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Physiochemical and Structural Characterization of Biosurfactant Produced by Halophilic *Pseudomonas aeruginosa* ENO14 Isolated from Seawater

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ABSTRACT

Objectives: The aim of this study was to isolate a potent biosurfactant producing bacterium and characterize the produced biosurfactant for its application in pharmaceutical, petrochemical, cosmetic and food industry. **Methods:** Enrichment method was carried out followed by serial dilution technique to isolate the bacteria from oil contaminated seawater. Various qualitative assays such as haemolytic assay, oil displacement assay, foaming index and emulsification index was carried out for the screening of the best isolate. Morphological, biochemical, physiological and 16S rDNA sequencing was used to identify the potent bacterium. For the production of biosurfactant, Bushnell Hass Broth (BHB), supplemented with 1% glucose was used. Various physiochemical and structural analysis of biosurfactant were carried out by different techniques. **Results:** The bacteria producing biosurfactant were isolated and screened for biosurfactant production using various qualitative assays from crude oil contaminated seawater. A potent bacterial isolate was selected on the basis of oil displacement activity and highest biosurfactant producing capability. Based on the morphological, physio-biochemical characteristics and sequencing of 16S rDNA of the isolate, ENO14, was revealed to be *Pseudomonas aeruginosa*. The biosurfactant exhibited high surface activity (critical micelle concentration, CMC=3.85 mg/ml) and excellent emulsifying activity against different hydrocarbons (emulsifying activity, EA_{24} =100% against crude oil). The biosurfactant showed stability over a temperature range of 20-70°C, pH range of 2-12 and salinity range of 2- 12% (w/v). Compositional analysis of the purified biosurfactant by chemical method as well as by Ultraviolet-Visible Spectroscopy (UV-Vis), Energy Dispersive X-ray Spectroscopy (EDX), Fourier Transform Infrared Spectroscopy (FT-IR), reveals it is a type of glycolipid biosurfactant. **Conclusion:** We conclude that *Pseudomonas aeruginosa* ENO14 is an efficient biosurfactant producer. The produced biosurfactant was well characterized for its application in pharmaceutical, petrochemical, food and cosmetic industry.

Keywords: *Pseudomonas*, Biosurfactant, CMC, Emulsification activity, EDX, FT-IR.

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INTRODUCTION

Crude oil and its derivatives are considered to be one of the most common pollutants in the environment, as they pose a growing problem of enormity around the world.[1] The components of crude oil may trigger multiple toxic effects including acute lethal toxicity, sub-lethal chronic toxicity depending on the type of exposure and the organism exposed.[2,3] Oil spills can often cause immediate and long-term environmental damage.[4] In addition, this problem is further aggravated by unsafe disposal methods due to higher cost associated with safe and proper disposal.[5] Therefore, these harmful hydrocarbon pollutants make the development of bioremediation technology essential for cleaning polluted sites. Compared to other strategies adopted to treat crude oil contamination, bioremediation is recognized as one of the most efficient, inexpensive and environmentally friendly technologies.[6] The bacteria, a free-living and ubiquitous micro-organism, have long been considered as one of the main agents for the hydrocarbon degradation.[7,8] An appropriate method that can be adopted to expedite the bioremediation of hydrocarbon contaminated sites (terrestrial or marine) is the involvement of biosurfactant producing hydrocarbon degrading micro-organism.

Biosurfactants are biological surface-active molecule released by living bacteria, fungi and yeast. They are principally of five kinds dependent on their structure, for example, glycolipid phospholipids, polymeric biosurfactants, lipopeptides and particulate biosurfactants.[9,10] Glycolipids comprising lipid and sugar in which sugar part is connected to a fatty acid.[11-13] The most common glycolipids are rhamnolipids, trehalolipids and sophorolipids.[14,15] Rhamnolipids are made of rhamnose (hydrophilic) moiety connected to fatty acids (hydrophobic), comprising of single or double bonded alkyl chains.[16] The chain length of fatty acid differs from 8-14 carbon atoms.[17-19] At present, synthetic surfactants are exclusively marketed.[9] However, there is a raising interest in biosurfactants particularly microbial biosurfactants for a few reasons. Firstly, they are non-toxic and biodegradable.[14,20] Secondly, they have distinct structural characteristics and it is essential for their realistic application to numerous industrial aspects, varying from biotechnology to environmental cleaning.[10,11] Lastly, they have higher frothing capability, higher specificity and specific activity at environmental conditions, for example, temperature, pH, salinity and apart from this it can be produced from renewable feedstocks.[15,21] Among the various biosurfactants, rhamnolipid, a kind of glycolipid is released from *Pseudomonas* sp. has been studied extensively and is known for many applications.[22]

Marine environment consists over seventy percent of our Earth's surface containing a rich chemical as well as biological diversity.[23] The

symbiotic relationship, highest gene transfer frequencies, competition amongst marine micro-organisms for nutrient and habitat, low or high pH, extreme salinity, temperature and pressure led a foundation for microbes for evolving new adaptive mechanism to survive in these extreme conditions. Previous studies have substantiated the isolation of novel biosurfactant-producing micro-organisms in perspective of the biosurfactant heterogeneity with the diversity of species.[24,25] In the recent past, considerable advances were made on the screening and isolation for biosurfactant generating isolates from marine ecosystems having unique physiological and metabolic capacities to flourish in extreme conditions and the capabilities to form novel metabolites.[26] Nevertheless, the biosurfactants secreted by marine bacteria have been less explored comparison with the bacteria isolated from soil.[27,28] The bacterial genera from marine habitats have been accounted to produce biosurfactants which include *Pseudomonas*, *Alteromonas, Bacillus*, *Acinetobacter*, *Myroides* and *Corynebacterium*.[26,28]

In the present investigation, we report a promising biosurfactant producing *Pseudomonas aeruginosa* ENO14 strain isolated from the coastal area of Ennore, Southern India. The physiological characterization like emulsification index and surface tension measurement of ENO14BS were carried out. Characterization of biosurfactant were analyzed by FTIR (Fourier Transform Infrared spectroscopy) and UV-Vis spectroscopy. Thermal decay and elemental composition were carried out by Thermo Gravimetric Analysis (TGA) and Energy Dispersive X-ray spectroscopy (EDX) respectively. Surface morphological studies were analyzed by Scanning Electron Microscopy (SEM).

MATERIALS AND METHODS

Isolation, screening and identification of potential biosurfactant generating marine micro-organisms

The seawater samples were collected after two months of the occurrence of an oil spill from the coastal area of Ennore, Tamilnadu, India (13°13′41″ N x 80°21′48″E). These samples were immediately brought to the laboratory in 100 ml sterilized glass bottles and stored at 4°C till further use. For the bacterial isolation, 1 ml of water sample was inoculated in 50 ml Zobel marine broth (ZMB) containing 1% (v/v) crude oil as a carbon source. The media pH was set to 7. Finally, the media was incubated at 37°C, at 100 rpm for 5 days. After 5 days, 1 ml of culture from the media was taken for serial dilution followed by spreading of 0.1 ml from the diluted sample (10^{-5} to 10^{-8}) on Zobel marine agar (ZMA) and the plates were incubated at 37°C for 24-48 hr. Different morphological bacterial colonies were selected and individually streaked on nutrient agar plates to get the pure bacterial colony. All the bacterial culture was maintained on nutrient agar slants, preserved in 40% glycerol and were stored at -80°C till further analysis. Various screen assays were performed for all the isolated bacterial strains, for example, oil displacement assay,[29] haemolytic assay,[26] foaming index,[30] emulsification index (E_{24})[31] for selecting the most promising bacterial strains. All these assays were done in triplicates with distilled water as the control. Based on the morphological, biochemical, physiological and 16S rDNA sequencing, the most promising strain was identified. The morphological and biochemical characterization were performed according to Bergey's manual of determinative bacteriology.[32] The genomic DNA was extracted and purified by HiMedia bacterial genomic DNA purification kit (MB505). The 16S rDNA sequence was amplified by polymerase chain reaction (PCR). The standard 16S r-RNA specific universal primers (purchased from GenScript, India), 8F (5′ AGAGTTTGATCCTGGCTCAG3′) and 1942R (5′ GGTTACCTTGTTACGACTT3′) were used for the amplification. The PCR was performed on a master cycler gradient (Eppendorf). The sequence homolog of potential isolate for the species level identification was analyzed by NCBI BLAST (www.ncbi.nlm.nih.gov) and the sequence was submitted to GenBank for generating accession number as a reference. Sequence data were aligned using ClustalW and phylogenetic analysis was performed by MEGA6 with 1000 bootstraps and neighbour joining algorithm.[33,34]

Production of biosurfactant and culture condition

For the production of biosurfactant the Bushnell Hass Broth (BHB), containing NH_4NO_3, 1.0 g; KH_2PO_4, 1 g; K_2HPO_4, 1 g; $MgSO_4$, 0.2 g; $CaCl_2$, 0.02 g and $FeCl_3$, 0.05 g per litre of distilled water, was used.[16] The pH of the media was set to 7 ± 0.2. Erlenmeyer flasks of 500 ml volume, containing 100 ml BHB media, supplemented with 1% (w/v) glucose as a carbon source, were inoculated by 2% (v/v) of bacterial culture (10^8 cells/ml) and was incubated at 37°C with rpm of 150 for 168 h. The cell growth was calculated by cell count method as explained by Thavasi *et al.*[35] Briefly, 1 ml of sample was taken at an interval 24 h (till 168 h) from the media, serially diluted and then plated on nutrient agar (2 % NaCl) for incubation at 37°C for 24 h. The developed colonies on the nutrient agar plate were calculated as Log CFU ml^{-1}. At the same time, five ml of cell-free culture broth was taken at 24 h intervals (till 168 h) for the extraction and quantification of extracellular biosurfactant based on the method explained by Thavasi *et al.*[36] with slight modification. Briefly, the broth was centrifuged at 6000 rpm for 20 min at 4°C. The supernatant was acidified to pH 2 by using 6N HCl and was stored at 4°C overnight. The refrigerated supernatant was extracted twice with methanol and chloroform (1:2, v/v). Then the solvent was evaporated by a rotary vacuum evaporator (Buchi, India) and the crude biosurfactant was purified by using a silica gel column (60-120 mesh). The column was eluted with methanol and chloroform ranging from 1:20 to 1:2 (v/v) in a gradient fashion. All the fractions were pooled and the solvent was removed again by an evaporator. The residue was dialysed against distilled water and it was freeze dried. This purified dried biosurfactant assigned as ENO14BS, was used for further characterization and bioremediation studies.

Biochemical characterization and cetyltrimethylammonium bromide agar (CTAB) agar plate assay

Biochemical characterization for the ENO14BS biosurfactant was performed using thin layer chromatography (TLC, for lipid detection), anthrone method (for sugar detection) and ninhydrin method (for protein detection) following the standardized protocol.[19,37,38] Total sugar was estimated by the phenol-sulfuric acid procedure as per Dubois *et al.*[39] The standard curve was analysed with D-glucose and the lipid content was calculated according to the method of Folch *et al.*[40] Furthermore, CTAB plate assay was performed to confirm the biosurfactant type.[41] Briefly, the methylene blue agar plates were prepared by adding 0.5 g CTAB, 0.5 g methylene blue (MB), 20 g of agar-agar and making the final volume up to 1 litre by mixing with distilled water. The media was sterilized at 121°C for 15 min at 15 lbs before pouring into the plates. Borer was used for cutting shallow wells on the agar plate surface, wherein 30 µl of the purified ENO14BS (5 mg/ml, dissolved in H_2O) was added. The plates were incubated at 37°C for 48 h. Formation of a dark blue halo against a light blue background around the well is considered as positive for glycolipid biosurfactant detection.

Critical micelle concentration (CMC) determination

The critical micelle concentration of the ENO14BS was calculated and compared with the chemical surfactants such as SDS (sodium dodecyl

sulfate) used as an anionic surfactant and T80 (Tween 80) used as a nonionic surfactant. The CMC values were measured by evaluating the surface tension of ENO14BS (prepared in distilled water) in the range of (0–10 mg/ml) using Tensiometer by the Du Nouy ring method (Tensiometer K100, Germany) at room temperature (30°C).[42]

Emulsifying activity (EA_{24})

This assay was performed according to the method of Copper and Goldenberg[31] with slight modification against eight hydrocarbon substrates, namely, soy bean oil, kerosene, toluene, engine oil, petrol, diesel oil, n-hexadecane and crude oil. Five ml of each hydrocarbon oil was mixed with 3 ml of ENO14BS solution (3mg/ml) in a glass tube. Then the mixture was vortexed for 2 min and stored at 30°C. The emulsification index was measured after 24 h as follows: Emulsifying activity (EA_{24}) = (H_e/H_t) × 100 where, H_e is the emulsified height and H_t is the total height of the solution.

Effects of pH, temperature and salt concentration on the surface activity

To evaluate the effect of pH, ENO14BS solutions having different pH ranging between 2-12 were made by using NaOH (0.05N) and HCL (6N). Thereafter, the emulsification activity (EA_{24}) was checked at each pH. To determine the effect of temperature on ENO14BS surface activity, 5 ml of the ENO14BS solution (4mg/ml) was treated at various temperatures 20°C, 30°C, 40°C, 50°C, 60°C, 70°C in hot water bath for 1 h. Once the solution cools down, the corresponding emulsification activity was determined to evaluate the thermal stability of ENO14BS. For determining the effect of salinity on the emulsification activity of ENO14BS, the ENO14BS solution was treated with various salt concentrations ranging between (2-10%). Finally, for calculating the emulsification activity of the ENO14BS solution, 5 ml of kerosene was mixed with 3 ml of ENO14BS solution. Then the mixture was vigorously shaken for few min and left undisturbed at room temperature for 24 h. Water was used as a control.

Thermogravimetric (TGA) and UV-Vis spectroscopy Analysis

TG and UV- Visible spectroscopy was performed according to the method described by Ohadi *et al.*[43] with slight modification. Thermo-Gravimetric (TG) analysis of ENO14BS biosurfactant was carried out by a thermal system (Perkin Elmer, Diamond TG/DTA). About 20 mg of dried biosurfactant sample was taken and TG thermograms were taken in the range of 40–700°C under the nitrogen air flow at the rate of 10°C/min. The graphical representation was displayed with weight (percentage) loss against temperature. The UV-Visible spectra of the purified biosurfactant sample (conc.1 mg/ml) was measured in the range of 200-800 nm by a UV-Visible spectrophotometer.

SEM-EDX analysis

For the quantitative elemental compositional analysis of the ENO14BS, Energy Dispersive X-ray (EDX) spectroscopy (OXFORD XMX N) was used. Samples weighing between 10-15 mg were mounted on an aluminium grid and observed at an acceleration voltage of 15 keV.[44] Surface texture analysis was done by scanning electron microscopy (SEM- JEOL Model JSM - 6390LV).

Fourier-transformed infrared spectroscopy (FT-IR) analysis

The functional groups present in the purified ENO14BS biosurfactant were determined by FTIR. The 5 mg of biosurfactant sample was added with dry KBr (potassium bromide) followed by compressing it into the pellet. The FTIR spectrum was measured in the wavelength ranges of 400–4000 cm^{-1} at a resolution of 4 cm^{-1} by using a FT-IR (Thermo Nicolet: 6700).[35]

Statistical Analysis

All the experiments were performed in triplicates and the values were given as mean ± standard deviation (SD) using Microsoft Excel 2010 (Microsoft, USA).

RESULTS

Isolation, Screening and Identification of potential biosurfactant producing marine micro-organisms

A few biosurfactants producing bacterial cultures were isolated from crude oil contaminated seawater samples by enrichment method. Based on the various qualitative assays such as haemolytic assay, oil displacement assay, foaming index, emulsification index, one of the best isolates for the production of biosurfactant was chosen for further analysis (Table S1). The bacterial isolate selected was assigned as ENO14. Basic morphological and biochemical properties of the isolated bacterial culture showed that ENO14 isolate was firmly related to *Pseudomonas* sp. (Table S2). On nutrient agar media, the isolated strain ENO14 produced green colored colonies. The observation of cells under a scanning electron microscopy (SEM) revealed their rod-shaped structure with cell size ranged from 1.93 to 2.12 µm in length (Figure 1A). The genotypic analysis based on partial 16S rRNA sequencing was analysed to find out the exact taxonomic position of the bacterial strain. The 16S rDNA sequences alignment of ENO14 with the sequences obtained from the BLASTn search shown 95% similarity to the *Pseudomonas aeruginosa* strain. (Figure 1B) The sequence was deposited to the NCBI and the accession number obtained was MH271625.

Production of biosurfactant, biochemical characterization and CTAB agar plate assay

The highest biosurfactant concentration of 3.65 mg/ml was found on the 5th day of incubation, when the bacterial cells almost reached in stationary phase. Maximum cell growth was found at 4th day (9.78 Log CFU/ml)

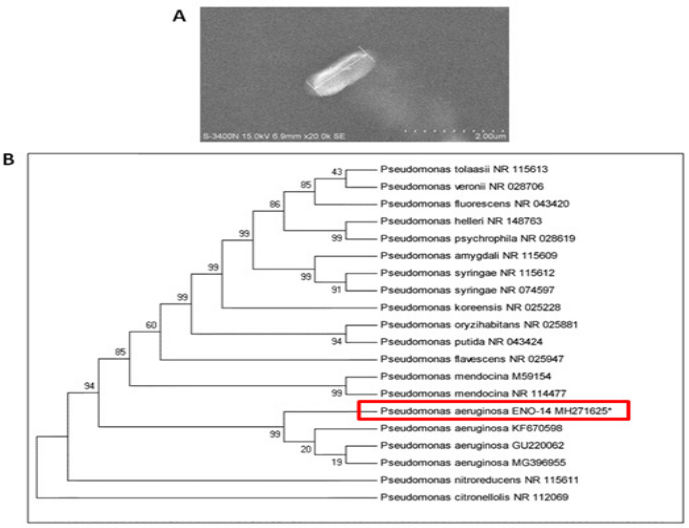

Figure 1: SEM imaging and phylogenetic analysis of the *P. aeruginosa* ENO14 (A) Scanning Electron Microscope (SEM) imaging of *P. aeruginosa* ENO14. (B) Phylogenetic relationship between the stain *P. aeruginosa* ENO14 and other strains or species based on the homology of 16S rDNA sequences.

(Figure S1A). In the TLC plate, yellow color spots were observed after iodine vapor exposure, which clearly indicates the presence of lipid in the extracted biosurfactant (Figure S1B). In the anthrone test, the formation of a bluish green color clearly indicates the presence of sugars in the biosurfactant (Figure S1C). In the ninhydrin test, the absence of the formation of purple color complex clearly indicating the absence of protein or amino acids in the biosurfactant molecule (data not shown). The quantification studies of the extracted and purified biosurfactant revealed that the biosurfactant was a type of glycolipid which was made of lipid molecule (~65%, w/w) and sugar molecule (~32%, w/w). Furthermore, the produced biosurfactant showed dark blue haloes against a light blue background in the CTAB agar plate assay (Figure S1D). Thus, from the above-mentioned biochemical and quantification assays, it can be inferred that the biosurfactant (ENO14BS) produced by the ENO14 strain is glycolipid in nature.

Critical micelle concentration (CMC) determination

The gradual decrease of surface tension was observed from 72.81 ± 1.12 to 29.22 ± 1.89 mN m-1 while increasing the ENO14BS sample concentration (Figure 2A). It was found that the surface tension became stable at the concentration higher than 3.85 mg/ml.

Emulsification activity (EA_{24})

Emulsification activity and stability of ENO14BS (1 mg/ml) was determined by using soybean oil, kerosene, toluene, engine oil, petrol, diesel oil, n-hexadecane and crude oil and their E_{24} activity were found to be 75.2, 85, 80, 83, 90, 92, 78 and 100 % respectively (Figure 2B).

Effects of pH, temperature and salt concentration on the surface activity

The emulsification activity for the ENO14BS was retained over a high pH range of 8-12. The emulsifying activity at pH 2, 4 and 6 was 16 %, 30 % and 52% respectively. The maximum emulsifying activity ($EA_{24,}$ 85%) was found at pH 8 (Figure 3A). Interestingly, the emulsifying activity showed gradual increase with the increase in pH from 2-8. Emulsifying activity was found to decrease gradually once the pH reached 10-12, it may be due to the structural changes of the ENO14BS at high pH.Biosurfactants are surface active molecules used as bioremediating agents. Therefore, it is crucial to study the stability of ENO14BS. The thermo-stability of ENO14BS was studied over a temperature range of 20-70°C. At 70°C ENO14BS solution showed little change in its emulsifying activity (65%). The maximum emulsification activity (EA_{24}, 85%) was observed at 40°C (Figure 3B). The emulsification activity slightly decreased from 80-65% when the temperature was in the range of 50-70°C.

The salinity stability on ENO14BS's activity was also studied (Figure 3C). Even with an increase of 8% NaCl concentration steady emulsification activity of 83% was observed. At 12% NaCl concentration, emulsifying activity decreased insignificantly to 79%.

Thermogravimetric (TGA) and UV-Vis spectroscopy Analysis

The analysis of the thermo stability of a biosurfactant is very much essential for its industrial application. Thermal decay of ENO14BS occurred by two clear differentiated steps as found in the TGA analysis graph (Figure S2A). Ten percent (10%) of weight loss was observed from 40 to 125°C due to loss of moisture and alcohol present in the

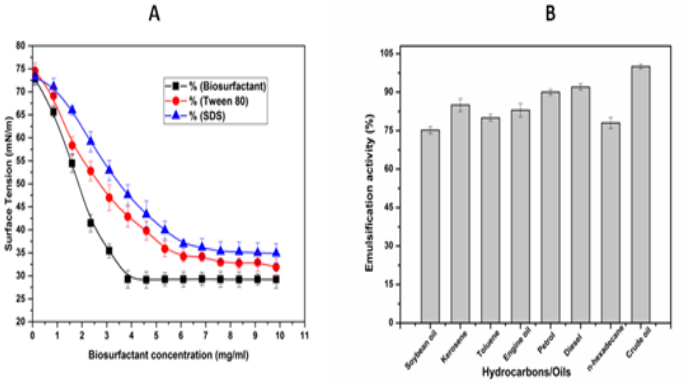

Figure 2: (A) Determination of CMC for ENO14BS biosurfactant. (B) Emulsification activity of ENO14BS against different hydrocarbon substrates.

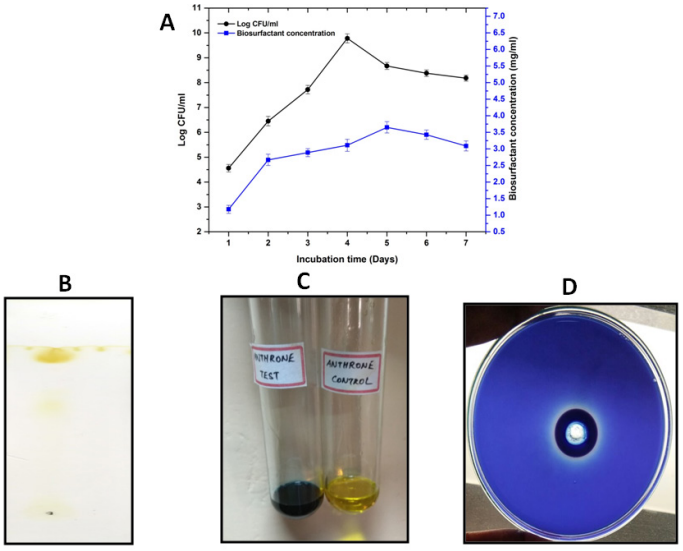

Figure S1: (A) Growth and biosurfactant production by *P. aeruginosa* ENO14 under basal condition. (B) TLC-yellow colored spots revealing the presence of lipids upon iodine vapor exposure. (C) Anthrone test showing the presence of sugar moiety (greenish blue) in the test sample. (D) Blue agar plate (CTAB agar plate), dark blue haloes against a light blue background clearly indicating the production of rhamnolipid biosurfactant.

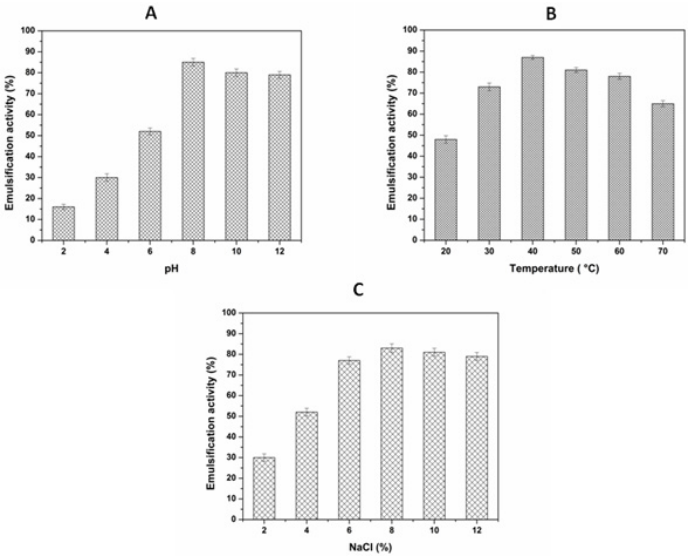

Figure 3: Effect of (A) pH, (B) Temperature and (C) Salinity on the surface activity of ENO14BS.

biosurfactant molecules followed by the second phase of thermal decay at 290°C, where, 53 % of weight loss was observed. The complete degradation of biosurfactant was found after 400°C.

In Figure S2B, the UV-Vis spectrum of the extracted ENO14BS biosurfactant is plotted. The results indicate that UV region absorbs the significant amount of light.

SEM-EDX analysis

The result of EDX analysis for the extracted ENO14BS revealed the presence of carbon, oxygen and potassium in a ratio of 62.57, 29.46 and 7.97 (Atomic %) respectively (Figure 4A). SEM (Scanning Electron Microscopy) is one of the very helpful technique to analyse the surface morphology of biomolecules which helps to understand the physical characteristics of the compounds. SEM micrographs of the ENO14BS revealed the presence of compact, dense irregular forms of clump-like structures (Figure 4B).

Fourier-transformed infrared spectroscopy (FT-IR) analysis

The FTIR spectrum of ENO14BS was measured for the detection of functional groups, in the spectral area of 400–4000 cm^{-1} (Figure 5). The peak at 1731 cm^{-1} denotes the presence of carbonyl ester bond in the molecule. The presence of stretching of COO– group was found at 1656 cm^{-1}. The absorption peak for the glycosidic bond (C-O-C) present in the biosurfactant molecule was found at 1068 cm^{-1}. A broad band was observed at 3389 cm^{-1}, for the presence of hydroxyl (-OH) group in the molecule. The presence of C–H bands (CH_2-CH_3) was observed at 2927 cm^{-1} and 2856 cm^{-1}.

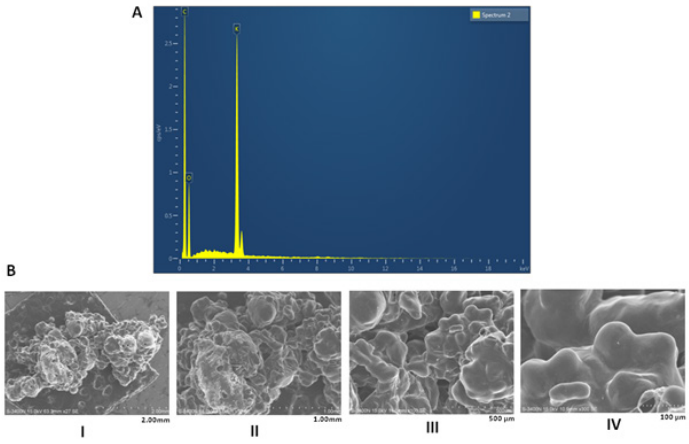

Figure 4: (A) EDX analysis of the purified ENO14BS. (B) SEM images of ENO14BS showing the microstructure and surface morphology at various magnifications. Scale bars denote 2 mm (I), 1 mm (II), 500 µm (III) and 100 µm (IV).

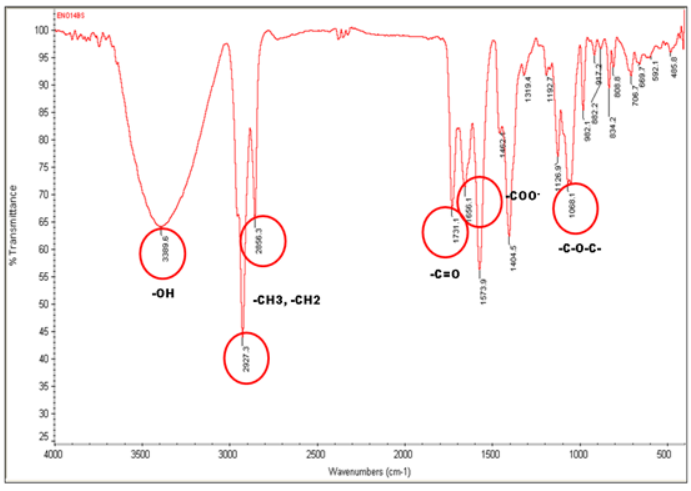

Figure 5: FT-IR spectrum of the biosurfactant ENO14BS showing the major functional groups present in ENO14BS.

DISCUSSION

Pseudomonas aeruginosa is the most well-known producers of glycolipid biosurfactants, isolated from oil contaminated seawater samples.[28,45] In

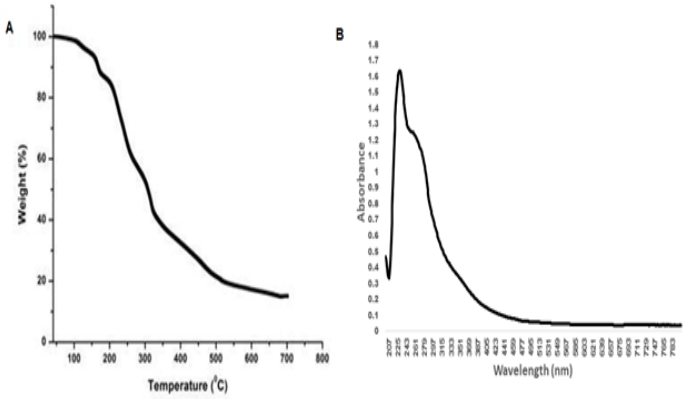

Figure S2: (A) TG thermogram of ENO14BS biosurfactant obtained from *Pseudomonas aeruginosa* ENO14 (B) UV-Vis spectrum of the purified biosurfactant ENO14BS.

Table S1: Qualitative assays for the biosurfactant production.

Test Isolates	Haemolytic assay	Foaming index (%)	Oil displacement assay (cm)	Emulsification index (% E_{24})
ENO-1	-	0	1.5 ± 0.1	0
ENO-2	-	0	1.4 ± 0.1	0
ENO-3	-	0	1.6 ± 0.1	0
ENO-4	+	8 ± 0.3	1.2 ± 0.11	12 ± 0.11
ENO-5	++	25 ± 0.5	1.4 ± 0.12	54 ± 0.12
ENO-6	-	0	1.3 ± 0.11	0
ENO-7	-	0	1.3 ± 0.1	0
ENO-8	++	36	1.5 ± 0.2	43 ± 0.3
ENO-9	-	15 ± 0.3	1.4 ± 0.11	23 ± 0.11
ENO-10	-	0	1.3 ± 0.06	0
ENO-11	-	32 ± 0.2	1.4 ± 0.05	48 ± 0.3
ENO-12	-	0	1.4 ± 0.08	0
ENO-13	-	38 ± 0.4	1.8 ± 0.2	52 ± 0.4
ENO-14	++++	69 ± 0.2	3.4 ± 0.12	78 ± 0.2
ENO-15	+++	46 ± 0.3	2.5 ± 0.2	57 ± 0.5

The zones of clearing were scored as follows: '–', no hemolysis; '+', incomplete hemolysis; '++', complete hemolysis with a diameter of lysis <1 cm; '+++', complete hemolysis with a diameter of lysis >1cm but <3cm; and '++++', complete hemolysis with a diameter of lysis >3 cm and green colonies.

Table S2: The biochemical and morphological characteristics of the isolated bacterial strain EN014.

Character/Tests	Results
Gram staining	Gram negative
Shape	Rod
Catalase test	Positive
Spore formation	Negative
Capsule staining	Positive
Motility Test	Motile
Indole production	Negative
MR test	Negative
VP test	Negative
Citrate utilization test	Positive
Starch hydrolysis	Negative
Urease Test	Positive
Lactose fermentation	Negative
Glucose fermentation	Positive
Utilization of galactose	Negative

this manuscript, we report the production of glycolipid biosurfactant by marine *P. aeruginosa* ENO14 strain isolated from oil spilled coastal area and characterized it for its application in industry. Characterization of biosurfactant is very crucial for its application-based studies. Chemical characterization helps to detect the chemical composition of biosurfactant by various different methods.[46,47] Hassan *et al.*[48] reported the confirmation of the glycolipid biosurfactant production by CTAB agar plate assay from *Pseudomonas* sp. On the other hand, critical micelle concentration of biosurfactant was determined to check the surface proprieties.[49] The purity of the biosurfactant and nature of solvent also affect the CMC value for a particular biosurfactant.[50] Furthermore, a good emulsifier must maintain the 50% emulsion of its original emulsion volume after 24 h of its formation.[51] In this study, the highest emulsification activity of the biosurfactant was 100% for crude oil, followed by other oils in the order of diesel oil, petrol, kerosene, engine oil, toluene, n-hexadecane and soy bean oil. It was observed that EN014BS was able to emulsify various hydrocarbons which confirms its applicability in degrading recalcitrant hydrocarbons thus can be used as a bioremediating agent.[47] To check the stability of biosurfactant, it was tested for various temperature, salt and pH range. It showed remarkable stability at high temperature. Such extreme stability was reported by Aparna *et al.*[47] These results clearly suggest that the extracted ENO14BS could be highly useful in bioremediation as well as microbial enhanced oil recovery process where high temperature prevails. ENO14BS biosurfactant was also quite stable at high salt concentration. These findings are in concurrence with Helvaci *et al.*[52] At high pH, ENO14BS showed excellent activity. A similar type of result was reported by Jain *et al.*[53] from *Cronobacter sakazakii* isolated from oil-contaminated wastewater.

UV-Vis spectroscopy was used to check the presence of a particular type function group. The absorption peak at 200-240 nm describe the existence of $\pi - \pi^*$ as well as $\pi-\sigma^*$ transitions of the different chemical bonds, for example, carbonyl, ester and carboxyl.[54] Moreover, the existence of a substituted aromatic structure is depicted in the region of 260-280 nm.[55] These above results clearly indicate that glycolipid biosurfactant is a very large and complex molecule which contains many UV-absorbing functional groups.

The existence of carbon (C) and oxygen (O) in a comparatively huge amount shows the presence of sugar and lipid molecule in the purified biosurfactant which was analysed by EDX. The existence of carboxyl and acetyl groups in the biomolecule indicated its anionic character which may be helpful for remediating the divalent cations.[56] SEM images showed irregular structure of biosurfactant. This irregular and smooth texture could be useful in cosmetic and food industries as gelling, thickening, texturing and stabilizing agents.

FT-IR spectroscopy is very helpful for identifying the functional groups present in a particular compound or a mixture of compounds.[57] There was no peak at 1552, 3246 and 3422 cm^{-1} indicated the absence of N–H bonds in the molecule. Therefore, it can be concluded from the above findings that the chemical structure of this biosurfactant produced by *P. aeruginosa* ENO14 is similar to those of earlier reported glycolipid biosurfactant which consist of rhamnose sugar linked with long hydrocarbon fatty acids chains. In our previous study, we showed, ENO14 BS biosurfactant is a very good candidate for anti-colon cancer activity.[58] Therefore, these additional characterizations of ENO14BS in this study will have greater impact on the pharmaceutical application-based work.

CONCLUSION

We conclude that ENO14BS biosurfactant produced by *Pseudomonas aeruginosa* ENO14 using glucose is an efficient producer. The biosurfactant was well characterized for its application in industry. The physiochemical properties of this organism have suggested its application to the *in-situ* bioremediation of sites polluted by petroleum hydrocarbon as well as the management of oil spills, whether marine or terrestrial. The ENO14BS biosurfactant, can also be find its applications in cosmetics, mining of minerals, pharmaceutical, food and textile industries after specific sectoral research.

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CONFLICT OF INTEREST

All the authors declare that there is no conflict of interest

ABBREVIATIONS

TLC: Thin layer chromatography; **FT-IR:** Fourier transform infrared spectroscopy; **ZMA:** Zobel marine agar; **ZMB:** Zobel marine broth; **PCR:** Polymerase chain reaction; **NCBI:** National Centre for Biotechnology Information; **BLAST:** Basic local alignment search tool; **TGA:** Thermo Gravimetric Analysis; **SEM:** Scanning Electron Microscopy; **EDX:** Energy Dispersive X-ray spectroscopy; **DNA:** Deoxy ribonucleic acid; **BHB:** Bushnell Hass Broth; **CFU:** Colony forming unit, **CTAB:** Cetyltrimethylammonium bromide; **MB:** Methylene blue; **CMC:** Critical micelle concentration; **SDS:** Sodium dodecyl sulphate; **HCL:** Hydrochloric acid.

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