing biosurfactant, various concentrations of sodium chloride (1–20% w/v) were employed [21].

2.11. Biochemical analysis of the biosurfactant

Total sugar present in the biosurfactant was determined by the phenol sulfuric acid method according to Dubois et al. [22]. The standard curve was prepared with D-glucose. Total protein content was measured by Lowry's method [23], standardized with bovine serum albumin. To determine the lipid content, 0.2 g biosurfactant sample was blended with chloroform–methanol mixture (2:1) and agitated acutely. Solvent phase was recovered by centrifuging at 10,000 rpm for 15 min. The extraction process was carried out three times. The whole solvent was collected, evaporated and dried under vacuum. The lipid content was determined by gravimetric estimation [24].

2.12. Characterization of the purified biosurfactant

2.12.1. Fourier transform infrared spectroscopy (FT-IR) analysis

FT-IR spectrum of the dried biosurfactant was recorded on a Thermo Scientific FT-IR spectrometer equipped with a mercury–cadmium–telluride (MCT) detector. About 2 mg of dried biomaterial was analyzed by FT-IR spectrum measurement in wave number range of 4000–400 cm^{-1}. The analysis of IR spectra was carried out by using Thermo software.

2.12.2. Mass spectrometric analysis of the biosurfactant

Biosurfactant was dissolved in methanol and mixed thoroughly. The mass spectrometric analysis of the biosurfactant was carried out in Shimadzu LC-MS 2010 spectrometer (Shimadzu, Japan) utilizing electrospray ionization (ESI). The mass spectra were detected in the conditions of negative ion mode and scanning was done at 50–1000 *m*/*z* range.

Table 1
Morphological and biochemical characterization of the bacterial isolate *Pseudomonas* sp. 2B.

Characters/test	Result
Gram staining	Gram negative
Shape of the cell	Rod
Capsule staining	Presence of capsule
Endospore staining	Absence of endospore
Motility test	Motile
Starch hydrolysis	Negative
Methyl red test	Negative
Voges–Proskauer test	Negative
Indole test	Negative
Citrate utilization test	Positive
Glucose fermentation	Positive
Sucrose fermentation	Negative
Lactose fermentation	Negative
Nitrate reduction test	Positive
Hydrogen sulfide production	Negative
Gelatin hydrolysis test	Positive
Oxidase test	Positive
Catalase test	Positive

2.13. Application of the biosurfactant in hydrocarbon contaminants removal from sand

Biosurfactant suitability for enhanced oil recovery was carried out using artificially contaminated sand with 10% of crude oil according to method described by Silva et al. [8]. Samples of 20 g of 0.1–0.2 mm fractions of the contaminated sand were transferred to 250 ml Erlenmeyer flasks, which were submitted to the following treatments: addition of 40 ml distilled water (control) or 40 ml of the cell-free broth or 40 ml of a solution of the isolated biosurfactant at 0.01 and 0.05% (w/v) concentrations. The samples were incubated on a rotary shaker (150 rpm) for 24 h at 27 °C and then were centrifuged at 9000 rpm for 20 min for separation of the laundering solution and the sand. The amount of oil residing in the sand after the impact of biosurfactant was gravimetrically determined as the amount of material extracted from the sand by dichloromethane [25].

3. Results and discussion

3.1. Isolation and identification of selected biosurfactant-producing bacterial strain

Several bacterial strains with the ability to produce biosurfactant were isolated from hydrocarbon contaminated soil samples by enrichment technique. Among the bacterial isolates assayed by CTAB–Methylene blue agar method, one of the strains demonstrated a positive reaction for biosurfactant production, this efficient bacterial strain which had rapid drop collapse activity and highest biosurfactant production was selected for further studies. The bacterial strain selected is designated as 2B.

The biochemical and morphological characteristics of the isolated bacterial strain revealed that strain was closely related to the species in genus *Pseudomonas* (Table 1). The isolated strain produced a diffusible green colored fluorescent pigment. The isolate formed irregular and glistening colonies. During the growth on nutrient agar medium, the diameter of the colonies ranged from 1 to 2 mm within 24 h growth at 37 °C. The genotypic analysis on the basis of partial 16S rRNA sequencing was examined to determine the precise taxonomic position of the strain. Alignment of the 16S rRNA gene sequences of 2B with sequences obtained by doing a Blast search revealed 95% similarity to *Pseudomonas* sp. The biochemical test and morphological characteristics also support that the bacterium is *Pseudomonas* sp. *Pseudomonas* sp. are the most common producers of biosurfactants, isolated from

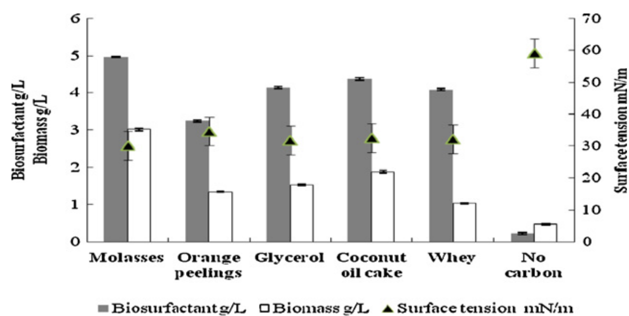

Fig. 1. The effect of carbon source on rhamnolipid production, cell growth and surface tension by *Pseudomonas* sp. 2B. Results are represented as means ± SEM ($n = 3$).

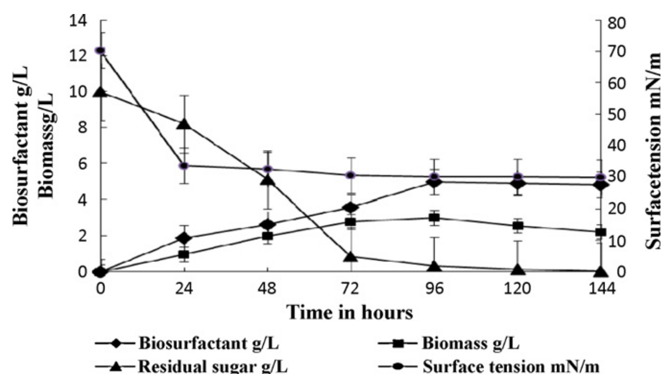

Fig. 2. Time course profile of extracellular biosurfactant synthesis, cell growth, surface tension and substrate utilization by *Pseudomonas* sp. 2B in 1% (v/v) molasses-containing modified PPGAS medium. Results are represented as means ± SEM ($n = 3$).

petroleum-contaminated soil samples [26–28]. In this paper, we report biosurfactant production by the novel *Pseudomonas* sp. 2B.

3.2. Biosurfactant production by Pseudomonas sp. 2B on different low-cost carbon sources

To determine the optimal conditions for highest biosurfactant production, the use effect of various low cost carbon substrates were analyzed. The organism was able to utilize all the carbon sources tested; growth was accompanied with biosurfactant production. The surface tension of the medium was lowered from 60 mN/m to 30 mN/m with every carbon source tested. Maximum biosurfactant production (4.97 g/L) was observed when molasses was used as the carbon source. The highest production in molasses containing modified PPGAS medium may be due to its high total sugar contents (mainly sucrose). However, the biosurfactant yield was affected by the type of carbon substrate used. The amounts of rhamnolipid produced were found to be 4.14 g/L, 4.38 g/L, 3.24 g/L and 4.09 g/L when grown on glycerol, coconut oil cake, orange peelings and whey, respectively. Least biosurfactant production (0.22 g/L) was observed in the medium without carbon source (Fig. 1). This suggests that biosurfactant production can be enhanced to economically viable values using renewable feedstock as carbon source, reiterating the importance of the utilization of industrial by-products and agricultural wastes as cost-effective alternative substrates for microbial growth and biosurfactant production [29–31]. Agro-industrial wastes are considered as the promising cost effective substrate for biosurfactant production and can alleviate many processing industrial waste management problems [32].

The kinetics growth curve indicated a parallel relationship between biosurfactant production, bacterial growth, surface tension reduction and substrate utilization, suggesting a growth associated biosurfactant production. The exponential growth of *Pseudomonas* sp. 2B was observed at about 24 h and after 96 h of cultivation, the maximum biomass (2.7 g/L) was reached. The biosurfactant production commenced at about 24 h, i.e., during the exponential phase, indicating its accumulation during growth phase. Maximum biosurfactant production (4.97 g/L) occurred at 96 h (Fig. 2). The surface tension of the cell free broth showed lowest value, i.e. 30.14 mN/m when molasses was used as the carbon source. It has been reported by various researchers that rhamnolipid type of biosurfactants are “primary metabolites” and their production coincides with the exponential growth phase [33,34].

3.3. Determination of critical micelle concentration (CMC)

The presence of a surfactant reduces the surface tension air/water, which is proportional to the concentration of the biosurfactant in the solution, until it reaches the CMC [35]. For practical purposes, it is important to distinguish between an effective biosurfactant and an efficient biosurfactant. Effectiveness is measured by the minimum value to which the surface tension can be reduced, whereas efficiency is measured by the biosurfactant concentration required to produce a significant reduction in the surface tension of water. The latter can be known from the CMC of the biosurfactant [18].

As seen in Fig. 3, the critical micelle concentration corresponded to a sudden change in the surface tension. The CMC for the isolated biosurfactant was 100 mg/L and the corresponding surface tension was 29.73 mN/m. The data showed the high effectiveness of rhamnolipids as they could lower the surface tension of water from 70 to 30 mN/m for the rhamnolipid. The biosurfactant proved to be highly efficient, since the CMC value for the biosurfactant was found to be 100 mg/L. Efficient surfactants have very low CMC values, i.e., less surfactant is required to decrease surface tension. Moreover, a range of CMC values between 10 and 230 mg/L have been reported for rhamnolipids from different microbial sources [36].

3.4. Substrate specificity of biosurfactant and determination of emulsifying activity (E24)

The emulsification property is an important factor of a surfactant. The isolated biosurfactant showed appreciable emulsification indices, as shown in Table 2, with sunflower oil, crude oil, gasoline, n-hexadecane, kerosene, hexane and benzene. Sunflower oil was the best substrate showing E24 value of 84 while benzene was the poorest with E24 value of 33. The emulsions of sunflower oil, crude oil and gasoline produced by the isolate were stable for a month compared to that of other hydrocarbons evaluated. The ability of biosurfactants to form stable emulsions with vegetable oils and fats suggests potential application as cleaning and emulsifying agent in

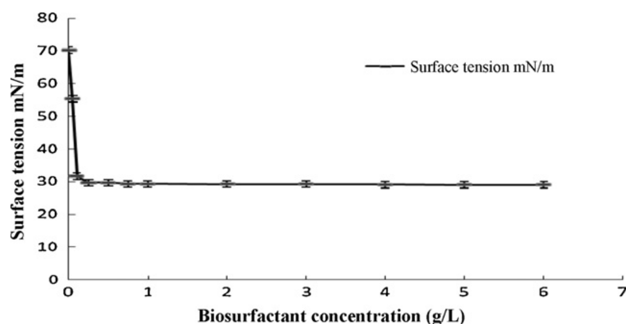

Fig. 3. Determination of critical micelle concentration of the biosurfactant produced by *Pseudomonas* sp. 2B. Results are represented as means ± SEM ($n = 3$).

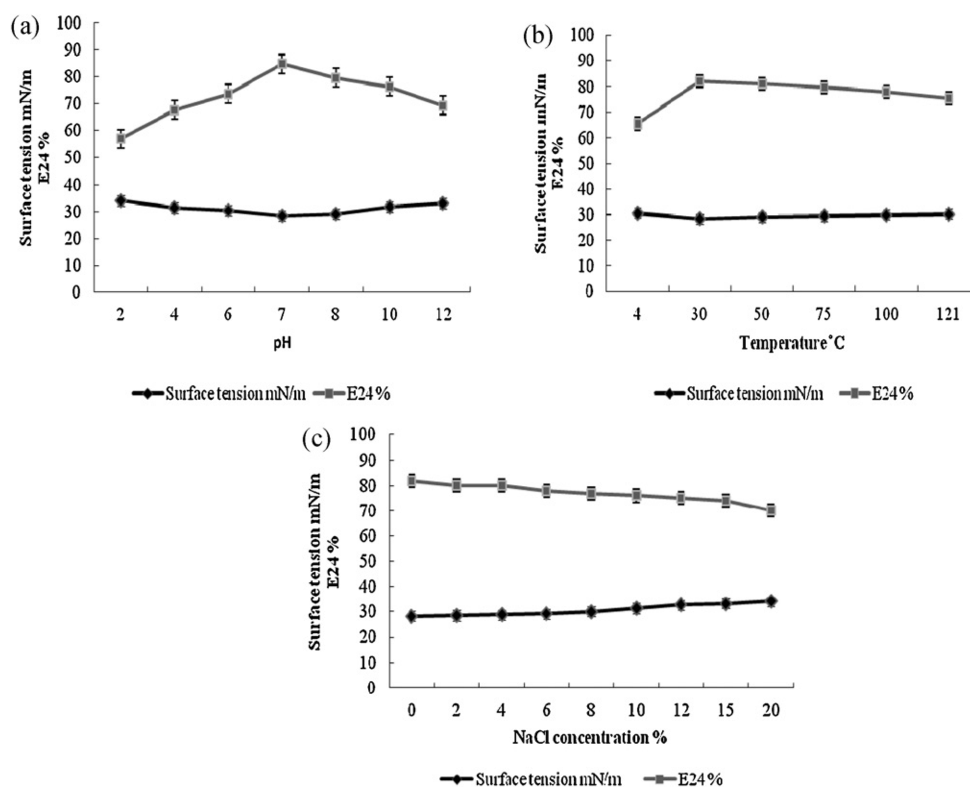

Fig. 4. Effect of (a) pH, (b) temperature and (c) salinity on the stability of biosurfactant. Data are represented as means ± SEM ($n = 3$).

the food industry [25]. Emulsification of hydrocarbons by the culture *Pseudomonas* sp. 2B was in the order of sunflower oil > crude oil > gasoline > hexadecane > kerosene > hexane > benzene. The results show that the biosurfactant could emulsify different hydrocarbons, which confirmed their applicability against different hydrocarbon pollution such that it enhances the availability of the recalcitrant hydrocarbons [37,38].

3.5. Studies on the effect of environmental factors on biosurfactant stability

Biosurfactants are "green chemicals" used in bioremediation purposes; therefore, the stability of the biosurfactant was studied at different temperature, salinity, and pH. The biosurfactant activity was retained over a pH range of 4–10 with minimal variation in surface tension and emulsification activity values (Fig. 4a). When the pH was acidic and set to 2, 4 and 6, the surface activities of the biosurfactant were 34.19, 31.48 and 30.39 mN/m, respectively. Correspondingly, the emulsification ability of cell-free broth was limited to acid to neutral pH and emulsification index up to 57%, 67% and 73%, respectively was obtained. The optimum surface activity (ST ~ 28.39 mN/m) and emulsification capacity (E24 ~ 84%) was observed at pH 7 With the increase of pH from 5 to 8, the negative charge of the polar head increases, this is reflected by increased aqueous solubility [8]. However, the emulsification activity of cell-free broth was relatively stable at pH 7 and linearly decreased from pH 8 to 12 which may be a result of some structural alteration of the surfactant under extreme pH conditions.

The stability of the biosurfactant was tested over a wide range of temperature (4–121 °C). The results obtained from thermal stability analysis of cell-free broth containing biosurfactant revealed the thermostability of biosurfactant (Fig. 4b). Heating of the cell-free supernatant up to 100 °C (or its autoclaving at 121 °C) caused no significant change on the biosurfactant performance and its emulsification capacity. Such extreme stability was reported by Abdel-Mawgoud et al. [39] and Kiran et al. [40] for the *Pseudomonas aeruginosa* strain and *Brevibacterium aureum* MSA13 respectively. This suggests that the biosurfactant isolated may be used in microbial enhanced oil recovery processes where high temperatures prevail. Loss of emulsification activity was observed when the cell-free broth was stored at 4 °C. The surface tension reduction and emulsification activity were stable, irrespective of operating temperature (ST ~ 28–30 mN/m; E24 ~ 78–83%).

The effect of salinity on the biosurfactant activity was tested (Fig. 4c). There is an urgent need to screen potential biosurfactants in bioremediation of contaminated marine environments, especially during oil spills. Steady surface tension of average 33 mN/m was noted even with an increase of 15% NaCl concentration. However, at concentration of 20% NaCl, emulsification capacity dropped to 70% and surface tension activity increased as well (34 mN/m). The results are in concurrence with Helvaci et al. [41]. They stated that electrolytes directly affect the carboxylate groups of the rhamnolipids. The solution/air interface has a net negative charge due to the ionized carboxylic acid groups at alkaline pH with strong repulsive electrostatic forces between the rhamnolipid molecules. This negative charge is shielded by the Na^+ ions in an electrical double layer in the presence of NaCl, causing the formation of a close-packed monolayer and consequently a decrease in surface tension values.

Table 2
Substrate specificity and emulsification activity of the biosurfactant produced by *Pseudomonas* sp. 2B. Results are represented as means ± SEM ($n = 3$).

Substrate	Emulsion index (E24)
Sunflower oil	84.0 ± 1.4
Crude oil	80.2 ± 2.9
Gasoline	76.7 ± 2.3
Hexadecane	69.3 ± 3.1
Kerosene	67.5 ± 3.5
Hexane	38.4 ± 3.7
Benzene	33.8 ± 4.1

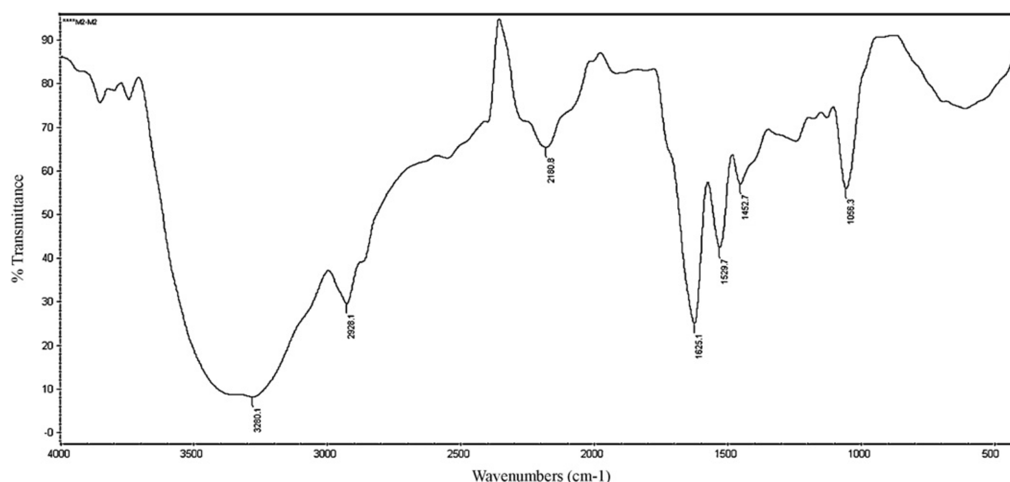

Fig. 5. FT-IR spectrum of the freeze-dried biosurfactant produced by *Pseudomonas* sp. 2B grown on molasses containing modified PPGAS medium.

Our findings indicate that potential application of the product over a wide range of temperature, pH values and saline environment. This finds application in situations where extreme conditions of temperature, pH and salinity prevail such as bioremediation of soil as well as marine environments. It can also be used for enhanced oil recovery operations.

3.6. Biochemical analysis of the biosurfactant

Compositional analysis of the purified biosurfactant revealed that the biosurfactant produced by *Pseudomonas* sp. 2B was a glycolipid, consisting of mixture of lipid and carbohydrate with a combination of 65%:32% (w/w) respectively.

3.7. Characterization of the partially purified biosurfactant

3.7.1. Fourier transform infrared spectroscopy (FT-IR) analysis

There are several literature reports of isolation and biosurfactant production by different species of the genus *Pseudomonas*. *P. aeruginosa*, which produces a rhamnolipid, has homologs that differ in the number of rhamnose molecules as well as in the length and composition of alkyl chain [26]. The molecular composition of the biosurfactant produced by *Pseudomonas* sp. 2B was evaluated by FT-IR. Fig. 5 represents the FT-IR spectra of the freeze-dried sample. The characteristic band at 3280 cm^{-1} indicates the presence of —OH bonds. Absorption around 2928 cm^{-1} is assigned to the symmetric stretch (—CH) of $—CH_2$ and $—CH_3$ groups of aliphatic chains. The absorption peak located at 1625 cm^{-1} indicates the presence of ester carbonyl groups (—C=O bond in —COOH). The ester carbonyl group was also proved from the band at 1238 cm^{-1} which corresponds to —C=O deformation vibrations, although other groups also absorb in this region. Protein-related weak bands the —C=O amide I (1625 cm^{-1}) and —NH/—C=O combination of the amide II bands (1529 cm^{-1}), were observed. It might be possible that the additional bands at 1625 cm^{-1} and 1529 cm^{-1} resulted from the contamination of polypeptides from cell debris's co-precipitated with the biosurfactant during extraction process [20]. The absorption peak around 1056 cm^{-1} indicates the presence of polysaccharide or polysaccharide-like substances in the biosurfactant. The absorption peak at 694 cm^{-1} indicates the presence of $—CH_2$ group. The above information from the respective wave numbers confirmed the glycolipid nature of the biosurfactant [42–44].

3.7.2. Mass spectrometric (MS) analysis of the biosurfactant

The mass spectrum (MS) of the *Pseudomonas* sp. 2B biosurfactant showed a mixture of rhamnolipid with a molecular weight between 333 and 678 with intense molecular ions at m/z 333, 479, 504, 505, 532, 650 and 678. The mass spectrometric analysis of the biosurfactant confirmed the above results with peaks observed at m/z = 333, 479, 504, 505 for lipids and at 650 and 678 for carbohydrate moieties [45,46]. The m/z values obtained were consistent with the molecular structure of Rha-C_{10}, Rha-C_{10}-C_{10}, Rha-C_{10}-C_{12} and Rha-Rha-C_{10}-C_{10}, respectively. Nine rhamnolipid homologs were identified in the present study (Table 3). In general, the results showed the presence of a relatively higher abundance of dirhamnolipid (L-rhamnopyranosyl-L-rhamnopyranosyl-3-hydroxydecanoyl-3hydroxydecanoate) than monorhamnolipid (L-rhamnopyranosyl-3-hydroxydecanoyl-3-hydroxydecanoate). As reported by Deziel et al. [45], peaks at 333.0 m/z (Rha-C_{10}) and 479.0 m/z (Rha-Rha-C_{10}) indicated fragments produced by cleavage of rhamnolipid molecules. The rhamnolipid composition and predominance of a particular type of congener depends on various factors like type of carbon substrate [46,47], culture conditions [48], age of the culture [49], and the strain *P. aeruginosa* [45].

3.8. Application of the biosurfactant in crude oil removal from contaminated sand

Application of biosurfactants in enhanced oil recovery is one of the most promising techniques to remove and recover a significant amount of the residual oil. The results obtained demonstrated that the *Pseudomonas* sp. 2B biosurfactant solution at 0.01% and 0.05% biosurfactant concentrations was capable to remove 89% and 92% of the oil adsorbed in the sand respectively, while the distilled water (control) and synthetic surfactant, sodium dodecyl sulfate

Table 3
Chemical composition of rhamnolipid mixture produced by *Pseudomonas* sp. 2B culture determined by MS analysis.

Rhamnolipid congeners	Pseudomolecular ion (m/z)
Rha-Rha-C_8-C_{10}	621.0
Rha-Rha-C_{10}-C_{10}	650.0
Rha-Rha-C_{10}-C_{12}	678.0
Rha-C_{10}-C_{10}	505.0
Rha-C_{10}-C_{12}	532.0
Rha-C_{10}	333.0
Rha-C_{10}-C_{10}	504.0
Rha-Rha-C_{12}-C_{10}	678.0
Rha-Rha-C_{10}	479.0

Table 4
Removal of crude oil from contaminated sand by the isolate grown on modified PPGAS medium supplemented with 1% molasses. Results are represented as means ± SEM ($n=3$).

Treatment	Removal of hydrocarbon pollutant from sand (%)
0.05% biosurfactant solution	92.4 ± 0.3
0.01% biosurfactant solution	89.7 ± 0.7
Cell free broth	81.3 ± 0.2
0.01% sodium dodecyl sulfate (SDS)	63.9 ± 0.8
Distilled water (control)	48.6 ± 0.5

(SDS), removed 48% and 63% of the contaminated oil respectively (Table 4). 81% of crude residual oil was removed using the cell-free broth containing biosurfactant produced by *Pseudomonas* sp. 2B. Similar results were obtained by Abu-Ruwaida et al. [34] for the cell-free broth containing a biosurfactant produced by *Rhodococcus* cells; 86% of crude residual oil adsorbed in the sand was removed. It could be observed in the present study that the isolated biosurfactant was more effective than the commercially available surfactant, SDS. The cell free broth containing biosurfactant and the isolated biosurfactant are almost equally effective in the removal of the crude oil pollutant. Thus cell free broth containing biosurfactant can be directly used without purification steps, which would further reduce the cost of production of biosurfactant. The biosurfactant produced by *Pseudomonas* sp. 2B, could be applied in enhanced oil recovery operations.

4. Conclusion

In the present investigation, *Pseudomonas* sp. 2B, a novel rhamnolipid-producing bacterium was isolated from petroleum hydrocarbon contaminated region. It produced glycolipid type biosurfactant from a range of low cost carbon sources. The isolated rhamnolipid was analyzed by FT-IR and LC–MS. It confirmed the presence of different species of rhamnolipids. The rhamnolipid produced showed stable surface tension reduction capacity and ability to emulsify different hydrocarbon substrates. The study of environmental factors on the biosurfactant stability indicates its stability in environments with varying pH, temperature and salinity, finding application in bioremediation and enhanced oil recovery operations. The application of this extracellular biosurfactant in bioremediation studies further adds to its value as an ecofriendly and biodegradable product.

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References

[1] J.D. Desai, I.M. Banat, Microbiol. Mol. Biol. Rev. 61 (1997) 47.
[2] E. Rosenberg, Crit. Rev. Biotechnol. 3 (1986) 109.
[3] S. Lang, D. Wullbrandt, Appl. Microbiol. Biotechnol. 51 (1999) 22.
[4] K. Muthusamy, S. Gopalakrishnan, T.K. Ravi, P. Sivachidambaram, Curr. Sci. 94 (2008) 736.
[5] J.C. Mata-Sandoval, J. Karns, A. Torrents, J. Chromatogr. A 864 (1999) 211.
[6] L. Guerra-Santos, O. Kapelli, A. Fiechter, Appl. Environ. Microbiol. 48 (1984) 301.
[7] C.N. Mulligan, Environ. Pollut. 133 (2005) 183.
[8] S.N.R.L. Silva, C.B.B. Farias, R.D. Rufino, J.M. Luna, L.A. Sarubbo, Colloids Surf. B: Biointerfaces 79 (2010) 174.
[9] I.D. Bushnell, H.E. Hass, J. Bacteriol. 41 (1941) 653.
[10] A.A. Bodour, R.M. Maier, J. Microbiol. Methods 32 (1998) 273.
[11] S.R. Rawlings, I. Piuz, W. Schlegel, J. Bockaert, L. Journot, Endocrinology 136 (1995) 2088.
[12] G. Muyzer, A. Teske, C.O. Wirsen, H.W. Jannasch, Arch. Microbiol. 164 (1995) 165.
[13] N.W. Gunther, A. Nunez, W. Fett, D.K.Y. Solaiman, Appl. Environ. Microbiol. 71 (2005) 2288.
[14] D.G. Cooper, B.G. Goldenberg, Appl. Environ. Microbiol. 53 (1987) 224.
[15] N. Samadi, N. Abadian, A. Akhavan, M.R. Fazeli, A. Tahzibi, H. Jamalifar, J. Biol. Sci. 7 (2007) 1266.
[16] M. Bonilla, C. Olivaro, M. Corona, A. Vazquez, M. Soubes, J. Appl. Microbiol. 98 (2005) 456.
[17] H. Kim, B. Yoon, C. Lee, H. Suh, H. Oh, T. Katsuragi, Y. Tani, J. Ferment. Bioeng. 84 (1997) 41.
[18] M. Parkinson, Biotechnol. Adv. 3 (1985) 65–83.
[19] N.H. Youssef, K.E. Duncan, D.P. Nagle, K.N. Savage, R.M. Knapp, M.J. Mcinerney, J. Microbiol. Methods 56 (2004) 339.
[20] T.B. Lotfabad, M. Shourian, R. Roostaazad, A.R. Najafabadi, M.R. Adelzadeh, K.A. Noghabi, Colloids Surf. B: Biointerfaces 69 (2009) 183.
[21] S. Joshi, C. Bharucha, S. Jha, S. Yadav, A. Nerurkar, A.J. Desai, Bioresour. Technol. 99 (2008) 195.
[22] M. Dubois, K. Gilles, J. Hamilton, P. Rebers, F. Smith, Anal. Chem. 28 (1956) 350.
[23] O.H. Lowry, N.J. Rosebrought, A. Farr, R.J. Randall, J. Biol. Chem. 139 (1951) 265.
[24] R.S. Makkar, S.S. Cameotra, J. Ind. Microbiol. Biotechnol. 20 (1998) 48.
[25] M. Nitschke, G.M. Pastore, Quim. Nova 25 (2002) 772.
[26] E. Deziel, G. Paquette, R. Villemur, F. Lepine, J.G. Bisaillon, Appl. Environ. Microbiol. 62 (1996) 1908.
[27] O. Pornsunthorntawee, P. Wongpanit, S. Chavadej, M. Abe, R. Rujiravanit, Bioresour. Technol. 99 (2008) 1589.
[28] N.P. Price, K.J. Ray, K. Vermillion, T.M. Kuo, Carbohydr. Res. 344 (2009) 204.
[29] R.M. Patel, A.J. Desai, Lett. Appl. Microbiol. 25 (1997) 91.
[30] Z.A. Raza, M.S. Khan, Z.M. Khalid, Process Biochem. 42 (2007) 686.
[31] A.B. Moldes, A.M. Torrado, M.T. Barral, J.M. Domianguez, J. Agric. Food Chem. 55 (2007) 4481.
[32] S. Maneerat, Songklanakarin J. Sci. Technol. 27 (2005) 675.
[33] A. Tabatabaee, M.M. Assadi, A.A. Noohi, V.A. Sajadian, Iran. J. Environ. Health Sci. Eng. 2 (2005) 6.
[34] A.S. Abu-Ruwaida, I.M. Banat, S. Haditirto, S. Salem, M. Kadri, Acta Biotechnol. 1 (1991) 315.
[35] E.Z. Ron, E. Rosenberg, Z. Naturforsch. 3 (2001) 229.
[36] M. Nitschke, S.G.V.A.O. Costa, J. Contiero, Biotechnol. Prog. 21 (2005) 1593.
[37] I.M. Banat, Bioresour. Technol. 51 (1995) 1.
[38] R.M. Maier, G. Soberon-Chavez, Appl. Microbiol. Biotechnol. 54 (2000) 625.
[39] A.M. Abdel-Mawgoud, M.M. Aboulwafa, N.A. Hassouna, Appl. Biochem. Biotechnol. 150 (2008) 289.
[40] G. Kiran, T.A. Thomas, J. Selvin, B. Sabarathnam, A.P. Lipton, Bioresour. Technol. 101 (2010) 2389.
[41] S.S. Helvaci, S. Peker, G. Ozdemir, Colloids Surf. B: Biointerfaces 35 (2004) 225.
[42] B.K. Tuleva, R.G. Ivanov, N.E. Christova, Z. Naturforsch. 57 (2001) 356.
[43] A. Tahzibi, F. Kamal, M.M. Assadi, Iran. Biomed. J. 8 (2004) 25.
[44] L.R. Rodrigues, J.A. Teixeira, H.C. van der Mei, R. Oliveira, Colloids Surf. B: Biointerfaces 53 (2006) 105.
[45] E. Deziel, F. Lepine, D. Dennie, D. Boismenu, O.A. Mamer, R. Villemur, Biochim. Biophys. Acta 1440 (1999) 244.
[46] K.S.M. Rahman, T.J. Rahman, S. McClean, R. Marchant, I.M. Banat, Biotechnol. Prog. 18 (2002) 1277.
[47] M. Benincasa, F.R. Accorsini, Bioresour. Technol. 99 (2008) 3843.
[48] S.G.V.A.O. Costa, M. Nitschke, R. Hadaad, M.N. Eberlin, J. Contiero, Process Biochem. 41 (2006) 483.
[49] E. Haba, A. Abalos, O. Jauregui, M.J. Espuny, A. Manresa, J. Surfactants Deter. 6 (2003) 155.