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Leveraging the Green Petroleum Hydrocarbon Remediation Potential of a Biosurfactant Producing Indigenous Oleophilic Bacterium Isolated from Hydrocarbon Soiled Environment

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ABSTRACT

The present investigation focused on the physicochemical characterization and bioprospecting of an indigenous oleophilic bacterium (OB) and its biosurfactant (BS) for bioremediation. Within 14 days of culture at 30°C with 2% (v/v) n-hexadecane, the OB could reduce the surface tension of the culture medium by up to 34.4 mNm^{-1}. Standard screening tests verified that the isolated OB produced BS and identified it as *Pseudomonas aeruginosa*. BS production was 434.7 $mg.L^{-1}$, with a CMC of 195.6 $mg.L^{-1}$, and was purified and characterized using standard chromatographic and spectroscopic techniques. FTIR analysis confirmed the glycolipid nature of BS. TLC of the partially purified BS revealed two homologues of rhamnolipid (RL), which were subsequently confirmed by NMR. Seven distinct RL congeners were identified using LC-MS, of which di-RLs constituted a notably large proportion. The surface and emulsification activities of BS demonstrated significant stability against various pH levels, temperatures, salinities, and metal ions. Furthermore, OB was able to utilize crude oil within 60 days, as confirmed by GC-MS. In the soil washing experiment, BS separated ≥80% of the crude oil from the contaminated sand at the CMC. The results suggest that the RLs and their producer isolated from automobile workshops in Mokokchung are not only the first report from Nagaland, India, but are also promising for various applications in the bioremediation of extreme and complex environments, including addressing regional environmental issues in Nagaland.

INTRODUCTION

There is mounting global concern over the degradation of the environment by various anthropogenic chemical contaminants that are continuously released into our surroundings, causing severe environmental degradation. Petroleum hydrocarbons are one of the most common contaminants that affect different components of the environment. These pollutants are released into the environment mainly through oil spills during extraction, transportation, and refinement leaks, raising serious issues for the environment, ecological systems, and public health, leading to both short- and long-term environmental harm (Chandankere et al. 2014, Gote et al. 2023). Heavy metals such As, Cd, Cu, Zn and Pb are another group of hazardous, non-biodegradable, and persistent environmental pollutants that cause prolonged ecological and environmental damage as they tend to pass and magnify from one food chain to another, obstructing biological pathways, disrupting cellular functions, and causing diseases and damage to various organs (Das et al. 2017). In the environment, these heavy metal pollutants are found in association with sub-surface soil sediments, which often results in their release into groundwater, leading to contamination (Yang et al. 2020).

Existing conventional physical and biological technologies used for the remediation of these environmental pollutants are often associated with several

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drawbacks, such as high costs, low efficiencies, and the production of secondary pollutants. Therefore, cleaner, eco-friendly, cost-effective, and efficient technologies, such as biologically derived treatment strategies, are needed to tackle these hazardous pollutants (Usman et al. 2016). One such environmentally benign approach is the use of BSs derived from microbes, such as bacteria, fungi, and yeast, for bioremediation (Rodrigues et al. 2006). The presence of BSs can potentially enhance the mineralization and biodegradation of hydrocarbon pollutants by solubilizing and emulsifying the contaminants and serving as an important mediator by increasing the bioavailability of hydrophobic contaminants for microorganisms (Parthasarathi et al. 2011, Ławniczak et al. 2013). BSs are also known to form non-ionic complexes with heavy metals in the soil, thereby reducing the interfacial tension between the soil and metal ions, resulting in the removal of heavy metals from contaminated environments (Santos et al. 2016).

BSs are amphiphilic compounds produced by microorganisms that adhere to cell surfaces or are secreted extracellularly into the surrounding medium (Chioma et al. 2013). They are amphiphilic organic compounds with hydrophobic (tails) and hydrophilic (heads) groups. The hydrophobic (non-polar) end of the BS is insoluble in water. It can contain long chains of fatty acids, hydroxyl fatty acids, or α-alkyl-β-hydroxy fatty acids. In contrast, the hydrophilic (polar) end may contain a phosphate, carboxylic acid, cyclic peptide, carbohydrate, amino acid, or alcohol (Chioma et al. 2013). Microorganisms produce a diverse range of BSs with different structural profiles, including polymeric, particulate, neutral lipids, glycolipids, lipopeptides, and lipoproteins (Wang et al. 2011).

Among the known BSs, rhamnolipids (RLs) have received considerable attention compared to chemically synthesized surfactants because of their unique physicochemical properties, low toxicity, biodegradability, and ecologically benign nature, which makes them potential candidates in the field of environmental remediation processes (Banat et al. 2010, Md Fakruddin 2012, Chandankere et al. 2014). RL belongs to the class of glycolipid BSs and is reported to have a significantly higher yield than other classes of BSs (Arutchelvi et al. 2010). RL consists of two molecules: lipid, sometimes referred to as the aglycon part, and rhamnose, commonly known as the glycon. A hydrophilic compound, the rhamnose moiety, is composed of mono- or di-(L)-rhamnose molecules connected by α-1, 2-glycosidic bonds (Thakur et al. 2021). The properties of RLs are influenced by the congener composition, which varies depending on the type of bacterial strain, growth conditions, and media composition (Costa et al. 2010).

The potential uses of RLs in the fields of environmental protection (Abdel-Mawgoud et al. 2008), petroleum extraction (Makkar & Cameotra 2002), synthesis of specific compounds (Das et al. 2013), agriculture (Sorhie et al. 2022), and the manufacturing of pharmaceuticals (Md Fakruddin 2012) and food (Cameotra & Makkar 1998) have sparked interest in them. Currently, there is growing interest in the market for RLs, but they cannot effectively compete with their chemical counterparts because of various factors, specifically their higher cost of production (Chrzanowski et al. 2012, Md Fakruddin 2012, Thakur et al. 2021). In this regard, screening for more efficient BS-producing native microbial strains can be an effective strategy to increase BS yield and prospects (Chandankere et al. 2014). Native BS-producing strains can be readily isolated from the natural environment, which might prove more efficient and cost-effective than mass culturing, maintaining, and effectiveness of an exotic microbial species in a foreign environment. Moreover, the use of native bacterial strains can address issues related to legitimate and biosafety concerns.

In the backdrop of the aforementioned information, the current research work was embarked upon with the prime objective to isolate a potential RL-producing bacterial strain from Mokokchung Town, Nagaland, India, for the first time, as per our information, with desirable physico-chemical properties to address the issues of soil contaminated with hydrocarbon and heavy metal pollutants.

MATERIALS AND METHODS

Isolation of Oleophilic Bacteria from Environmental Samples

Several bacteria were isolated from oil-contaminated soil samples collected from different automobile workshops in Mokokchung Town, Nagaland, India. Bacterial isolation was performed using the enrichment culture technique, where 5 g of each soil sample was inoculated into a 250 mL Erlenmeyer flask containing 100 mL of Bushnell-Haas medium with 2% (v/v) n-hexadecane as the sole carbon source. For the first cycle of incubation, the cultures were shaken in an orbital shaker for 14 days at 140 rpm at 30°C ±1. An aliquot of 1mL from each of the original cultures was transferred to 250 mL flasks containing fresh media with n-hexadecane and incubated for another 14 days for the second cycle. This process was repeated up to the fifth cycle. After the fifth cycle, the viable bacterial populations were isolated from the culture broth using the serial dilution technique in Bushnell Haas agar plates supplemented with n-hexadecane (2% v/v).

Culturing and Screening of BS-Producing Oleophilic Bacteria

The bacterial isolates obtained after five cycles of enrichment culture were later grown in mineral salt medium (MSM), and the pH of MSM was adjusted to 6.8 using 6 N HCl (Bharali et al. 2011). The culture was supplemented with 2% (v/v) n-hexadecane as the sole carbon source, and the temperature and pH were adjusted and maintained at 6.8 and 30°C, respectively, in an orbital shaker with an agitation rate of 140 rpm for 14 days (Bharali et al. 2022). The cell-free culture supernatant (CFCS) of bacterial cultures was used for the screening of BS production through various standard methods, such as surface tension (ST) measurement (Bodour & Miller-Maier 1998), oil displacement assay (Thavasi et al. 2011), drop collapse test (Bodour & Miller-Maier 1998), penetration assay (Kumar et al. 2017), CTAB agar test (Aparna et al. 2012), and hemolysis test (Mulligan et al. 1984, Youssef et al. 2004). The isolate that demonstrated the best results in the screening assays was selected for further characterization of its surface activity.

Characterization of Selected Bacterial Isolate

The morphological features of the selected bacterial isolate were examined using Bergey's Manual of Determinative Bacteriology (Bergey 1994). Standard biochemical testing was conducted as outlined by Cappuccino & Sherman (2013). Molecular characterization of the selected bacterial isolate was performed using a standard 16S rRNA gene sequencing approach. Based on the BLAST results, the evolutionary distance of the isolate with its most closely related strains was computed, and the sequence obtained was deposited in the NCBI GenBank database. Evolutionary analyses were conducted using MEGA 11 software with the maximum likelihood approach and 1000 bootstrap replicates (Tamura et al. 2021).

Growth Kinetics and BS Production

The chosen bacterial strain was cultivated in MSM enriched with 2% (v/v) n-hexadecane at 30°C in an orbital shaker operating at 140 rpm for 22 days (Bharali & Konwar 2011). Using a UV-Vis spectrometer (PerkinElmer UV/VIS Lambda 365), the optical density (OD) of the selected bacterial culture was recorded at 600 nm wavelength after every 24 h of incubation. For biomass quantification, the increase in dry bacterial biomass after every 24 h of incubation was determined gravimetrically. BS production in the culture medium was tracked every 24 h using the standard orcinol method (Rahman et al. 2010).

Isolation of BS

Bacterial cells were separated from the culture supernatant by centrifugation at 10000 rpm for 10 min. The CFCS was precipitated by acidifying it to pH 2 using 6 N HCl and stored overnight at 4°C. Acid-precipitated BSs were centrifuged for approximately 15 min at 10000 rpm and washed twice with phosphate-buffered saline (PBS). After washing, the recovered pellets were dried in a hot-air oven to remove moisture. The dried BS was then measured gravimetrically using a laboratory balance.

For the partial purification of the BS, the acidified precipitated CFCS was extracted three times using ethyl acetate at room temperature. The solvent was extracted from the organic phase and concentrated using a rotary evaporator with a round-bottom flask. A sticky, honey-colored BS was obtained at the end of the process (Bharali et al. 2011).

Purification of Partially Purified BS

Thin-layer chromatography (TLC) was used to identify and purify the BS. Chloroform: methanol: water (65:15:2) (v/v/v) was used as the mobile phase system (Bharali & Konwar 2011). Developing agents such as anthrone and iodine fumes were used to detect the presence of carbohydrate and lipid moieties of glycolipid-type BSs, respectively (George & Jayachandran 2013).

Approximately 5 g of a partially purified BS was dissolved in 10 mL of chloroform and loaded onto a glass column (26 cm × 3.3 cm), which was previously packed with 50 g of column-grade silica gel, followed by cleaning the column with chloroform to remove the neutral lipids. Subsequently, fractions were collected at a flow rate of 1 mL min^{-1}, with 20 mL portions obtained using chloroform: methanol mobile phases in a sequence of 50:3 v/v (1000 mL), 50:5 v/v (200 mL), and 50:50 v/v (100 mL). A final rinse with a 1:1 mixture of chloroform and methanol was used to remove residual RLs from the column. The collected fractions were evaporated to dryness under vacuum using a rotary evaporator (Mishra et al. 2019).

Physical Characterization of Isolated BS

Determination of Critical Micelle Concentration (CMC)

Various concentrations of BS solutions were prepared in water from the stock solution of the BS. The ST of the dilutions was measured at room temperature to determine the CMC. CMC was determined by plotting the ST as a function of the BS concentration. Three replicas of these studies were conducted (Chandankere et al. 2014).

Emulsification Index

The emulsification index (E_{24}) was determined by adding 2 mL of CFCS and an equal amount of different hydrocarbon oils, including diesel, kerosene, petrol, vegetable oil, crude oil, and waste vegetable oil, in different test tubes and vortexed for 2 min. The mixture was incubated for 24 h at room temperature (Sarubbo et al. 2006, Câmara et al. 2019). The percentage of emulsification after 24 h, E_{24} (%), was calculated using the following formulae,

E_{24} (%) = (Height of emulsion layer)/(Total height of the solution) × 100

Chemical Characterization of Isolated BS

Fourier-Transform Infrared Spectroscopy (FT-IR)

FT-IR analysis of the partially purified BS was performed in the 400–4000 cm^{-1} range. A translucent disc was prepared using a mixture of 10 mg of the extracted BS and 100 mg of spectral purity KBr at 25 Mpa for 30 second (Zhao et al. 2019). The resultant KBr discs were analyzed using a Perkin Elmer FTIR spectrophotometer (Perkin Elmer-Spectrum Two).

Liquid Chromatography and Mass Spectroscopy

The RLs were characterized using an MS Q-TOF Mass Spectrometer (model G6550B). The separation was based on reverse-phase chromatography using a Zorbax Eclipse XDB-C18 column (3.0 × 150 mm, 3.5 µm) at a temperature of 40°C. The flow rate was 0.2 mL min^{-1}. The mobile phase gradient consisted of 10 mM aqueous ammonium formate buffer with 0.05% formic acid (A) and acetonitrile (B). The gradient started at 20% B from 0 to 2 min, increased to 50% B from 2 to 10 min, and reached 100% B from 10 to 15 min. It remained constant at 100% for 5 min, then decreased to 20% over 8 to10 min, and remained constant until equilibration was achieved. The total run time was 30 min, and the injection volume of sample was 2 µL (Behrens et al. 2016).

Nuclear Magnetic Resonance

The samples were prepared by dissolving approximately 3 mg of BS in a 100% $CDCl_3$ solution and analyzed using an NMR spectrometer (Advance DPX 500 & 300 MHz FT NMR Spectrometer, Bruker). Proton and carbon NMR chemical shifts were measured in parts per million (ppm) with respect to the solvent shift, which served as the chemical standard. Using previously published data, the peaks were predicted and compared (Sharma et al. 2015).

Stability Studies

Effect of Environmental Variables on E_{24}%

The effect of time duration on the E_{24}% of the CFCS in diesel was calculated after 24 h, 15 days, and 30 days to determine the stability of the emulsification layer over time. The CFCS was exposed to 121, 100, 80, 40, 25, and 4°C for 60 min and emulsified with diesel to determine the effect of temperature on the E_{24}% of the BS. The pH of the CFCS was adjusted to 3, 5, 7, and 10 and emulsified with diesel oil to determine the effect of pH on the E_{24}% of the BS. Different concentrations of NaCl salt, 2%, 5%, 8%, and 10% (w/v), were added to CFCS to check the variation in the E_{24}% against diesel. Different heavy metallic salt, *viz*. $NaAsO_2$, $CdCl_2$, and K_2CrO_4 were added to the CFCS to determine the variation in the E_{24}% of the BS. For temperature, pH, NaCl, and heavy metal salts, the emulsion mixture was incubated for 24 h at room temperature. E_{24} (%) was calculated using the formula as described above.

Effect of Environmental Variables on Surface Activity

The reduction in the ST of the CFCS was checked after exposing it to a wide range of temperatures, namely, 121°C, 100°C, 80°C, 40°C, 25°C, and 4°C for 60 min to determine the effect of temperature on surface activity. To determine the effect of pH on surface activity, the pH of the CFCS was adjusted to 3, 5, 7, and 10. The effect of salinity on surface activity was evaluated by adding different concentrations of NaCl *i.e*., 2%, 5%, 8%, and 10% (w/v), to the CFCS. Different heavy metallic salts, *viz*. $NaAsO_2$, K_2CrO_4, and $CdCl_2$ were added at different concentrations (100 ppm, 300 ppm, and 500 ppm) to the CFCS to determine the variation in surface activity. The reduction in ST was measured using a surface tensiometer based on the *du Noüy* ring method.

Bioremediation Application

Soil washing experiment: 10% (w/w) of low viscosity crude oil was added to the acid-washed sand and allowed to sit at room temperature for seven days. Then, 5 g of sand samples were added to 100 mL of CFCS containing different concentrations of BS (at CMC, below CMC, and above CMC) in a 250 mL conical flask and rotated in an orbital shaker at 200 rpm for 24 h at 30°C. The aqueous solutions were decanted, and the sand was dried for 24 h at 50°C to evaporate the remaining solvent. The amount of oil that remained was measured gravimetrically, and the following equation was used to obtain the percentage of oil removed:

Crude oil removed (%) = (Oi – Or)/Oi × 100%

where Oi is the initial oil in the sand sample before washing and Or is the oil remaining in the sand sample after washing (Costa et al. 2010).

Crude oil degradation: 1 mL of the freshly grown culture supernatant of the bacterial strain was inoculated into 250 mL containing 2 mL of crude oil and 100 mL of MSM. The cultures were incubated at 30°C in an orbital shaker for 15, 30, 45, and 60 days. After each incubation period, the

cultures were centrifuged at 4500 rpm for 10 min to separate the bacterial biomass, and the residual hydrocarbon oil fractions in the CFCSs were extracted twice using dimethyl chloromethane (DCM) in a ratio 1:1. The contents of the residual crude oil extracted over 15, 30, 45, and 60 days were analyzed using Gas Chromatography-Mass Spectroscopy (Singh et al. 2016). Before being elevated to 280°C for crude oil analysis, the column temperature was maintained at 50°C for five min. A split ratio of 20:1 was used in all the studies. Helium was used as the carrier gas, and the flow rate was 0.8 mL min^{-1}. The injector was adjusted to a temperature of 250°C (Bharali et al. 2022).

RESULTS

Isolation of Oleophilic Bacteria from Environmental Samples

Thirty different numbers of soil samples were collected from ten hydrocarbon-contaminated sampling sites in the Mokokchung Town area of Nagaland, India (Fig. 1).

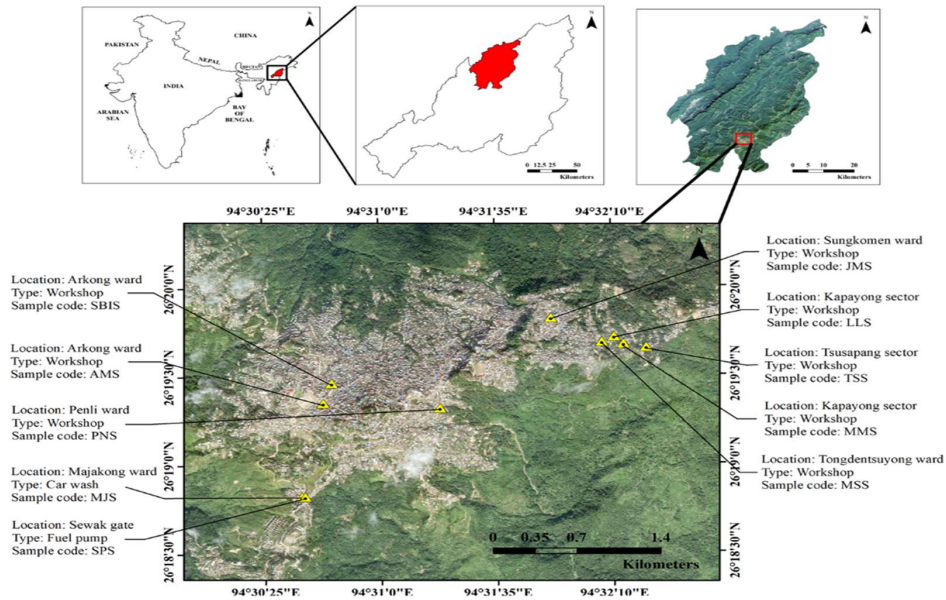

Fig. 1: Map illustrating the geographical position of the research region within Mokokchung town and the spatial layout of the sampling locations.

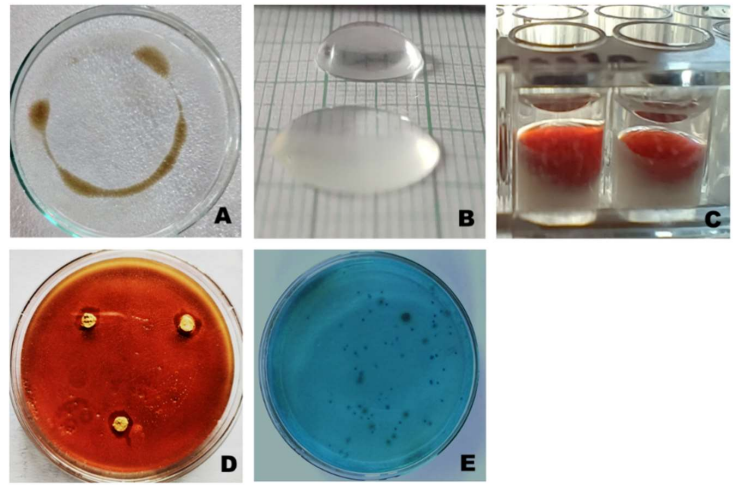

Fig. 2: Screening and confirmation of BS production by the selected isolate (A) oil displacement test, (B) drop-collapse test, (C) penetration assay, (D) hemolysis, and (E) CTAB agar assay.

From the environmental samples, approximately 60 bacterial isolates were obtained after the 5th cycle of enrichment culture of the soil samples using the serial dilution technique. The morphological characteristics of the obtained bacterial isolates were then recorded.

Culturing and Screening of BS-Producing Oleophilic Bacteria

The obtained bacterial isolates were further grown in MSM supplemented with 2% (v/v) n-hexadecane for screening and characterization. The reduction in the ST of the CFCS of the obtained bacterial isolates was determined. Isolates showing the best surface activity in terms of ST reduction measurement of the culture medium were selected for the second level of screening. Screening assays, such as oil displacement, drop collapse, and penetration assays, were performed to screen for BS production. The results of the screening assays of the selected bacterial isolate are presented in Fig. 2A, B, and C.

Potential strain was further screened using hemolysis and CTAB agar assays. The results are shown in Fig. 2D and E. The clear zone around the filter paper disc in the hemolysis assay indicated BS production. The anionic nature of the BS was confirmed using the CTAB agar test, which showed dark blue halos around the bacterial colonies.

Characterization of Selected Bacterial Isolate

The selected bacterial isolate was morphologically characterized using standard methods. The shape, size, and surface texture of the selected bacterial isolate were determined, and pure culture plates were prepared (Fig. 3A).

Table 1: Standard biochemical tests of the selected hydrocarbonoclastic bacterial isolate.

Biochemical Tests	Result
Gram staining	Negative rods
Spore staining	Negative
Catalase test	Positive
Oxidase test	Positive
Motility test (SIM)	Positive
Urea test	Negative
Indole test	Negative
MR test	Negative
VP test	Negative
Citrate test	Positive
TSI test	Alkaline
H_2S Test (SIM)	Negative
Nitrate test	Positive with Gas
Carbohydrate fermentation after 24 hrs	
Glucose	Positive
Sucrose	Negative
Lactose	Negative
Mannitol	Negative

The results of the standard biochemical characterization of the selected bacterial isolate are presented in Table 1.

The selected bacterium was identified as *Pseudomonas aeruginosa* (AMS1a) through 16S ribosomal RNA gene

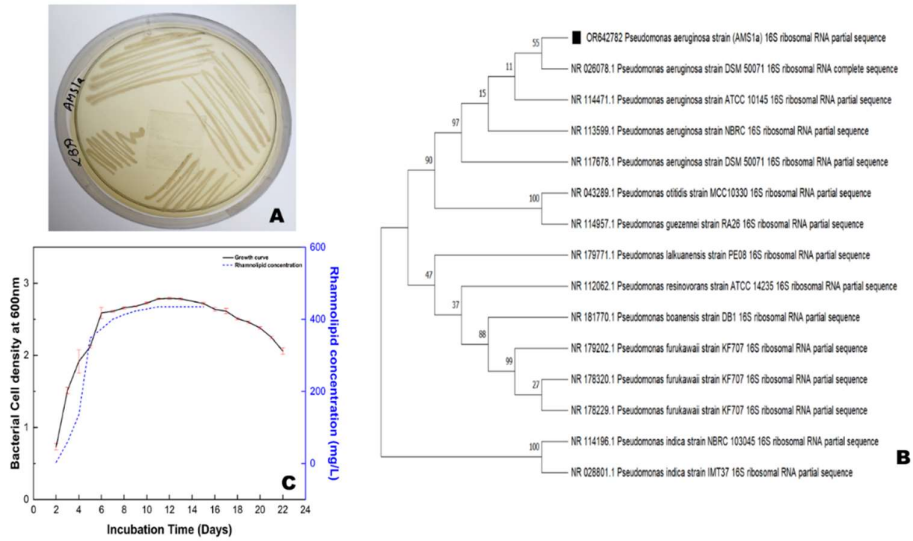

Fig. 3: (A) Colony morphology of pure culture on nutrient agar plate, (B) Phylogenetic tree of the selected bacterial isolate illustrating evolutionary relationships with closely related species, and (C) Growth and BS production curve of the selected bacterial isolate on MSM with n-hexadecane. Results are presented as the mean ± S.D. of three individual experiments.

sequencing, and the obtained sequence was submitted to NCBI with the accession number OR642782. In the phylogenetic tree analysis, the topology with the highest log-likelihood value was chosen. Fourteen nucleotide sequences were identified in this study (Fig. 3B).

Growth Kinetics and BS Production

The growth curve of the bacterial strain *P. aeruginosa* (AMS1a) was determined under constant temperature (30°C) and agitation (140 rpm) for 22 days. Fig. 3C shows the typical growth curve of the selected bacterial strain. It is evident from the growth curve that the bacteria were in the lag phase until the 3rd day and went into the exponential phase from the 4th day, indicating a rapid increase in bacterial population, which was continuous up to the 11th day. The time duration between the 2nd-3rd, 4th-8th and 9th-12th days represents the early, mid and late stationary phases, respectively. The stationary phase was sustained until the 15th day. Following the 15th day of incubation, the density of bacterial cells in the culture declined at a steady rate until the 22nd day, indicating the death phase.

It was found that the RL production increased with the increase in bacterial growth in the culture medium and accelerated in the exponential phase. Fig. 3C shows the pattern of BS production by the selected bacterial strain over 15 days. The RL concentration remained steady during the stationary phase. The final yield of RL reached a concentration of approximately 434.7 µg mL^{-1} after two weeks of incubation.

Isolation of BS

The CFCS containing the BS was acidified to pH 2 for approximately 24 h at 4°C, which caused the BS in the supernatant to protonate, resulting in the precipitation of the BS in the solution. The precipitated solution was then extracted using ethyl acetate as the solvent to separate the BS from the aqueous phase. The same is shown in Fig. 4A, 4B and 4C.

Purification of Partially Purified BS

TLC was used to determine the purity of the partially purified BS using a chloroform-methanol-water (65:35:2, v/v/v) solvent system. The separated compounds were observed under UV light (Fig. 4i). Two major spots and one minor spot were developed after exposure to iodine fumes (Fig. 4ii) and spraying with acidified anthrone reagent (Fig. 4iii) separately. The spots appeared at R_f values of 0.3, 0.5, and 0.7, as shown in Fig. 4iii.

The neutral lipids present in the partially purified BS were eluted from the column using chloroform. A solvent system comprising chloroform: methanol in a ratio of 50:3 (v/v) was used to separate the first fraction of the BS with a R_f value of 0.7. This was followed by a mobile solvent system comprising chloroform: methanol in a ratio of 50:5 (v/v) to separate the BS fraction with an R_f value of 0.5. Finally, a mobile system consisting of a 1:1 v/v ratio of chloroform: methanol was used to separate the final BS fraction, with an R_f value of 0.3. All the fractions of BS were confirmed by TLC before the introduction of the subsequent

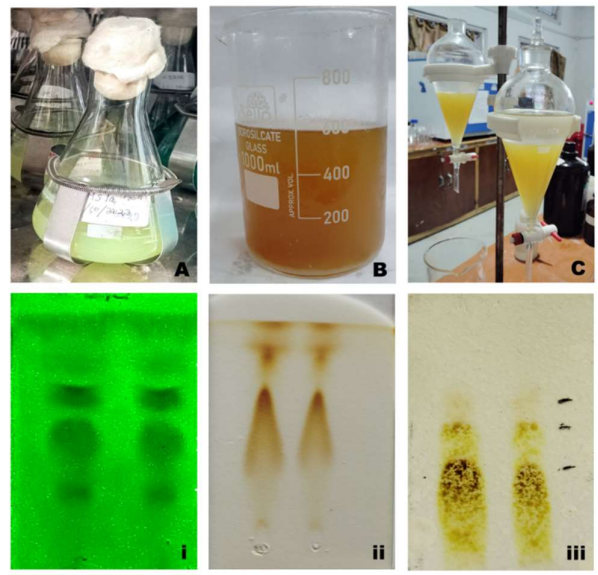

Fig. 4: (A) Matured MSM culture supplemented with n-hexadecane, (B) Acid ppt of crude BS from CFCS, (C) Solvent extraction of BS. TLC chromatogram of partially purified BSs after exposure to (i) UV lamp, (ii) iodine fumes, and (iii) anthrone reagent.

mobile system. The solvent system present in the separated BS fractions was evaporated and recovered using a rotary evaporator.

Physical Characterization of Isolated BS

Determination of CMC: The CMC of the BS produced by *P. aeruginosa* isolated from oil-contaminated sites of Mokokchung town, Nagaland, was found to be 195.6 mg L^{-1}, corresponding to a reduction in the ST of distilled water from ~72 mNm^{-1} to 34.4 mNm^{-1}. Fig. 5A shows the CMC of the isolated BS.

Emulsification activity: The emulsification indices of the tested BS against six different hydrophobic substrates were determined. The BS formed a stable emulsion with all the hydrophobic substrates used in the experiment, as evident from the height of the emulsion, as shown in Fig. 5B.

Chemical Characterization of Isolated BS

FTIR

The functional groups of the BS were further examined using FTIR spectroscopy. The results are presented in Fig. 6.

The presence of an aliphatic long fatty acid chain was indicated by the presence of distinctive peaks at 878, 1402, 2856, and 2931 cm^{-1}. The distinctive groups found in the BSs were the significant functional groups, namely the carbonyl group (C–O) (1260 cm^{-1}), alkene (C = C) (1644 cm^{-1}), and OH bond (3410 cm^{-1}). The vibration at 1124 cm^{-1} was observed to correlate with C–O stretching, which predicted the existence of the sugar moiety, while the presence of a more significant band at 1735 cm^{-1} implied the presence of a carboxyl group, which showed the linking group between the sugar and fatty acid. The FTIR results indicated that the isolated BS may belong to the glycolipid family.

Nuclear Magnetic Resonance

As shown in Fig. 7A and 7B, the presence of a chemical shift value in ^{1}H NMR at 1.24 ppm indicates the methyl group ($-CH_3$) corresponding to the sugar moiety.

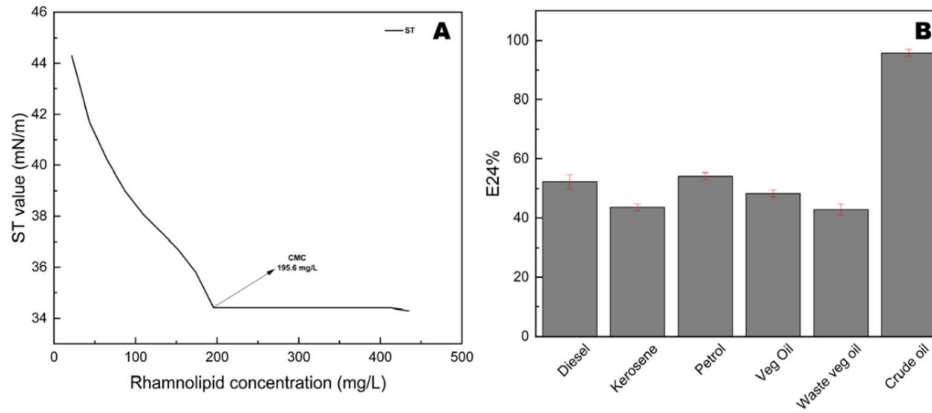

Fig. 5: (A) Determination of the CMC of the isolated BS and (B) emulsification activity (E_{24}%) of the BS toward selected hydrophobic substrates. Results represent mean ± S.D. of three individual experiments.

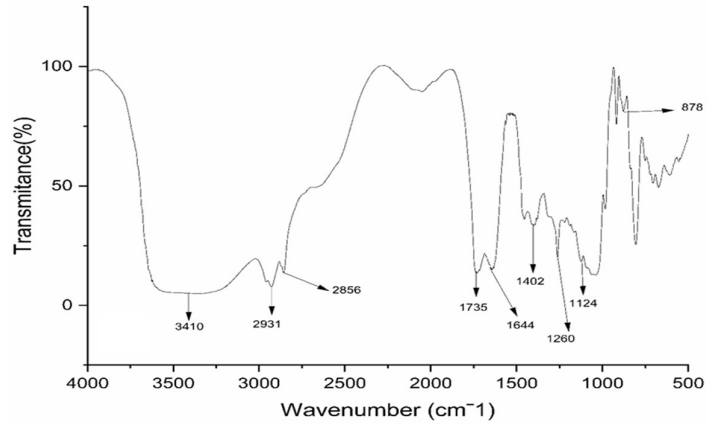

Fig. 6: FTIR spectrum of the isolated BS.

The peak at 0.86 ppm corresponds to the (–CH_3) group of the long-chain aliphatic group. The presence of the sugar moiety was confirmed by the presence of peaks at 4.93 (-1'-H), 3.63 (-5'-H), and 4.14-4.29 ppm (-2,' 3,' 4'-H). The sharp peaks at 1.24 ppm correspond to the long-chain hydrocarbon of the lipid group. Other characteristic peaks were also observed for -COO-CH- (5.81 ppm), -O-CH- (4.30 ppm), and -CH_2-COO- (2.32, 2.8 ppm), which confirms the presence of mono-RL with two long-chain fatty acids. Similarly, characteristic peaks at 0.82 (-CH_3), 1.24-1.21 ppm {-$(CH_2)_n$ and -CH_3 (ring)}, 5.34 (-COO-CH-), 4.16 ppm (-O-CH-), 2.35, 2.47 ppm (-CH_2-COO-) confirm the structure of di-RL.

In Fig. 7C and 8D, 170.85 and 171.61 ppm show the ester group (C=O) of mono and di-RL, respectively. The

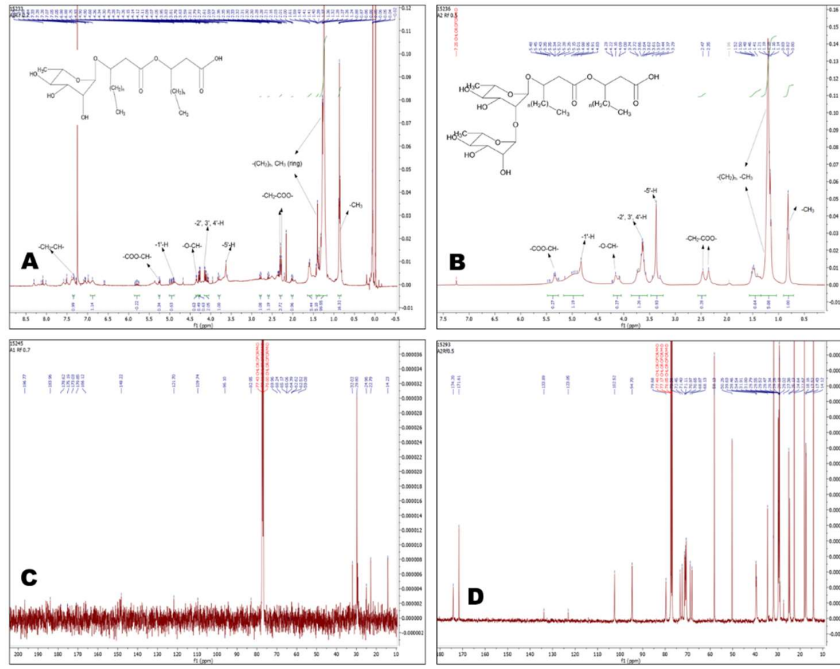

Fig. 7: (A and B) ^{1}H spectra of isolated mono-RL and di-RL, and (C and D) ^{13}C NMR spectra of isolated mono-RL and di-RL.

Table 2: The different RL congeners present in the isolated BS and their peak assignments.

Sl. No.	RT	m.z^{-1} $[M-H]^-$	Rhamnolipid/ HAA	Characteristic fragments								
				Rha-Rha-FA1	Rha-Rha-FA2	Rha-FA1	Rha-FA2	FA1-FA2	FA2-FA1	FA1	FA2	Others
1	22.06	621.37	Rha-Rha-C_8-C_{10}, Rha-Rha-C_{10}-C_8	-	479.27	-	-	-		141.1	169.13	204.9, 205.08, 247.1
2	23.05	649.4	Rha-Rha-C_{10}-C_{10}	479.27	-	-	-	--		169.2	-	163.07, 205.08, 247.10
3	23.5	675.423	Rha-Rha-C_{10}-$C_{12:1}$	479.27	-	-	-	-		169.2	195.1	247.1, 205
4	24.24	531.379	Rha-C_{10}-C_{12}	-	-	333.21		-		169.13	197.17	163.07, 205.08
5	24.5	703.45	Rha-Rha-C_{10}-$C_{14:1}$	479.27	-	333.21	-	-		169.2	197.17	163.07, 204.98, 248.97
6	24.9	705.4701	Rha-Rha-C_{10}-C_{14}, Rha-Rha-C_{14}-C_{10}	479.27	535.33	-	-	-		169.2	225.20	205.08, 163.07, 247.10
7	24.9	705.4701	Rha-Rha-C_{12}-C_{12}	507.3058	-	-	-	-		197.17	-	205.08, 163.07, 247.10

Note: RT - Retention time, Rha - Mono-RL, Rha-Rha - Di-RL, FA1-Fatty acid short chain, FA2-Fatty acid long chain.

presence of a carboxylic group was confirmed by the peaks at 173 ppm for mono-RL and 174.20 ppm for Di-RL. Peaks at 62.52-82.95 ppm and 68.13-73.36 ppm correspond to the rhamnose rings in both mono and di-rhamnolipid, respectively.

Liquid Chromatography and Mass Spectroscopy

LC-MS/MS was performed using the purified BS. The total ion chromatogram (TIC) obtained from LC-MS showed seven dominant peaks with retention times ranging from 22.059 to 24.907 min (Table 2).

These peaks detected in the TIC corresponded to a mixture of mono- and di-RLs. The peak detected at m/z 531 corresponded to Rha-C_{10}-C_{12}, while the rest of the signals were found to be di-RL, showing the m/z [M-H]- at 621.387 for Rha-Rha-C_8-C_{10}/ Rha-Rha-C_{10}-C_8, Rha-Rha-C_{10}-$C_{12:1}$ (m/z 675.423), Rha-Rha-C_{10}-C_{10} (m/z 649.4), Rha-Rha-C_{10}-$C_{14:1}$ (m/z 703.45), Rha-Rha-C_{10}-C_{14}/Rha-Rha-C_{14}-C_{10} (m/z 705.4701), and Rha-Rha-C_{12}-C_{12} (m/z 705.4701). The different assignments for the pseudo-molecular ion peaks and other characteristic peaks shown by the cleavage of the rhamnose moieties are presented in Table 2. In the case of

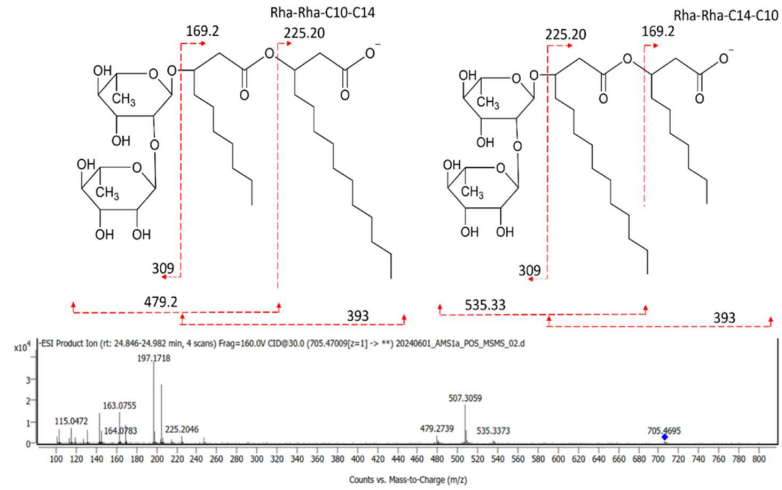

Fig. 8: ESI product ion of abundantly present Rha-Rha-C_{10}-C_{14} and Rha-Rha-C_{14}-C_{10} molecules.

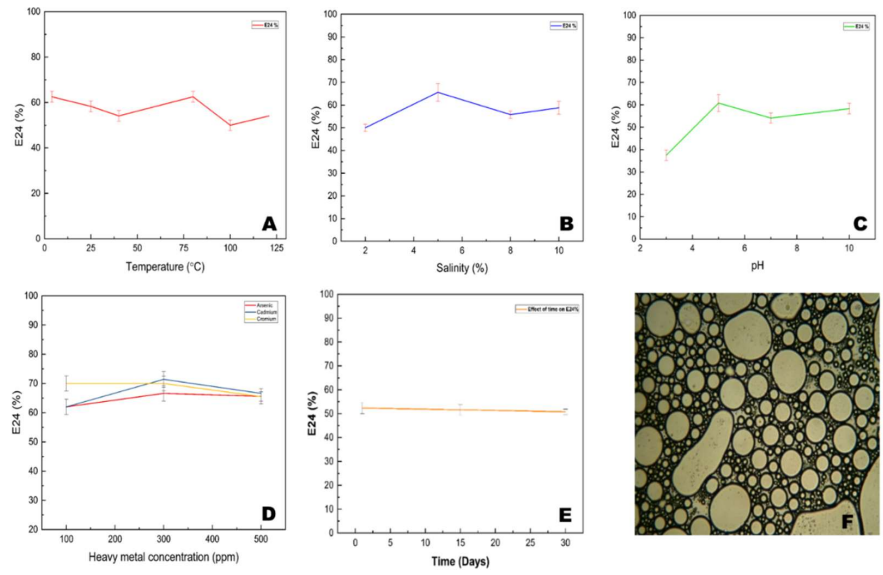

Fig. 9: Effect of varying (A) temperature (4-121ºC), (B) salinity (2-10%), (C) pH (3-10), (D) heavy metals (As, Cd, and Cr) at varying concentration (100-500 ppm), (E) time period (0-30 days) on the emulsifying activity of isolated BS, and (F) 10X magnification view of 30th day stable emulsion. Results represent mean ± S.D. of three individual experiments.

di-RLs such as Rha-Rha-C_{10}-C_{14}/Rha-Rha-C_{14}-C_{10} with m/z 705.4694, the fragmentation of the two fatty acid chains led to two peaks at m/z 225 and m/z 169.2. Another cleavage at the ether linkage of rhamnose and fatty acid chains gives two characteristic peaks at m/z 309 for the di-rhamnose moiety and m/z 393 for the two fatty acid chains, as shown in Fig. 8.

Stability Studies

Effect of environmental variables on E_{24}%: The BS exhibited and retained a stable E_{24}% within 54.1% to 62.5 at temperatures ranging from 4ºC to 121ºC, demonstrating the absence of any significant effect of temperature on the emulsification activity, and the same is presented in Fig. 9A.

At varying salinities (2%-10%), the BS was able to maintain a stable E_{24}% of 50% to 65.6%, highlighting the stable nature of the BS and the same is presented Fig. 9B. The BS was able to maintain a stable E_{24}% of 54% to 60% at the pH range of 5 to 10 and the same is presented in Fig. 9C. However, at an acidic pH of 3, the emulsification activity decreased to 37.5%. The BS showed a stable emulsification activity with an E_{24}% value of 62% to 71.4 with all the three types of heavy metal used (As, Cd, and Cr) at varying concentration (100-500 ppm), as shown in Fig. 9D. Over 30 days, no significant change in emulsification activity was observed (Fig. 9E). The BS maintained an average emulsification index value of approximately 51.6% ± 0.07 for 30 days of incubation. A compound microscopic image of the stable emulsion at 10 X magnification is presented in Fig. 9F.

Effect of environmental variables on surface activity: The BS was able to maintain a consistent reduction of ST of distilled water at an average of approximately 35 mNm^{-1} ± 0.05 at a temperature range of 25ºC to 121ºC. However, a slight increase in the ST value was observed, 36.1 ± 0.15 mNm^{-1} at 4°C, as shown in Fig. 10A.

As shown in Fig. 10B, the BS was able to maintain the reduction of ST value within the value of 34.2 ± 0.15 to 35.6 mNm^{-1} ± 0.05 at the salinity range of 2% to 10%. A small but steady reduction in ST values was observed with an increase in salinity. At pH values ranging from 3 to 10, the ST of the BS increased marginally and consistently with increasing pH. The maximum reduction in ST of 31.5 mNm^{-1} ± 0.1 was observed at pH 3, which steadily increased to 36.6 mNm^{-1} ± 0.05 at pH 10, as shown in Fig. 10C. The BS maintained a stable reduction in ST within the range of 34.4 mNm^{-1} ± 0.05 to 35.8 mNm^{-1} ± 0.11 with all the three types of heavy metals used (As, Cd, and Cr) at varying concentration (100-500 ppm), as shown in Fig. 10D.

Bioremediation Application

Soil washing experiment: In the crude oil washing experiment, BS performed much more consistently and efficiently after 24 h of washing (Fig. 11A). The average removal rates of the natural surfactants at CMC, below CMC,

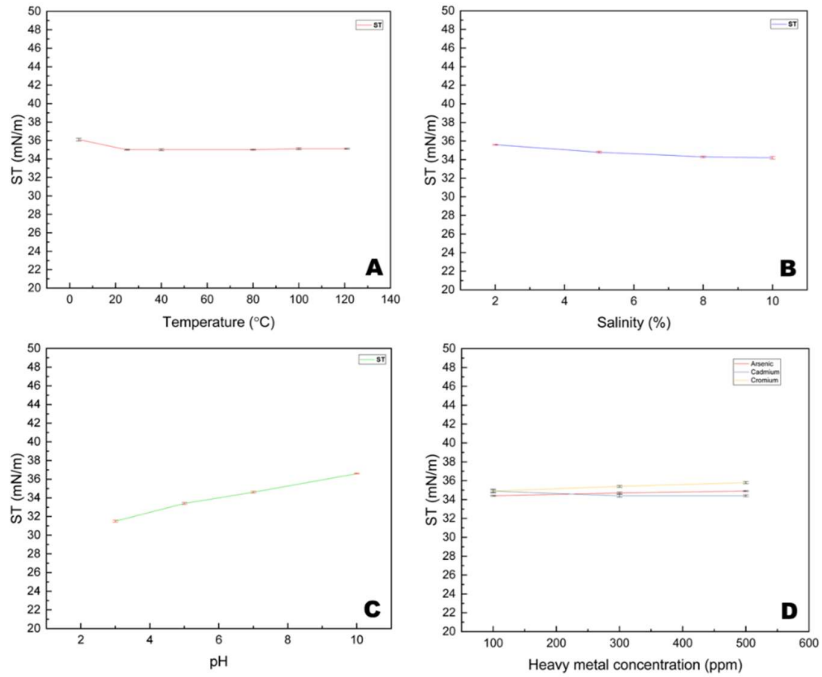

Fig. 10: Effect of varying (A) temperature (4-121ºC), (B) salinity (2-10%), (C) pH (3-10), and (D) heavy metals (As, Cd, and Cr) at varying concentration (100-500 ppm) on the surface activity of the isolated BS. Results represent mean ± S.D. of three individual experiments.

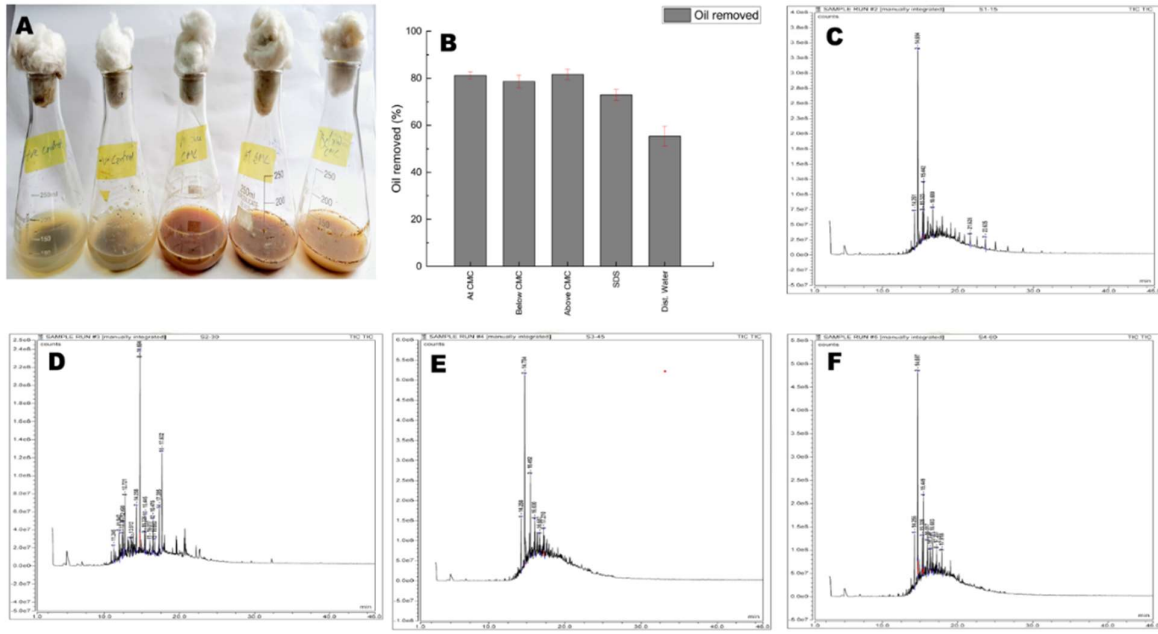

Fig. 11: (A) Soil washing experiment, (B) Effect of BS concentration, SDS, and water on soil washing. Results represent mean ± S.D. of three individual experiments and degradation pattern of crude oil by the selected bacterial strain after (C) 15 days, (D) 30 days, (E) 45 days, (F) 60 days.

and above CMC were 81.2% ± 1.5, 78.6% ± 2.7, and 81.6% ± 2.2, respectively. This is illustrated in Fig. 11B. The synthetic surfactant, SDS (0.1% w/v), was able to remove an average of 72.9% ± 2.3 of the crude oil, and the control, distilled water, was able to remove approximately 55.4% ± 4.2 of the oil.

Crude oil degradation: The ability of the selected bacterial strain to degrade crude oil was validated using GC-MS. Fig. 11C, D, E, and F display the chromatograms for 15, 30, 45, and 60 days, respectively. The results demonstrated that only specific medium to long-chain hydrocarbons were degraded into simpler and shorter molecules throughout the degradation process. The use of a complex hydrocarbon mixture as the exclusive carbon source resulted in the formation and accumulation of by-products during the 60-day degradation process, leading to the emergence of multiple additional compounds.

DISCUSSION

Isolation of Oleophilic Bacteria from Environmental Samples

In the present study, 30 different hydrocarbon-contaminated soil samples were collected from ten different automobile servicing stations within Mokokchung town of Nagaland, India. A total of sixty hydrocarbonoclastic bacterial isolates were obtained from the oil-contaminated soil samples at the end of the enrichment culture method. In accordance with the present investigation, Bekele et al. (2022) isolated two potent diesel-degrading strains *of P. aeruginosa* (AAUW23 and AAUG11) and one strain of *B. subtilis* (AAUG36) using the enrichment culture technique. Using an enrichment culture technique and burnt motor oil and diesel as the only sources of carbon, Hossain et al. (2022) used an oil sample collected from a petrol pump disposal site. They identified three distinct bacterial isolates closely linked to *Enterobacter* sp., *Pseudomonas* sp., and *Acinetobacter* sp. During isolation and screening, the enrichment culture approach promotes the proliferation of specific microorganisms with the characteristics of interest and increases the number of such target species. The selection of species unique to the metabolism of specific compounds using a certain carbon and/or energy source is one of the simplest methods for isolating novel species (Bhatt et al. 2023). Rahman et al. (2002a) documented a crude oil-metabolizing *Pseudomonas* sp. DS10-129 was isolated from soil samples obtained from gasoline and diesel stations. Rahman et al. (2002b) successfully obtained 130 bacterial isolates capable of degrading oil using the enrichment culture approach, out of which two *Pseudomonas* sp. were identified as proficient in using crude oil and producing BS. Fleck et al. (2000) and Bharathi & Vasudevan (2001) used the enrichment culture approach to isolate several bacterial strains that have the ability to produce BSs and metabolize crude oil.

Culturing and Screening of BS-Producing Oleophilic Bacteria

The OB obtained from the enrichment culture approach was

further inoculated into MSM supplemented with n-hexadecane as the sole carbon source. This approach confirmed the hydrocarbonoclastic nature of the OB isolates. The same strategy was previously used by Bordoloi & Konwar (2008) and Bharali et al. (2022) to isolate potential hydrocarbon-utilizing bacterial strains. The selected hydrocarbonoclastic OB were screened for their BS production capacity using the *du Noüy* ring method, crude oil displacement test, drop collapse test, penetration assay, and CTAB agar assay. The du-Noüy-Ring technique is widely employed for screening BS-producing bacteria because of its simplicity and reliability. It measures the force required to detach a platinum ring from the interface or surface of a liquid (Walter et al. 2010). Cooper (1986) stated that a culture is considered potential if it can decrease the ST of a liquid medium to 40 mNm^{-1} or below. The extent of the clear zone due to oil displacement upon the addition of CFCS was directly related to the activity of the BS. Morikawa et al. (2000), Cheng et al. (2017), and El-Housseiny et al. (2020) have established that the oil spreading approach is a dependable method for quantifying the surface activity of BSs. Furthermore, they demonstrated that this methodology is highly sensitive and capable of detecting even small quantities of BSs.

When a surfactant is present, the liquid drop expands or even collapses owing to the reduction in the force or interfacial tension between the liquid drop and the hydrophobic surface (Jain et al. 1991). The integrity of the drops is contingent upon the quantity of surfactants and is correlated with the degree of tension on the surface and interface (Walter et al. 2010). The penetration assay is appropriate for high-throughput screening and is based on the interaction between two insoluble phases, leading to a color change (Maczek et al. 2007, Walter et al. 2010). BSs can induce rupture of red blood cells (Walter et al. 2010). The selected bacterial isolate exhibited β-hemolysis and was confirmed to be positive for BS production. Mulligan and Cooper (1984) suggested using the blood agar assay as an initial assessment technique, which should be supplemented by additional techniques that rely on the measurement of surface activity. The CTAB agar plate method is a convenient screening technique used to identify the presence of exogenous glycolipids and other anionic surfactants (Siegmund & Wagner 1991). When bacteria in the culture medium produce anionic surfactants, they react with the cationic surfactant CTAB and the methylene blue dye to produce a dark blue, insoluble ion pair. BS-producing bacterial colonies were encircled by dark blue halos (Mulligan et al. 1984, Tuleva et al. 2002, Walter et al. 2010).

Characterization of a Selected Bacterial Isolate

Clustal Omega alignment tests showed that the isolate AMS1a sequence was 100% similar to various 16S rRNA sequences across *Pseudomonas* spp., particularly *P. aeruginosa*. The results of this analysis indicated that all 16S rRNA sequences obtained from NCBI were closely related. Specifically, the AMS1a sequence exhibited considerable homology (> 97%) compared to other Pseudomonas sequences. Phylogenetic analysis revealed that the AMS1a sequence was more closely related to the *P. aeruginosa* DSM 50071 and ATCC 10145 sequences. *P aeruginosa* ATCC 10145 was selected as the root of the phylogenetic tree because it was found to be closely related to AMS1a in the alignment test conducted using Clustal Omega. *P. aeruginosa* is a prevalent Gram-negative bacterial species among hydrocarbonoclastic oleophilic prokaryotes. It is recognized for its versatile metabolism and capacity to inhabit numerous environments (Mielko et al. 2019). Various species of Pseudomonas, capable of generating BSs, have been reported from diverse polluted environments such as petroleum-hydrocarbon contaminated fields (Barathi & Vasudevan 2001, Bordoloi & Konwar 2008, Saravanan & Vijayakumar 2012), metal-contaminated sites (Santos et al. 2024), pesticide-affected regions (Wang et al. 2022), and industrial effluent-laden habitats (Al-Ansari et al. 2021, Wang et al. 2023).

Growth Kinetics and BS Production

The current study showed a roughly sigmoidal growth curve over a 22-day incubation period, which closely aligns with the previously documented growth curves of other *Pseudomonas* species (Janek et al. 2013, Goswami et al. 2015, Ramírez et al. 2015). In comparison to earlier published studies, the selected bacterial strain exhibited optimal growth at 30˚C. This may be attributable to the abiotic factors at the contaminated sampling site from which the bacterial strain was isolated. According to records, the temperature in the Mokokchung township region does not exceed 32ºC during summer, with an average summer temperature of 27ºC (Central Ground Water Board 2013). Santos et al. (2024) isolated a strain of *P. aeruginosa* BM02 from acidic soil in a Brazilian municipality with temperatures ranging from 28 ± 6ºC, which is very near the ideal temperature for BS synthesis. Wei et al. (2005) and Chen et al. (2007) reported optimum RL-production by *P. aeruginosa* J4 at 30-37ºC, which decreased with further increase in temperature. Temperatures between 28-40ºC have been reported for the production of RLs by various strains of *P. aeruginosa* (Henkel et al. 2012, Müller et al. 2012). Such discrepancies in the reported optimum temperature for *P. aeruginosa* strains indicate their apparent physiological variations.

The bacterial strain exhibited progressive development from 1st to 14th day, persisted throughout the fermentation process, and entered the death phase after 14th day. It has

been proposed that cells enter the death phase due to the consumption of available nutrients in the culture medium, resulting in the accumulation of toxic substances and a restricted supply of dissolved oxygen, which may impede development (Lan et al. 2015). A spike in BS biosynthesis was observed even after the bacterial population entered the stationary phase. This could be attributed to the biosynthesis of BSs as secondary metabolites (Rahman et al. 2002a). The growth of hydrocarbonoclastic oleophilic can be accompanied by the production of BSs, which can aid in the attachment of cells to hydrophobic substrate molecules and facilitate their metabolism (Barathi et al. 2001). ST measurements in our experiments showed a decrease, indicating the presence of surface-active compounds such as RLs. Multiple studies have demonstrated that some bacteria can decrease the ST of their culture media by producing BSs during the shift from the exponential to the stationary growth phase (Yin et al. 2009, Safari et al. 2023).

Purification of Partially Purified BS

Previous studies have reported that different strains produce RLs with different compositions (Abdel-Mawgoud et al. 2010). The spot with a higher R_f value contains the mono-RLs, whereas the lower R_f value contains the di-RLs (Lotfabad et al. 2010). Moreover, in this study, a minor lower spot with an R_f value of 0.3 was also observed. A similar observation was reported by Lotfabad et al. (2010), where a lower R_f value of 0.31 referred to the di-RL structure, while the higher spot for mono-RLs had an R_f value of 0.76. Cheng et al. (2017) characterized the BS produced by *P. aeruginosa* ZS1 isolated from petroleum-sludge in Zhoushan islands, China, through the TLC technique. They were able to separate mono-RL, Rhamnose-C_{10}-C_{10}, di-RL, and Rhamnose-Rhamnose-C_{10}-C_{10} homologues from an RL mixture. Present findings are consistent with those of other studies by Abdel-Mawgoud et al. (2008), George and Jayachandran (2009), and Lotfabad et al. (2009). Abdel-Mawgood et al. (2008) obtained a lower spot (di-RL) and a higher spot (mono-RL) with respective R_f values of 0.4 and 0.68. El Housseiny et al. (2020) characterized the BS from *P. aeruginosa* P6 using TLC analysis and showed two spots, one major spot with a R_f value of 0.56 and another minor spot with a R_f value of 0.71, which corresponded to mono-RLs and di-RLs, respectively. Both mono-RLs and di-RLs are the two main types of RLs produced by most *P. aeruginosa* species (Lang et al. 1999, Maier & Soberon-Chavez 2000). Column chromatography was performed to separate the individual RL homologs using a chloroform-methanol solvent system at different ratios. Many compounds can be efficiently purified, and their structural and functional analyses can be performed using this chromatography technology. Perfect outcomes are obtained in the identification and isolation of RL congers using this approach (Sim et al. 1997). Column chromatography uses a cheap, disposable stationary phase that can be disposed of after use to avoid deterioration and cross-contamination. Additionally, recovering the mobile phase for later use is much simpler.

Physical Characterization of Isolated BS

Determination of CMC: According to the findings, the CMC of the extracted BS was measured to be 195.6 mgL^{-1}, which is equivalent to a ST of 34.4 mNm^{-1}. The CMC of RLs is significantly lower than that of synthetic surfactants, such as SDS, which has a CMC of 2200 mgL^{-1} (Khademolhosseini et al. 2019). The CMC values of mono- and di-RLs, as well as their combinations, produced by different techniques, have been reported to vary between 1 and 400 mgL^{-1} (Kopalle et al. 2022, Arkhipov et al. 2023). Higher CMC values indicate that a greater quantity of surfactant is required to reduce the ST. Therefore, a smaller quantity of BSs is required to achieve maximal ST reduction. This is an important aspect that contributes to the greater utility of BSs compared to chemical surfactants. According to Manivasgan et al. (2014), a fine-quality BS can decrease the ST of water from 72.75 mNm^{-1} to 35 mNm^{-1}. According to Zhang and Miller (1992), effective BSs can reduce the ST of water to less than 40 mNm^{-1}.

Moreover, variations in the purity and content of the BSs may have contributed to the disparate CMC values. For RLs derived from different microbial sources, CMC values ranging from 10 to 230 mgL^{-1} have been reported (Zhang & Miller 1992, Nitschke et al. 2005, Abdel-Mawgoud et al. 2010). It is well known that the distribution of homologs affects the characteristics of RLs. In general, the CMC tends to decrease as the length of the surfactant chain increases and the di-RL content increases (Perfumo et al. 2006). However, this trend was not observed in the current study. A possible explanation for this phenomenon is that the BSs examined in this study are not composed of one primary congener but rather contain an assortment of congeners with varying chain lengths in addition to the predicted congeners with a certain chain length. Additionally, the LC-MS results for the extracted BS indicated the presence of a combination of di-RL and mono-RL congeners, which may potentially impact its CMC values.

Emulsification activity: Emulsification activity is regarded as a critical parameter among the physicochemical qualities used to assess the commercial usability of BSs (Mendes et al. 2015). This is regarded as an indirect approach to evaluate BS production. The findings demonstrated a varied emulsifying capacity against a wide range of hydrocarbon

oils, reflecting the capability of the selected bacterial strain to produce BSs that improve the interaction of hydrophobic substances with water. According to Patel & Desai (1997) and Lovaglio et al. (2011), RLs have a great ability to emulsify a wide range of n-alkanes, aromatic hydrocarbons, petroleum-derived compounds, crude oil, and vegetable oils. According to an investigation conducted by Sun et al. (2018), *Pseudomonas* sp. CQ2 from the Changqing oil field, China, produces a BS with an $E_{24\%}$ of up to 61.5%. As reported by Kumari et al. (2012), the BS generated by *Pseudomonas* sp. BP10 exhibited a high emulsification activity of up to 75%. Hydrocarbons are pseudo-solubilized or emulsified at varying rates by the majority of microbial surfactants, which are substrate-specific (Etoumi et al. 2008). Several studies have found emulsifying indices ranging from 50% to 75% for various RL mixtures (Wei et al. 2005, Abdel-Mawgoud et al. 2008, Benincasa & Accorsini 2008, George et al. 2009, Lotfabad et al. 2009, Bharali et al. 2011, Abbasi et al. 2012, Aparna et al. 2012). The resulting water-oil (WO) emulsions were dense and persisted for over four weeks at room temperature, indicating the potential use of BS in bioremediation to improve the bioavailability of intractable hydrocarbons. In addition, as noted by Maier & Soberon-Chavez (2000) and Abdel-Mawgoud et al. (2008), the ability of BS to emulsify fossil fuel products, particularly kerosene, n-hexadecane, octadecane, diesel, and lubricating oil, may facilitate their microbial assimilation.

Chemical Characterization of Isolated BS

FTIR

The recovered BS was analyzed using FTIR to determine its functional groups. The FTIR investigation identified significant absorption bands in the tested BS at certain wavenumbers *i.e*. 3410, 2931, 2856, 1735, 1644, 1402, and 1206 cm^{-1}. The results provide compelling evidence that the detected wavenumbers align with the various functional categories of RL, as reported in the literature (Cortés-Sánchez et al. 2013, Araujo et al. 2020). Lan et al. (2015) and Ibrahim (2018) reported that the aforementioned primary chemical structure groups align with the structural features of RL, as determined by Fourier transform infrared (FTIR) analysis. Wavenumbers below 1200 cm^{-1} encompass many types of C–H, C–O, and $–CH_3$ vibrations that cannot be further classified (Zhao et al. 2013). It is also crucial to note that slight differences were observed in the infrared spectra compared to the previously published spectra. This variation may be ascribed to the specific constitution of RL mixtures obtained from various strains of *Pseudomonas* sp. and differences in the culture and purification conditions employed.

NMR

The signals identified in the ^{1}H and ^{13}C NMR spectra corresponded to the functional groups of RL, as described in the literature (Cortés-Sánchez et al. 2013, Araujo et al. 2020). Therefore, according to the explanations obtained through NMR studies, it can be concluded that BS is composed of RL. The inadequate clarity of the NMR spectra suggests that the substance being studied may include a combination of several RL congeners. The current results are consistent with those of Monteiro et al. (2007) and Wei et al. (2005), who used ^{1}H and ^{13}C NMR analyses to determine the chemical composition of a BS.

LC-MS/MS

The mass spectrometry data indicated the presence of both mono- and di-rhamnose groups, as well as mono- and di-lipid groups. There was a significant variation in the length and composition (saturated or unsaturated) of the lipid chains, with a particular focus on the presence of saturated C_{10} and C_{12} fatty acids in mono-RL and saturated C_8, C_{10}, C_{12}, and C_{14} fatty acids in di-RL. Previous investigations have reported that the most prevalent *P. aeruginosa* RL comprises fatty acids ranging from C_8 to C_{14}, with C_{10} being the most common. In their study, Christova et al. (2011) identified mono- and di-RL congeners as anions with m/z values of 503 and 649, respectively. These values corresponded to the deprotonated molecules of Rha-C_{10}-C_{10} (mono-RL) and Rha_2-C_{10}-C_{10} (di-RL), respectively. Déziel et al. (1999) and (2000) observed that the ions with the greatest abundance were found at m/z 649.9 and 503.6, respectively. These ions were identified as (Rha-C_{10}-C_{10}) and (Rha-C_{10}-C_{10}) congeners, respectively. It is important to note that there was no estimation of each congener; thus, it is possible that some of them, particularly the less frequent ones, might also be present in minimal proportions. The NMR investigation did not reveal the existence of unsaturated chain congeners, regardless of the weak signals in the 7.0–8.2 ppm region. However, these congeners were identified using mass spectrometry.

Stability Studies

Effect of Environmental Variables on E_{24}%

The efficacy of BS relies on its capacity to maintain stability in diverse environments, accounting for fluctuations in pH, temperature, and salinity (Gudina et al. 2010). The current study assessed the strength of the obtained RL by exposing the CFCS to severe environmental variables such as pH, temperature, salt, and heavy metals. BS demonstrated stability throughout all evaluated variables, with no precipitate formation or significant decline in surface-active behavior. The findings indicated that the BS being tested

exhibited robustness, as it was able to continue operating and retain its active property within a wide temperature range of 4–121˚C. Moreover, there was minimal variation in the $E_{24\%}$ when subjected to varying temperatures, with overall values ranging between 50% and 65.6%. In a similar study, Santos et al. (2024) examined the effects of RL isolated from *P. aeruginosa* BM02 on its ability to endure a wide range of temperatures. The results showed that RL can thrive even at extreme temperatures, ranging from 40 to 120˚C, with minimal variations in its $E_{24\%}$ value, which remained between 65% and 71%. RLs undergo precipitation at low temperatures (4˚C), which adversely affects their surface activity (El-Housseiny et al. 2020). Following autoclaving, there was a minimal reduction in the surface activity. We also examined the impact of elevated temperatures on the emulsifying activity of CFS and found that autoclaving led to a marginal decrease in the emulsification index (El-Housseiny et al. 2020). The proficient temperature stability and strong emulsifying activity of the investigated RLs render them suitable for implementing Microbial Enhanced Oil Recovery (MEOR) and bioremediation in oil-contaminated areas characterized by elevated temperatures (Zhou et al. 2019).

RL in its crude form (CFS) eliminates the need for expensive extraction procedures. Furthermore, employing unrefined RLs is advantageous as it will reduce expenses typically given away to the steps of separation and purification processes (El-Housseiny et al. 2020).

The optimal surface activity was achieved within a pH range of 6–8. However, a substantial decrease in surface activity was observed under extremely acidic (pH 3) or strongly alkaline (pH ≥ 8) conditions. This phenomenon arises due to the precipitation of RLs in highly acidic conditions, resulting in structural distortion and a diminished capacity to decrease ST. Under high acidic settings, the groups with negative charges at the polar ends of RL molecules undergo protonation (El-Housseiny et al. 2020).

The impact of ionic concentration on the surface-active properties was also examined, revealing that the surface activity was markedly diminished at NaCl concentrations exceeding 9% w/v. Reddy et al. (2016) conducted a study to examine the impact of different levels of salinity (2, 6, 10, 15, and 20% w/v) on the stability of RL BSs generated by *P. aeruginosa* DR1, utilizing mango kernel oil. The ST exhibited a progressive increase from 30 to 38 mNm^{-1} when the salt content increased from 6 to 20%. However, salt concentrations of 2-3% render commercial surfactants ineffective (Abdel-Mawgoud et al. 2008). Thus, the obtained RLs have demonstrated their superiority as a preferred option for bioremediation processes in high-salinity areas, such as polluted coastal environments.

Consistent with the present investigation, Santos et al. (2024) found that BS derived from *P. aeruginosa* BM02 remained stable under all tested conditions. The lowest ST value (~27 mNm^{-1}) was observed at pH 3.0, 2% NaCl concentration, and 80˚C. Joshi et al. (2008) found that the stability of the BS was maintained at a temperature of 80˚C, and within pH and NaCl ranges of 6 to 12 and 1 to 7%, respectively. However, the surface activity of BS declined as the NaCl concentration increased to 10 percent and the pH level decreased to the acidic range, resulting in the formation of precipitates. Khademolhosseini et al. (2019) conducted a study on a RL BS produced by *P. aeruginosa* HAK01. They revealed that the BS possessed excellent stability under various conditions, including temperatures ranging from 40 to 121˚C, pH values between 3 and 10, and salt levels of up to 10% (w/v) NaCl. Based on their findings, they proposed that BSs might be utilized in Microbial Enhanced Oil Recovery (MEOR) applications. Multiple researchers have reported similar findings, demonstrating that the activity of RLs remains consistent throughout a broad spectrum of temperature, salinity, and pH levels (Abdel-Mawgoud et al. 2008, El-Housseiny et al. 2020, Zhou et al. 2019, Zhao et al. 2018).

Effect of Environmental Variables on Emulsification Activity

The excellent emulsification activity demonstrated by RL against different hydrocarbon oils credits it as a powerful emulsifying agent that can be potentially used in environmental applications such as MEOR and oil mobilizing agents (El-Housseiny et al. 2020). Furthermore, the bioremediation of petroleum-contaminated settings may benefit from the capacity of BSs to emulsify various hydrocarbon oils, as this may facilitate the assimilation of such oils by BS-producing microorganisms (Maier & Soberon-Chavez 2000, Abdel-Mawgoud et al. 2008). The stability and ability of the BS to retain its properties can be determined from emulsification and surface activity stability tests against varying environmental factors, which underpin their potential bioremediation applications (Chandankere et al. 2014, Abdel-Mawgoud et al. 2008, Datta et al. 2018). It was noteworthy that it performed remarkably well even after autoclaving at 121ºC for 10 min (Abdel-Mawgoud et al. 2008). The CFCS exhibited stable and consistent emulsification activity with diesel oil for 30 days. A stable oil-water emulsion is typically used as a surface activity indicator (Hao et al. 2008). The stability of the emulsification activity of the CFCS containing BSs was also reviewed against varying temperatures, pH, and salinity. The results of the stability tests underscored the ability and efficacy of the BS under study to form stable emulsions with numerous oils over varying temperatures (5-80ºC), pH

(3-10), salinity (2-10%), and time (1-30 days). The stable water-oil emulsions also indicate the potential use of RL in the bioremediation process to improve the accessibility of resistant hydrocarbons.

Bioremediation Application

Soil washing experiment: The percentage of crude oil removed from the sand by the CFCS was relatively higher than that removed by SDS. Similar findings regarding the efficient crude oil removal capacity of RLs produced by *P. aeruginosa* have been reported in a previous study by Aparna et al. (2012). This shows that the BS from *P. aeruginosa* is highly effective for crude oil removal and can be an efficient alternative for the bioremediation of hydrocarbon oil contamination. The removal of oil from soil using BSs can be explained by two proposed mechanisms: mobilization and solubilization. Mobilization occurs when the surfactant concentration is below the CMC. In this phase, the capillary forces, surface and interfacial tension, contact angle, and wettability were reduced. Below the CMC, surfactants lower the surface and interfacial tensions between water, oil, air, and soil systems. When surfactants interact with the oil-soil system, they increase the contact angle and reduce the capillary forces that hold the oil to the soil by decreasing interfacial tension. The mobilization step also depends on the ionic charge of the surfactant, as surfactants are adsorbed onto the soil. This adsorption can lead to a reduction in surfactant concentration, making it less effective or even ineffective in soil treatment. When the concentration exceeds the CMC, the oil solubility increases significantly because of the formation of surfactant micelles. These micelles have hydrophobic ends that cluster inside and hydrophilic ends that face water. This structure provides a suitable environment for hydrophobic organic molecules, a process referred to as solubilization (Urum et al. 2004). Thus, it can be concluded that the main mechanism involved in the removal of crude oil by RL in the current study was greatly attributed to the mobilization phenomenon, as there was no substantial increase in the oil removal capacity at concentrations above the CMC. Gaur et al. (2021) demonstrated the excellent oil recovery capacity of BS produced by *P. aeruginosa* from soil contaminated with engine oil through centrifugation at 6000 rpm for 20 min. Bharali et al. (2022) also conducted a study on the efficiency of BSs produced by different strains of *P. aeruginosa* to recover residual crude oil from petroleum sludge and reported that the BSs were able to recover about 73.5–63.4% of residual oil from the sludge.

Crude oil degradation: The oil degradation potential of the selected bacterium was confirmed by GC-MS analysis. Selected complex and long-chain hydrocarbons are broken down into simpler and shorter compounds during degradation. As a result of the utilization of the complex mixture of hydrocarbons as the sole carbon source, the generation of bacterial by-products was observed during the entire degradation process of 60 days, leading to the formation of new major compounds such as erucic acid, cis-10-Nonadecenoic acid, 5,8,11,14-Eicosatetraynoic acid, methyl ester, 9-Hexadecenoic acid, and eicosyl ester. Additionally, the degradation of large hydrocarbon molecules leads to their breakage and the generation of several smaller molecules, which further serve as precursors for the formation of other complex and stable compounds. It is also evident from the GC-MS data that the bacterium was unable to breakdown the complex and highly branched hydrocarbons such as Pentadecane, 2,6,10-trimethyl, Heptadecane, 2,6,10,14-tetramethyl, Tetradecane, 2,6,10-trimethyl-, Heptadecane, 2,6,10,15-tetramethyl, Dodecane, 2,6,10-trimethyl, Octadecane, 3-ethyl-5-(2-ethylbutyl), Heptadecane, 9-hexyl, Tricyclo[7.4.1.1(2,7)] pentadeca-2,4,6,9,11,13-hexaene-8-ol, 10-Acetoxy-2-hydroxy- 1,2,6a,6b,9,9,12a-heptamethyl-1,3,4,5,6 etc. even after a period of 60 days. Previous studies have established that every bacterial strain has its limitations, and it can degrade only a selected number of hydrocarbons (Rehman et al. 2021). This restriction is the result of several reasons. Primarily, crude oil is composed of a wide variety of hydrocarbons with unique chemical structures and traits, making its composition extremely complicated. Second, nutrient availability and environmental variables can influence the efficacy of bacterial decomposition. For instance, some hydrocarbons may be more susceptible to bacterial enzymes, whereas others may exhibit greater resistance to breakdown owing to their chemical characteristics or spatial configuration within the oil matrix. Moreover, toxic chemicals in crude oil, including heavy metals and aromatic and polyaromatic compounds, can impede bacterial growth and catalytic activity, thereby restricting their capacity to completely break down the oil (Chuah et al. 2023). Hence, for the effective degradation of crude oil components, it is preferred to use a consortium of bacteria with different degradation potentials (Primeia et al. 2020).

Limitations

The constraint arises from the multitude of factors and their interconnectedness that define the production of microbial surfactants. The inability to identify the most economical conditions for producing BS in bioreactor systems for BS commercialization is one of the main limitations of the present study. In addition to the microbial producer, the fermentation and purification processes impact the physicochemical properties of the BS. Comprehending these attributes is necessary for accurately determining

their industrial use. Further industrial applications of this strain, such as the synthesis of enzymes and other bioactive compounds, may be expanded by thorough molecular investigation.

Additionally, this study did not assess the specific uses of the isolated BS other than bioremediation. To determine the precise biodegradation capacity of the chosen strain, a comprehensive molecular examination of its hydrocarbonoclastic characteristics is essential. Additionally, the strain was initially cultured in crude oil to verify its ability to utilize the different components of crude oil as the sole source of carbon and energy. However, studies on its innate ability to use and break down certain components of crude oil, such as aliphatic, aromatic, polyaromatic, and NSO-containing compounds, have yet to be conducted. All of these flaws would be seen as opportunities for further research on the topic in the future.

CONCLUSIONS

The current study revealed the isolation, characterization, and identification of *P. aeruginosa* AMS1a strain, a potential hydrocarbonoclastic, oleophilic, and BS-producing strain from Mokokchung, Nagaland, India. The strain was discovered outside an automobile repair shop in polluted soil and demonstrated a great capacity to degrade crude oil. When the BS production capability was assessed, the strain was found to produce BS that significantly reduced ST and exhibited effective emulsifying properties. Structural characterization using TLC, FTIR, ^{1}H and ^{13}C NMR, and LC-MS/MS verified that the BS generated by the selected strain comprised a combination of mono- and di-RLs. BS demonstrates significant surface-active properties and stability throughout a broad spectrum of temperature, pH, salinity, and heavy metals, rendering them appropriate for many commercial applications, including the pharmaceutical, cosmetics, food, and remediation technology sectors. Furthermore, the strain has the potential to be employed in bioremediation processes, which involve the biodegradation of petroleum-based pollutants and the removal of crude oil from contaminated soils by washing. However, given the costs associated with extraction and purification, the yield is rather low, which essentially prevents its industrial application. Further research and development are required to improve the culture conditions and increase the quality, yield, and efficiency of BS, which will increase the number of potential applications. To increase BS production on a wide scale, cutting-edge extraction techniques that use affordable, renewable carbon feedstocks must be investigated. Through further investigation into the optimization of growth parameters and exploration of novel genetically engineered microbes, researchers can fully realize the potential of BS, which will open up novel applications in disciplines such as biomedicine, environmentally benign remediation technology, organic compound synthesis, and nanotechnology. Additionally, to promote sustainability, various government organizations should implement policies to promote and encourage the use of greener and more environmentally friendly natural products, such as BS, to achieve the Sustainable Development Goals set by the UN.

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