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Full Length Research Paper

Production and characterization of biosurfactant from *Pseudomonas aeruginosa* PBSC1 isolated from mangrove ecosystem

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In this present study, biosurfactant-producing microorganisms *Pseudomonas aeruginosa* PBSC1, was isolated from mangrove ecosystem in Pichavaram (Boat house), Tamil Nadu, India. The biosurfactant production was done using a minimal salt medium (MSM) with crude oil as the hydrocarbon. The microbial growths were investigated, and the best cultivation times for the biosurfactant production by *P. aeruginosa* PBSC1 was found to be 144 h. The biosurfactant was extracted and characterized. The critical micelle concentrations (CMCs) of extracted biosurfactant was found to be 78 mg l^{-1}. Stability studies were done at various pH, temperature and NaCl concentrations. The emulsification activity was stable at lower and higher pH and temperature respectively. NaCl concentration does not affect the emulsification activity of the biosurfactant. The emulsion formed by the biosurfactant against each hydrocarbon was stable for one month. Further characterization of biosurfactant using Fourier transform infrared spectroscopy (FTIR) revealed it as a rhamnolipid.

Key words: Mangrove ecosystems, *Pseudomonas aeruginosa*, biosurfactant, critical micelle concentration (CMC), FT-IR fourier transform infrared spectroscopy (FTIR).

INTRODUCTION

Microbial biosurfactants are extracellular compounds produced by microbes such as bacteria, fungi and actinomycetes when grown in culture medium containing hydrophobic/hydrophilic substrates. Biosurfactants are surface active molecules having hydrophilic and hydrophobic moieties as their constituents which allow them to interact at interfaces and reduce the surface tension. They are classified based on their chemical composition into many groups such as fatty acids, glycolipids, glycolipopeptides, glycoproteins, lipopeptides, phospholipids, polymeric and particulate biosurfactants (Desai and Banat, 1997).

The biosurfactants properties includes excellent detergency, emulsification, foaming, dispersing traits,

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wetting, penetrating, thickening, microbial growth enhancement, metal sequestering and resource recovering (oil) which make surfactants replace some of the most versatile process chemicals (Rosenberg, 1986). They 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 (Mercade and Manresa, 1994). The establishment of waste-based medium for biosurfactant production also faces another problem; that is, the kind and the properties of final product depend on the composition of culture media (Besson and Michel, 1992).

Mangrove ecosystem is a bridge between terrestrial and marine ecosystem and harbours unique microbial diversity. Mangroves are present in the coastal areas of tropical countries and supports abundant life through a food chain that starts with the trees and the micro-biota (Smith et al., 1991). Although mangrove ecosystem is rich in organic matter, by and large they are nutrient-deficient especially in nitrogen and phosphorus. Diversity of microbial communities inhabiting this unique swampy, saline, partially anaerobic environment is useful as it provides clue of the microorganism and their adaptability in such habitats (Semenov et al., 1999). The biosurfactant producing organisms are isolated from the mangrove ecosystems.

In this present study, biosurfactant-producing micro-organism *P. aeruginosa* PBSC1 was isolated from mangrove ecosystems. The production of biosurfactant was carried out using the MSM. The cell free culture broth was extracted for the biosurfactant and quantified. To confirm the type of biosurfactant, the characterization study was conducted.

MATERIALS AND METHODS

Isolation, screening and identification of the microorganism

The mangrove soil samples were collected from various places of Cuddalore district of Tamil Nadu, India. The places are Pichavaram (boat house), South Pichavaram, Kodiyampalayam, Muzhukuthurai and Artificial Mangrove forest. From these five places, a total of 21 soil samples were collected from sediments and rhizosphere of the mangrove plant. One hundred gram of freshly collected soil samples was taken in a 250 ml Erlenmeyer flask. It was added to 10 ml of crude oil (to selectly enrich the biosurfactant producers) and thoroughly mixed and kept for incubation at room temperature (28 ± 2°C) for 30 days. The samples were moistened with water to avoid desiccation when necessary. The screening of bacterial isolates was done using hemolytic assay (Mulligan et al., 1984), drop collapse assay (Jain et al., 1991; Bodour and Miller-Maier, 1998), oil speading test (Morikawa et al., 2000), and emulsification (Cooper and Goldenberg, 1987). Best biosurfactant producing strain was identified and used for the production and characterization.

Identification of bacterial isolates

The isolated bacterial strains were identified using standard biochemical and sugar fermentation test. The species level identification was done using 16S rRNA sequencing.

16S rRNA sequencing

It is important to use a pure cultivated bacterium for identification. The purification of PCR products were done by removing unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). The sequencing was carried out by purifying the PCR products of approximately 1,400 bp and was sequenced using 2 primers. Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA).

The partial sequencing of the 16S rRNA gene was commercially carried out at the Yazhl Xenomics, Chennai, using universal amplification and sequencing primers and Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems), following the protocols provided by the manufacturer.

Phylogenetic analysis

The partial sequencing was analyzed and compared with nucleotide sequence databases in the National Center for Biotechnology Information (NCBI) website using basic local alignment search tool (BLAST) program (http://www. ncbi.nlm.nih.gov/BLAST), in order to confer percentage sequence similarities. The evolutionary history of SOL-10 strain was inferred using the Neighbor-Joining (NJ) method. The evolutionary distances were computed using the maximum composite likelihood (MCL) method. Phylogenetic analyses were conducted in Molecular Evolutionary Genetics Analysis (MEGA) software (Version 4.0).

Growth and biosurfactant production

Bacteria were grown in Minimal Salt Medium (g L^{-1}) containing 1.0 K_2HPO_4, 0.2 $MgSO_4.7H_2O$, 0.05 $FeSO_4.7H_2O$, 0.1 $CaCl_2.2H_2O$, 0.001 $Na_2MoO_4.2H_2O$, and 30 NaCl was added with crude oil (1.0%, w/v). Flasks containing sterilized MSM were inoculated with a loopful of bacteria and flasks were maintained in an Orbital shaker for seven days at 120 rpm, 30°C. After seven days of incubation, culture broth from each flask was centrifuged at 6000 rpm, 4°C for 15 min and the supernatant was filtered through 0.45 µm pore size filter paper (Millipore, India). This cell free culture broth was used for drop collapse assay, oil spreading assay, emulsification assay and surface tension measurement. All the screening experiments were performed in triplicates (until otherwise mentioned) and the mean values were recorded.

Biosurfactant extraction and characterization

The MSM broth with the culture inoculums was centrifuged at 10,000 × *g* for 30 min to discard the cells 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. The crude biosurfactant was expressed in g L^{-1} (Thavasi et al., 2011).

The critical micelle dilution (CMD) is defined as the solubility of a surfactant in an aqueous phase and is commonly used to measure the efficiency of a surfactant (Desai and Banat, 1997). The extracted biosurfactant was dissolved in distilled water at concentrations ranging from 1.0 to 200 mg L^{-1} for calculation of critical micelle concentration (CMC). This is a direct measurement of surfactant concentration corresponding to the concentration of an amphiphilic component at which the formation of micelles is initiated in the solution (Abouseoud et al., 2008). The CMC of the produced biosurfactant was determined following standard methods (Kim et al., 1997; Bonilla et al., 2005). CMD^{-1} and CMD^{-2} were determined by measuring the surface tensions of cell free supernatant diluted 10-times and 100-times in distilled water (Kosaric, 1993).

Carbohydrate moieties in the biosurfactant molecule were assayed using rhamnose (Dubois et al., 1956) and Molisch's test. The rhamnose test was performed by adding 0.5 mL cell supernatant to 0.5 ml 5% phenol solution and 2.5 ml sulfuric acid and incubating the sample for 15 min before measuring absorbance at 490 nm. Molisch's test was performed by adding 3 mL cell free supernatant to 1 mL 10% α-naphthol. This was followed by the addition of 1 mL concentrated sulfuric acid to the sample without disturbing it.

The crude biosurfactant extracted with chloroform: methanol was analyzed by thin layer chromatography (TLC). The TLC tank was filled with a solvent mixture of chloroform:methanol:acetic acid:water (25:15:4:2 v/v/v/v). The chromatogram was sprayed with α-naphthol and sulfuric acid.

Activity characterization

Foam was produced by hand shaking a two-day-old culture supernatant for a few minutes. The stability of the foam was monitored by observing it for 48 h. To determine the thermal stability of the biosurfactant, cell-free broth of the isolate was maintained at a constant temperature range of 20-100°C for 15 min, followed by cooling at room temperature (28 ± 2°C). The effect of pH and salinity on stability of the biosurfactant was evaluated by altering the pH (2-12) and the concentration of NaCl (0-1%, 5%) of the cell free culture supernatant and measuring the surface tension and Emulsification index (E_{24}, %) (Rashmi et al., 2012).

Emulsification

Cell free culture broth was used as the biosurfactant source to check the emulsification of crude oil. 1 ml of cell free culture broth was added to 5 ml of 50 mM Tris buffer (pH 8.0) in a 30 ml screw-capped test tube. Five milligram of hydrocarbon was added to the above solution and vortex-shaken for 1 min and the emulsion mixture was allowed to stand for 20 min. A negative control was maintained only with buffer solution and crude oil and Triton X-100 was used as the positive control. Various hydrocarbons like xylene, crude oil, benzene, kerosene, coconut oil, heptanes, n-hexadecane, diesel and petrol were used to check the emulsification activity of the isolate.

$$E_{24}\ (\%) = \frac{\text{The height of emulsion layer}}{\text{The height of total solution}} \times 100$$

FTIR

The FT-IR spectra was recorded in a Thermo Niocolet, AVATAR 330 FT-IR system, Madison WI 53711-4495, in the spectral region of 4000-400 cm^{-1} using potassium bromide (KBr) pellets. The air dried biosurfactant sample was ground with a purified potassium bromide salt to remove scattering effects from large crystals. This powdered mixture is then pressed in a mechanical press to form a translucent pellet through which the beam from spectrometer passed.

Statistical analysis

All the results related to determination of emulsification activity, biosurfactants quantity and CFU counts were the average of three replicates of two separate experiments for each cultural condition. They were statistically analyzed by SPSS software (version 100) using the Duncan test performed after analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Among the 21 soil samples, the higher microbial population was observed with Pichavaram (boat house) soil samples. All the 63 bacterial strains isolated were subjected to screening for their biosurfactant production. Results on identification of 63 bacterial strains revealed that out of 63 isolates, 29 strains belong to Gram positive and 34 strains to Gram negative group represented by 5 genera. The genera were as followed: *Bacillus* (18), *Escherichia coli* (6), *Klebsiella* (5), *Lactobacillus* (3), *Proteus* (6), *Pseudomonas* (21) and *Staphylococcus aureus* (4). Species dominance results showed that among the 63 strains isolated, 21 strains belong to the genus *Pseudomonas* (33.3%). From 63 bacterial strains tested, 34 (53.97%) strains were positive for hemolysis and the promising isolate PBSC1 showed the maximum hemolytic activity of 2.9 cm followed by isolate KBSB1 (2.5 cm). Among the 63 strains screened, 41 (65.1 %) strains were positive for drop collapse activity. 12 isolates showed positive to hemolytic and negative to the drop collapse test. The isolate PBSC1 reduced the surface tension greatly to 30.20 mN/m, which was selected as the most potent biosurfactant producer.

16S rRNA sequencing

The isolate PBSC1 was initially identified using standard biochemical and sugar fermentation test and further subjected to 16S rRNA sequencing. The isolate PBSC1 was identified by the 16S rRNA sequence as *Pseudomonas aeruginosa* PBSC1and deposited under the accession no JQ314422 in the Gen bank. The isolate PBSC1 has 99% similarity with the *P. aeruginosa*. The Figure 1 represents the phylogentic tree of *P. aeruginosa* PBSC1.

Estimation of growth and biosurfactant production

The biosurfactant production was studied using 2.0% crude oil supplemented with 1% mannitol in the MSM medium. Figure 2 shows the time-course of biosurfactant production by *P. aeruginosa* PBSC1 with crude oil as the

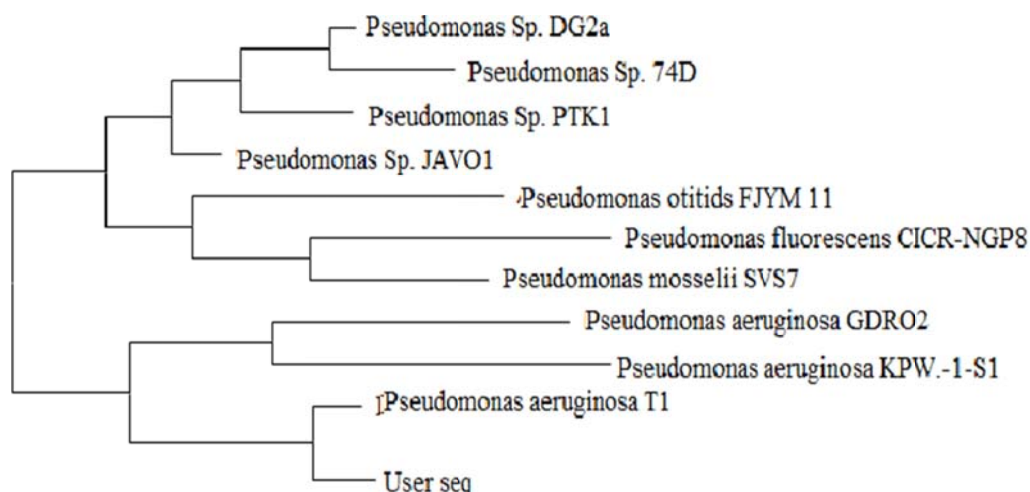

Figure 1. 16S rRNA sequence analysis of isolate PBSC1 and phylogenetic tree.

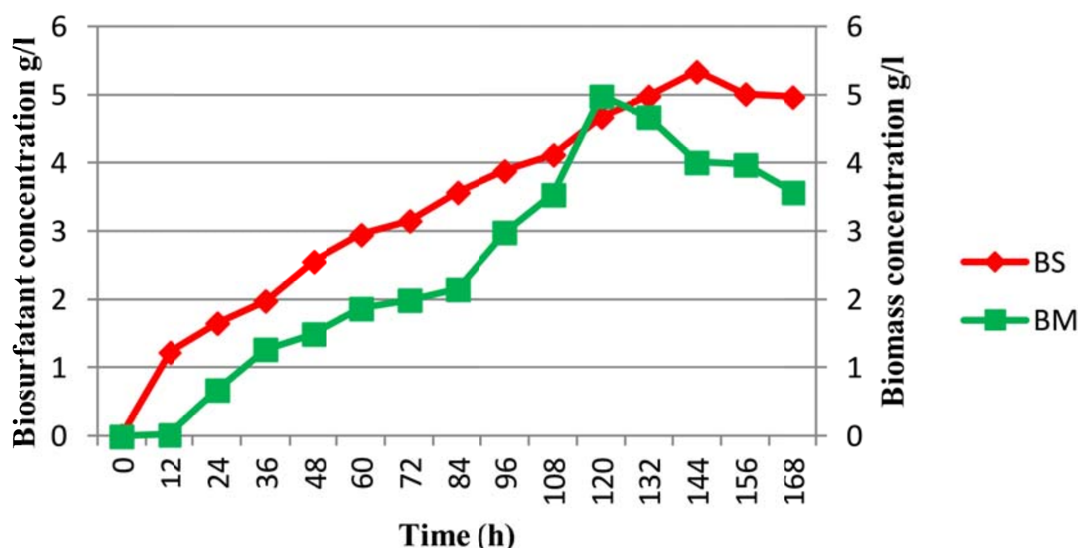

Figure 2. Growth kinetics of biosurfactant production by *Pseudomonas aeruginosa* PBSC1 under optimized conditions.

substrate. Maximum biosurfactant concentration of biosurfactant of 5.34 g L^{-1} occurred at 144 h of incubation, when the cells reached their early stationary phase. Maximum biomass was observed at 120 h (4.98 g L^{-1}). The biosurfactant was extracted from the cell free broth and further characterized to confirm the biosurfactant as rhamnolipid (a type of glycolipid) biosurfactant.

Characterization of biosurfactant

The CMC value of *P. aeruginosa* PBSC1was found to be 78 mg L^{-1}. Biosurfactant concentrations above the CMC could not decrease the surface tension further, indicating that biosurfactant molecules had begun to aggregate (Karsa et al., 1999). In the case of *P. aeruginosa* SP4, the excreted biosurfactant in the culture supernatant could decrease the surface tension of pure water from 72.0 to 28.3 mN m^{-1}, and the CMC was estimated to be 120 mg L^{-1} (Pornsunthorntawee et al., 2008). For *Pseudomonas fluorescens*, the CMC recorded for the isolated biosurfactant was 290 mg l^{-1} and the corresponding surface tension was 32 mN m^{-1}. The biosurfactant produced by *P. aeruginosa* PBSC1 exhibits better properties in terms of higher surface tension reduction and a lower CMC. The results of CMD^{-1} and CMD^{-2} of the biosurfactant containing cell-free medium were 30.3 and 42.0 mN m^{-1}, respectively, depicting non-significant change in efficiency. The results suggest that a sufficient amount of biosurfactant was present in the

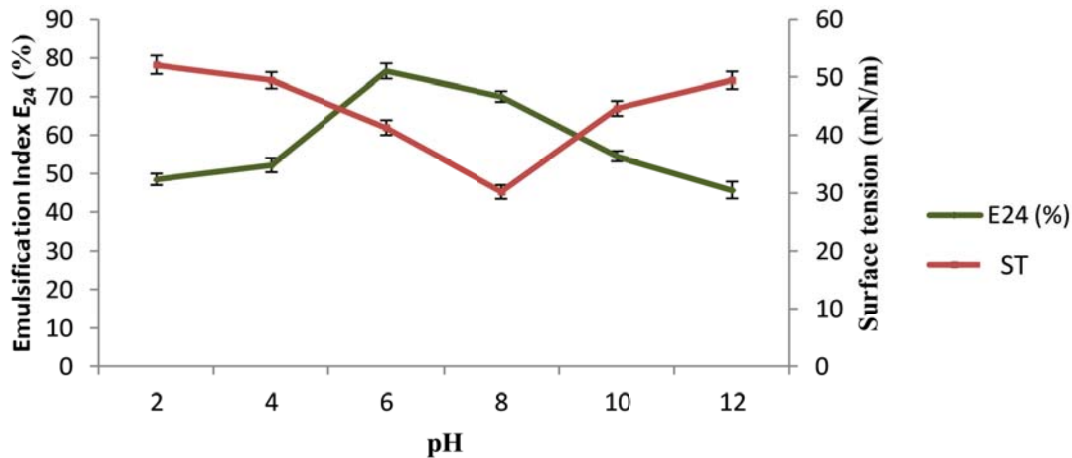

Figure 3. Stability of biosurfactant towards the changes in pH.

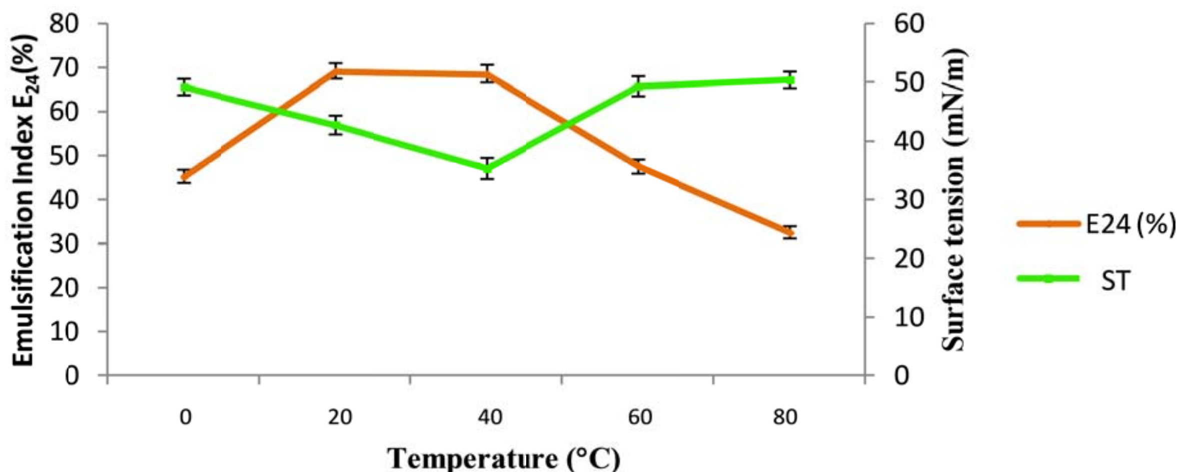

Figure 4. Stability of biosurfactant towards the changes in temperature.

culture medium, and thus its surface activity was retained even at such a high dilution.

Molisch's test showed a clear purple ring between the layers of solvent and the sample, indicating that the sample contained sugar moieties. The rhamnose test was positive, indicating that the separated biosurfactant could be of the glycolipid type. Red spots appeared on the TLC plate after spraying with α-naphthol and sulfuric acid, indicating the presence of carbohydrates in the sample. The production of glycolipid-type biosurfactant was previously reported for *Pseudomonas* sp. (Wilson and Bradley, 1996).

Activity characterization

Biosurfactant containing culture supernatant showed good foaming stability. The foam produced was stable for 48 h. Stable foam indicates that the produced biosurfactant can be used as a good foaming agent. Similar findings were reported for 48-h-old culture of *P. aeruginosa* PTCC 1561 grown in nutrient broth, which showed foam stability for 48 h (Noudeh et al., 2010).

In the case of stability of the biosurfactant towards the variations in the pH may alter the emulsification activity of the surfactant (Figure 3). pH range between 6-8, the highest emulsification activity was found. In the pH level of 2 and 12 the emulsification activity was greatly reduced and moderate activity was observed with the pH 4 and 10. The temperature may also have a significant role in the activity of emulsification of biosurfactant (Figure 4). In the lowest and highest temperature the activity was greatly reduced but good activity was observed with 20 and 40°C. When compared with pH and temperature the sodium chloride concentration does not produce any major differences in the emulsification activity (Figure 5). 5%

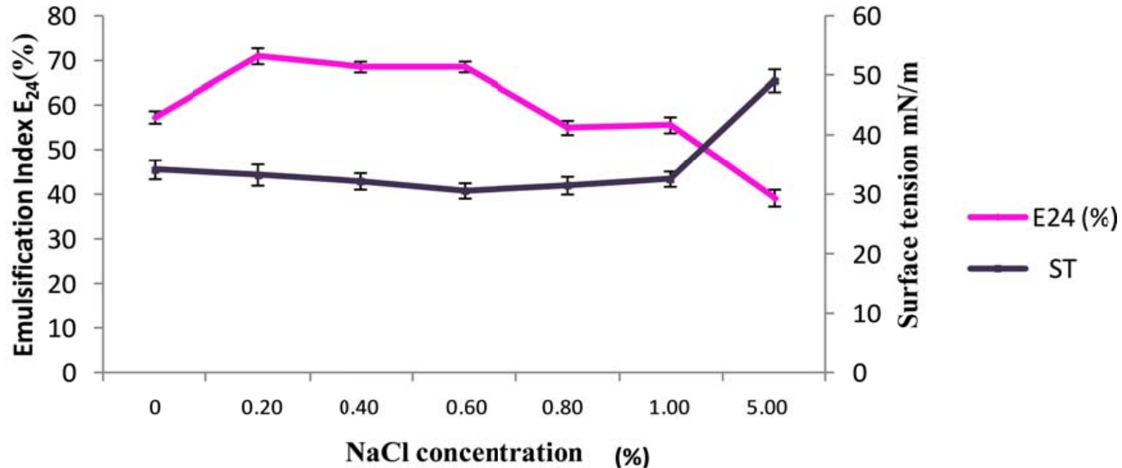

Figure 5. Stability of biosurfactant toward the changes in NaCl Concentration.

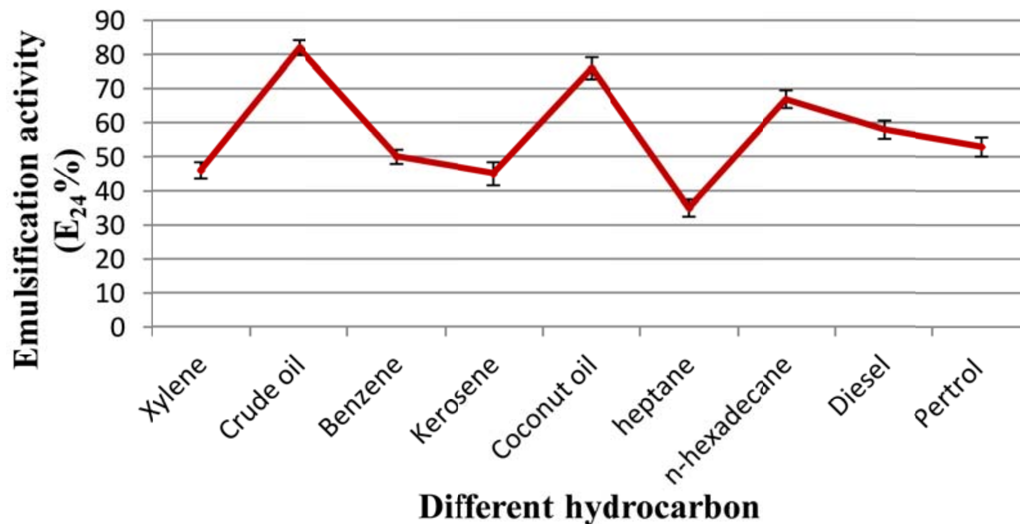

Figure 6. Emulsification activity of *P. aeruginosa* PBSC1 with various hydrocarbon.

concentration of NaCl showed highest emulsification activity followed by 10 and 15 %. From these results the biosurfactant was stable in the different pH levels with various temperatures and various concentration of sodium chloride.

This indicated that Surface tension and the E_{24} were stable even at a high temperature, in contrast to synthetic surfactants such as Sodium Dodecyl Sulphate, which exhibits a significant loss of emulsification activity above 70°C (Kim et al., 1997). Similar findings were reported for *P. aeruginosa* isolate Bs20, which exhibited excellent stability at high temperature (heating at 100°C for 1 h and autoclaving at 121°C for 10 min), salinities up to 6% NaCl, and pH values up to pH 13 (Abdel-Mawgoud et al., 2009).

Emulsification assay

Bio surfactant isolated from *P. aeruginosa* PBSC1 showed maximum emulsification activity against crude oil. Emulsification activities of the biosurfactant with different hydrocarbons were illustrated in Figure 6. The emulsion formed by the biosurfactant against each hydrocarbons were stable for one month.

Fourier transform Infrared (FT-IR) spectral analysis

The organism *P. aeruginosa* PBSC1 produced a rhamnolipid biosurfatant and it was confirmed with the FT-IR analysis based on the presence of functional

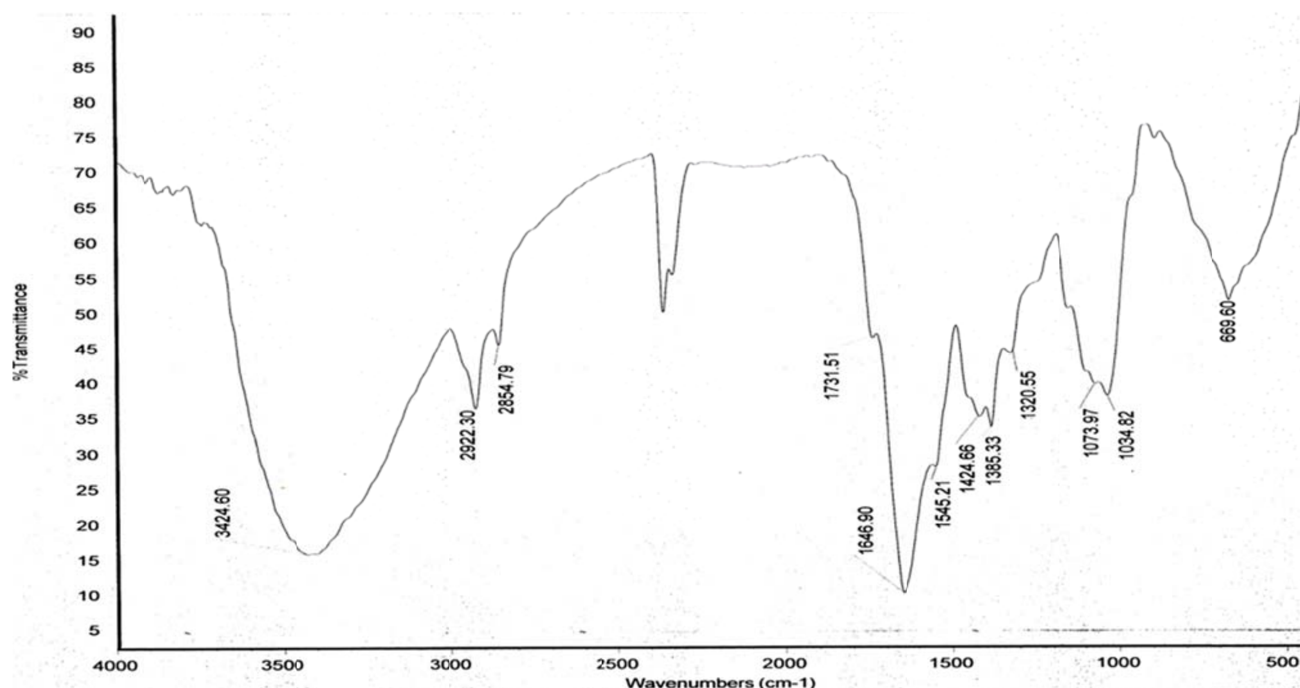

Figure 7. FT-IR spectra of biosurfactant produced by *P. aeruginosa* PBSC1.

group. FT-IR spectrum (Figure 7) revealed that, the most important adsorption bands were located at 3424.60 (OH bond, typical polysaccharides), 2922.30 and 2854.79 (CH band: CH2-CH3, hydrocarbon chains), 1731.51 and1646.90 cm^{-1} (for C=O, C=O ester bond), 1424.66 cm^{-1}(C-N amide groups). The C-O stretching bands at 1034.82-1320.55 cm^{-1} confirm the presence of bonds formed between carbon atoms and hydroxyl groups in the chemical structures of the rhamnose rings and 669.60 (for the CH2 groups) . According to Govindammal and Parthasarathi (2013) the most important adsorption bands were located at 3466.24 cm^{-1} which indicate the presence of OH bond, 2926.45 and 2856.23 cm^{-1} (CH band: CH2-CH3, hydrocarbon chains), and 1743.47 and1601.26 cm^{-1} (for C=O, C=O ester bond). The C-O stretching bands at 1162.26 to 1232.88 cm^{-1} showed the presence of bonds formed between carbon atoms and hydroxyl groups in the chemical structures of the rhamnose rings and 846.93 and 652.05 for CH_2 groups.

Conclusion

The isolated *P. aeruginosa* PBSC1 is a potent biosurfactant-producing native strain. The amount of crude biosurfactant recovered (5.34 g L^{-1}) from the culture medium. The CMC value (78 mg l^{-1}) of the produced biosurfactant is superior to many other biosurfactants. The tensioactive properties and stability of the biosurfactant to high temperature, pH and salinity reveal good prospects for this product in industrial applications. The emulsifying and foaming activity of the biosurfactant indicate that it can be used as a good emulsion-forming and foaming agent in different industries. The FT-IR spectrum reveals that the produced biosurfactant is a rhamnolipid. This strain can be further used in large scale production with alternative agroindustrial substrates for the better yield of biosurfactant. The optimization of the substrate may be studied elaborately using Response Surface Methodology (RSM).

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Conflict of Interests

The author(s) have not declared any conflict of interests.

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