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Research Article

MICROBIAL PRODUCTION OF BIOSURFACTANTS AND THEIR BIOCHEMICAL CHARACTERIZATION

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ABSTRACT

Objective: The present work was undertaken to isolate an efficient biosurfactant-producing bacterium, optimize its production under different environmental and nutritional conditions, and characterize the structural and functional properties of the biosurfactant for prospective industrial use.

Methods: Environmental samples from soil and wastewater were subjected to preliminary screening through hemolytic activity, oil displacement, and emulsification index (E24) assays. The most promising isolate was identified by morphological and biochemical tests, followed by molecular confirmation using 16S rRNA gene sequencing. Production parameters were optimized by varying carbon and nitrogen sources, culture pH, temperature, and incubation period. Crude biosurfactant was recovered through acid precipitation and solvent extraction. Its functional attributes were examined through surface tension reduction, critical micelle concentration (CMC) determination, emulsification potential, and stability across pH, temperature, and salinity gradients. Structural features were analyzed by Fourier-transform infrared spectroscopy (FTIR) and gas chromatography–mass spectrometry.

Results: The identified *Bacillus subtilis* strain effectively reduced surface tension from 72.0 to 28.6 mN/m and exhibited a high emulsification index (75.4±1.2% with kerosene). Optimal production occurred with glucose (2% w/v) and ammonium nitrate (0.5% w/v) at pH 7 and 30°C after 72 h, yielding 4.85±0.15 g/L of crude biosurfactant. The CMC was estimated at 35 mg/L. The compound retained stability between pH 4–10, at temperatures up to 100°C, and in salinity up to 10% NaCl. FTIR and GC–MS confirmed a glycolipid nature, composed mainly of long-chain fatty acids and sugar moieties.

Conclusion: The biosurfactant derived from *B. subtilis* displayed strong surface-active properties, appreciable yield, and robustness under diverse physicochemical conditions. These features underline its promise for applications in petroleum recovery, bioremediation, effluent treatment, and biopharmaceutical formulations, underscoring its potential as a safe and sustainable alternative to synthetic surfactants.

Keywords: Biosurfactant, *Bacillus subtilis*, Emulsification index, Surface tension, Glycolipid, Eco-friendly surfactant.

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INTRODUCTION

Surfactants are amphiphilic compounds containing both hydrophilic and hydrophobic domains, which allow them to localize at phase boundaries and lower the surface and interfacial tension between liquids, solids, and gases. Although synthetic surfactants have long been the mainstay in industrial applications, their non-biodegradable nature, ecological toxicity – particularly to aquatic systems – and reliance on petrochemical feedstocks have raised significant concerns regarding their environmental impact and long-term sustainability. This has led to increasing global interest in biosurfactants – microbially derived surface-active compounds that offer several advantages including biodegradability, low toxicity, functional diversity, and effectiveness under extreme environmental conditions [1,2].

Biosurfactants are synthesized by diverse microorganisms including *Pseudomonas aeruginosa, B. subtilis, Starmerella bombicola*, and *Candida tropicalis*. Based on their structural makeup and microbial source, they are broadly categorized into glycolipids, lipopeptides, phospholipids, fatty acids, and polymeric forms [3,4]. Among the various classes, rhamnolipids, sophorolipids, and surfactin are the most extensively investigated. These molecules exhibit exceptional surface-active properties, frequently reducing the surface tension of water from approximately 72 mN/m to below 30 mN/m, while also showing strong emulsification efficiency [5,6]. These properties enable biosurfactants to function as emulsifiers, foaming agents, wetting agents, and dispersants, with broad industrial relevance.

The application potential of biosurfactants spans multiple sectors. In environmental biotechnology, they are employed in the bioremediation of hydrocarbon-contaminated soils and oil spill management due to their ability to solubilize hydrophobic pollutants and enhance bioavailability for microbial degradation [7,8]. In the petroleum industry, biosurfactants are integral to microbial enhanced oil recovery (MEOR), where they reduce oil-water interfacial tension and improve mobilization of residual oil [9]. The food and cosmetic industries utilize biosurfactants for their emulsifying properties and biocompatibility, while in the biomedical sector, they have demonstrated antimicrobial, anti-biofilm, antioxidant, and even anticancer activities, positioning them as candidates for drug delivery systems and medical coatings [10,11].

Despite their versatility, large-scale commercial adoption of biosurfactants faces challenges. The primary limitation is the relatively high production cost compared to synthetic alternatives, attributed to low yields in unoptimized fermentation processes and expensive

substrates. Strategies to overcome these challenges include optimization of growth media, selection of cost-effective renewable substrates (such as agro-industrial waste), and genetic or metabolic engineering of high-yield microbial strains [12,13]. In addition, physicochemical stability – resistance to degradation under extremes of pH, temperature, and salinity – is a critical determinant of biosurfactant suitability for industrial applications.

Biochemical characterization is essential for understanding biosurfactant functionality. Key parameters include surface tension reduction capability, critical micelle concentration (CMC), emulsification index (E24), and stability under variable environmental conditions. Advanced analytical tools, including Fourier-transform infrared spectroscopy (FTIR) and gas chromatography–mass spectrometry (GC–MS), are widely employed to identify molecular structures and functional groups of biosurfactants. These techniques provide critical information for classification and help in predicting their potential industrial and biomedical applications [14,15].

The present study is designed to address the dual objectives of isolation and optimization of biosurfactant production from potent microbial strains, and comprehensive biochemical characterization of the extracted products. Environmental samples from soil and wastewater were explored as microbial sources, given their diverse and often extremophile populations capable of producing robust biosurfactants. Optimization experiments investigated the effects of carbon and nitrogen sources, pH, temperature, and incubation period on biosurfactant yield. The physicochemical and structural properties were then evaluated to determine industrial feasibility. By integrating production optimization with advanced characterization, this study aims to identify biosurfactants that combine high yield, strong surface activity, and environmental stability – key attributes for large-scale sustainable applications.

METHODS

Sample collection

Environmental samples were obtained from oil-contaminated soils, industrial wastewater outlets, and automobile service stations within the study area. Soil samples (50–100 g) were collected aseptically from a depth of 5–10 cm using sterile spatulas, while wastewater samples (500 mL) were taken in sterile bottles. All collected samples were kept at 4°C during transport and processed within 24 h to ensure retention of microbial viability [14].

Isolation of microorganisms

Microorganisms were isolated from environmental samples using the serial dilution technique. Each sample was serially diluted (10^{-1}–10^{-6}) in sterile physiological saline (0.85% NaCl; HiMedia, Mumbai, India), and 100 µL aliquots from appropriate dilutions were spread onto nutrient agar (NA, HiMedia, India) and mineral salts agar (MSA, HiMedia, India) plates supplemented with 1% (v/v) crude oil (Indian Oil Corporation, India) as the sole carbon source. Plates were incubated at 30±2°C for 48–72 h in a bacteriological incubator (Innova 42, New Brunswick Scientific, USA).

Distinct colonies differing in morphology were carefully selected and subjected to repeat streaking on NA plates until pure cultures were obtained. Pure isolates were preserved for long-term storage in nutrient broth containing 20% (v/v) glycerol and maintained at –20°C in a deep freezer (Thermo Scientific, USA).

The supplementation of crude oil as the exclusive carbon source functioned as an enrichment strategy, ensuring the selective recovery of hydrocarbon-degrading microorganisms. Such organisms are predisposed to biosurfactant production since amphiphilic metabolites enhance the solubilization and assimilation of hydrophobic substrates. This enrichment-based approach is well recognized in microbial screening protocols to increase the likelihood of isolating potent biosurfactant producers from diverse environmental samples [2,16].

Screening for biosurfactant production

Initial screening of the isolates for biosurfactant production was carried out using a combination of qualitative and quantitative assays. For the hemolysis test, isolates were streaked on blood agar plates containing 5% defibrinated sheep blood (HiMedia, Mumbai, India) and incubated at 30±2°C for 48 h in a bacteriological incubator (Innova 42, New Brunswick Scientific, USA). The presence of β-hemolysis surrounding the colonies was interpreted as a positive indication of biosurfactant activity [17].

The oil displacement assay was performed by layering 20 µL of crude oil (Indian Oil Corporation, India) onto the surface of 40 mL of distilled water in a sterile Petri dish, followed by the addition of 10 µL of cell-free culture supernatant prepared by centrifugation at 10,000 × g for 20 min at 4°C in a refrigerated centrifuge (Eppendorf 5810R, Eppendorf AG, Hamburg, Germany). The diameter of the clear zone formed on the oil layer was measured using a digital Vernier caliper (Mitutoyo, Japan) as a quantitative measure of biosurfactant activity [18].

For the drop-collapse assay, 25 µL of culture supernatant was carefully dispensed onto a glass slide pre-coated with a thin film of crude oil. Droplet spreading or collapse was recorded as evidence of surface tension reduction and thus biosurfactant activity [19].

Quantitative evaluation was carried out using the emulsification index (E24). Equal volumes (2 mL each) of cell-free culture supernatant and kerosene were mixed in a borosilicate test tube (Borosil, India) and vortexed for 2 min using a vortex mixer (REMI CM-101, REMI Instruments, India**)**. The tubes were left undisturbed at room temperature for 24 h, after which the emulsification index (E24) was calculated as the percentage of the height of the emulsion layer relative to the total height of the liquid column [20].

Molecular identification of the potent isolate

To establish the taxonomic identity of the most potent biosurfactant-producing microorganism, genomic DNA was isolated from a fresh overnight culture using a silica-membrane based bacterial DNA extraction kit (Qiagen, Germany) following the manufacturer's guidelines. DNA integrity was verified by 1% agarose gel electrophoresis, and concentration was measured spectrophotometrically at 260/280 nm.

The nearly complete 16S ribosomal RNA gene was amplified using the universal bacterial primer pair 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). Polymerase chain reaction (PCR) amplification of the 16S rRNA gene was performed in a thermal cycler (Eppendorf Mastercycler Nexus, Eppendorf AG, Hamburg, Germany). Amplicons were resolved by agarose gel electrophoresis using a gel electrophoresis unit (Bio-Rad Sub-Cell GT, Bio-Rad Laboratories, Hercules, CA, USA) and visualized under UV illumination in a gel documentation system (Bio-Rad Gel Doc XR+ Imaging System, Bio-Rad Laboratories, USA. Amplification was carried out in a thermal cycler under the following conditions: initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 45 s, annealing at 55°C for 45 s, extension at 72°C for 90 s; and a final extension at 72°C for 10 min. Amplicons were examined on a 1.2% agarose gel, yielding a product of approximately 1.5 kb, consistent with the expected 16S rRNA gene size.

The PCR product was purified using a gel extraction kit and sequenced bidirectionally by Sanger dideoxy sequencing. Raw chromatograms were manually inspected to eliminate ambiguous base calls, and a consensus sequence was generated using BioEdit software. The final sequence was compared against the National Center for Biotechnology Information (NCBI) nucleotide database using the BLASTn algorithm [15-21]. Phylogenetic affiliation was determined on the basis of maximum sequence similarity with type strains deposited in GenBank.

The isolate exhibited 99.6% sequence identity with *B. subtilis* strain (Bbv 57), confirming its taxonomic placement within the genus *Bacillus.* The sequence, 1460 bp in length, corresponds to a partial 16S ribosomal RNA gene and has been deposited in the NCBI GenBank repository under the accession number MW282917.1 [15].

To further validate the taxonomic assignment, the sequence was cross-checked using the Ribosomal Database Project Classifier, which confirmed its placement within the family Bacillaceae. Reporting both the GenBank accession number and associated metadata ensures transparency, facilitates reproducibility, and allows future comparative analyses with other *B. subtilis* biosurfactant-producing strains.

Optimization of biosurfactant production

Biosurfactant production was optimized using a one-variable-at-a-time approach [22]. Mineral salts medium (MSM; HiMedia, Mumbai, India) was prepared and supplemented with different carbon sources including glucose, sucrose, glycerol, crude oil (Indian Oil Corporation, India), and molasses (procured locally), each at a final concentration of 2% (w/v). Nitrogen sources tested included ammonium nitrate, sodium nitrate, yeast extract, and peptone (all from HiMedia, India), each at 0.5% (w/v).

The effect of physicochemical parameters was also evaluated by varying the pH (5–9, adjusted using a digital pH meter, Mettler Toledo SevenCompact, Mettler-Toledo International Inc., Columbus, USA), incubation temperature (25–45°C, maintained in a bacteriological incubator shaker, Innova 44, New Brunswick Scientific, USA), and incubation period (24–120 h).

All experiments were conducted in 250 mL Erlenmeyer flasks (Borosil, India) containing 100 mL of medium inoculated with 2% (v/v) of an actively growing overnight culture. Cultures were incubated at 150 rpm under controlled shaking conditions to ensure proper aeration and mixing. At the end of each experimental run, the cultures were harvested, and biosurfactant production was quantified gravimetrically after recovery and extraction as described earlier. Yields were expressed as mean±standard deviation (SD) from triplicate experiments.

Extraction of biosurfactant and recovery

After incubation, cultures were centrifuged at 10,000 × g for 20 min at 4°C using a refrigerated high-speed centrifuge (Eppendorf 5810R, Eppendorf AG, Hamburg, Germany) to obtain cell-free supernatants. The pH of the supernatant was adjusted to 2.0 with 6 N HCl (Merck, Darmstadt, Germany) and stored at 4°C overnight to facilitate biosurfactant precipitation. Precipitates were collected by centrifugation under the same conditions, resuspended in distilled water, and extracted using a chloroform–methanol (2:1, v/v) mixture (Sigma-Aldrich, St. Louis, USA). The organic phase was evaporated under reduced pressure in a rotary evaporator (Buchi Rotavapor R-210, Büchi Labortechnik AG, Flawil, Switzerland) to yield crude biosurfactant [23,24].

Biochemical characterization

Surface tension measurement, CMC, and stability testing

Surface tension of the biosurfactant solutions was measured at room temperature using a Du Noüy ring tensiometer (Krüss K6, Krüss GmbH, Hamburg, Germany), calibrated before each measurement, with distilled water (72.0 mN/m) used as the reference control [11,12]. To determine the CMC, surface tension values were plotted against a series of increasing biosurfactant concentrations (5–100 mg/L). The concentration corresponding to the inflection point of the curve, where further addition of biosurfactant did not result in a significant decrease in surface tension, was taken as the CMC.

The stability of biosurfactant activity was evaluated by exposing aqueous biosurfactant solutions (1 g/L) to varying physicochemical conditions [25]. pH stability was assessed across a range of pH 2–12, adjusted using either 1 N HCl (Merck, Darmstadt, Germany) or 1 N NaOH (Sigma-Aldrich, USA), with pH values confirmed using a digital pH meter (Mettler Toledo SevenCompact, Mettler-Toledo International Inc., Columbus, USA). Thermal stability was examined by incubating samples at 4°C (Refrigerator, Samsung, South Korea), 30–100°C (hot-air oven, Memmert UN55, Memmert GmbH, Germany), and by autoclaving at 121°C for 15 min (Tuttnauer 3870 EL, Tuttnauer Co., Breda, Netherlands). Salinity tolerance was determined by supplementing solutions with NaCl (HiMedia, India) at concentrations ranging from 1–15% (w/v).

After 1 h of treatment under each condition, surface tension of the treated solutions was measured again using the tensiometer to assess residual surface activity, and results were expressed as mean±SD of triplicate experiments.

Structural characterization by FTIR and GC–MS

The structural properties of the purified biosurfactant were investigated using FTIR and GC–MS [1].

For FTIR analysis, dried biosurfactant samples were finely ground with spectroscopic-grade potassium bromide (KBr; Merck, Darmstadt, Germany) and pressed into translucent pellets using a hydraulic press (Specac Atlas 15T, UK). Spectra were recorded in the transmission mode on a Shimadzu IRTracer-100 FTIR spectrophotometer (Shimadzu Corporation, Kyoto, Japan**)** within the range of 4000–400 cm^{-1}, at a spectral resolution of 4 cm^{-1}, and averaged over 32 scans to enhance signal-to-noise ratio. Baseline correction and spectral interpretation were performed using LabSolutions IR software (Shimadzu, Japan). Functional groups were assigned by comparing absorption bands with standard reference data to identify characteristic peaks corresponding to hydroxyl, aliphatic, ester, and glycosidic linkages, thereby elucidating the glycolipid nature of the compound.

For GC–MS analysis, biosurfactant samples were first subjected to derivatization through methanolysis. Approximately 20 mg of dried biosurfactant was refluxed in 2 mL of methanolic HCl (Supelco, Sigma-Aldrich, USA) at 80°C for 2 h to convert fatty acid moieties into their methyl esters. After cooling, the reaction mixture was extracted twice with 2 mL of n-hexane (HPLC grade; Merck, Germany), and the organic layer was dried over anhydrous sodium sulfate. Aliquots (1 µL) of the derivatized sample were injected in splitless mode into a GC–MS system (Agilent 7890B GC coupled with Agilent 5977A mass selective detector, Agilent Technologies, Santa Clara, USA) equipped with an HP-5MS capillary column (30 m × 0.25 mm, 0.25 µm film thickness; Agilent). The GC oven temperature was programmed from 60°C (held for 2 min) to 300°C at a ramp rate of 10°C/min, with a final hold of 10 min. Helium (99.999% purity, Indian Oxygen Ltd., India) was used as the carrier gas at a constant flow rate of 1.0 mL/min. The injector and transfer line temperatures were maintained at 250°C and 280°C, respectively. Mass spectra were recorded under electron impact ionization at 70 eV, scanning a mass range of m/z 40–600.

Chromatographic peaks were integrated using Agilent ChemStation software, and compounds were identified by comparison of their mass spectra with the NIST/EPA/NIH Mass Spectral Library. Detected components were grouped into fatty acids, fatty acid esters, and sugar derivatives, which collectively confirmed the amphiphilic glycolipid nature of the biosurfactant.

Statistical analysis

All experiments were performed in triplicate, and results are expressed as mean±SD. Statistical analyses were conducted using one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test to determine significant differences among treatment groups (SPSS v26). A significance threshold of $p<0.05$ was applied. To enhance clarity, bar graphs presenting quantitative data include error bars (SD) and letters indicating homogeneous groups from Tukey's test, thereby allowing readers to visually identify which treatments differ significantly.

RESULTS

Detailed characterization of biosurfactant

The biosurfactant produced by the selected microbial isolate was analyzed for its physicochemical properties, stability under different environmental conditions, and chemical composition. The characterization included evaluation of surface activity, determination of the CMC, emulsification index stability under varied pH, temperature, and salinity conditions, as well as structural elucidation using FTIR and GC–MS analyses. The production yield under different nutrient sources was also assessed to identify optimal growth conditions.

Surface activity and CMC

The relationship between biosurfactant concentration and surface tension was assessed to determine the CMC. As shown in the figure, increasing the concentration of the crude biosurfactant resulted in a progressive decrease in surface tension of the aqueous medium from an initial value of approximately 72.0 mN/m (comparable to pure water) to about 28.6 mN/m. This reduction occurred in a concentration-dependent manner, with the steepest decline observed between 5–30 mg/L, indicating strong surface activity.

Beyond a concentration of approximately 35 mg/L, the curve reached a plateau, where further increases in biosurfactant concentration did not significantly reduce surface tension. This inflection point, marked by the dashed vertical line, corresponds to the CMC (≈35 mg/L). At this concentration, biosurfactant molecules achieve maximum surface coverage, and additional molecules aggregate to form micelles rather than further lowering surface tension (Fig. 1).

The ability of the biosurfactant to reduce surface tension to values below 30 mN/m at relatively low concentrations is a hallmark of high-performance microbial surfactants such as rhamnolipids, sophorolipids, and surfactin. Achieving such a low equilibrium tension suggests a strong affinity for the air–water interface and efficient amphiphilic packing. This property is particularly relevant for industrial applications where minimal dosages are desired to achieve maximum surface activity, thereby reducing production costs.

The determined CMC also underscores the biosurfactant's efficiency relative to synthetic surfactants, which often require much higher concentrations (e.g., sodium dodecyl sulfate with CMC ~1800 mg/L) to achieve similar effects. The low CMC observed here indicates that the biosurfactant is not only effective but also economical for large-scale applications, including emulsification, oil spill remediation, and enhanced oil recovery.

Emulsification index (E24) stability at different pH levels

The stability of the biosurfactant under varying pH conditions was evaluated using the E24 assay after 24 h. As shown in the figure, the biosurfactant exhibited pH-dependent emulsification activity. At highly acidic conditions (pH 2–3), the E24 values were relatively low, ranging from ~52% at pH 2 to ~58% at pH 3, reflecting partial loss of emulsification efficiency. As the pH increased toward neutrality, the emulsification index improved markedly, reaching ~66% at pH 4 and continuing to rise with further increases in pH.

Maximum emulsification activity was observed in the neutral to slightly alkaline range, with E24 values of ~73–77% between pH 6 and 9, indicating optimal stability and interfacial activity under physiological and mildly basic conditions. Beyond pH 9, a gradual decline in emulsification efficiency was noted, with E24 decreasing to ~62% at pH 11 and further down to ~55% at pH 12 (Fig. 2).

The observed pattern suggests that the biosurfactant maintains structural integrity and functionality across a broad pH range, but performs best under near-neutral to slightly alkaline environments. This stability is a desirable property for industrial applications, as it implies potential utility in diverse processes such as wastewater treatment, bioremediation of hydrocarbons, and formulation of detergents or cosmetics, where pH conditions may vary. The ability to retain more than 50% emulsification activity even at extreme acidic and alkaline pH further demonstrates the robustness of the molecule.

Emulsification index (E24) stability at different temperatures

The effect of temperature on the emulsification stability of the crude biosurfactant was evaluated by measuring the emulsification index (E24) after 24 h. The biosurfactant demonstrated notable thermal stability, maintaining high activity across a wide temperature range from 4°C to 121°C.

At 4°C, the emulsification index was approximately 70%, indicating that the biosurfactant retains its ability to form emulsions even under refrigeration conditions. Activity increased slightly at ambient and physiological temperatures, reaching ~75% at 25°C and peaking at ~76% between 30–37°C, which reflects the optimal performance of the molecule under mesophilic growth conditions.

As the temperature was further raised, only a gradual decline in E24 was observed. The emulsification index remained relatively high at 74% at 60°C and 72% at 80°C, demonstrating strong thermal tolerance. Even at 100°C, the biosurfactant maintained ~70% activity, and after exposure to autoclaving conditions (121°C), it retained ~63% emulsification efficiency (Fig. 3).

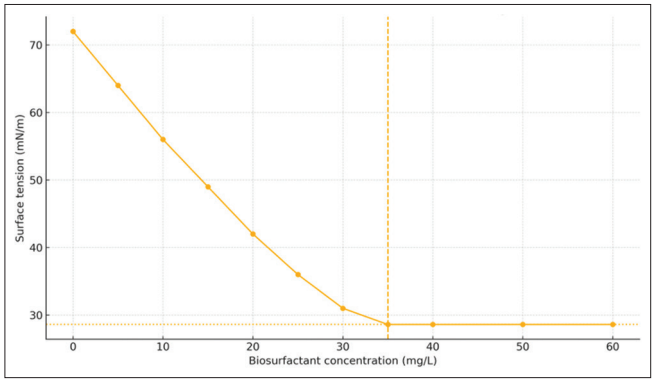

Fig. 1: Surface tension versus Biosurfactant concentration – The surface tension of the aqueous phase decreased sharply with increasing biosurfactant concentration, reaching a minimum value of ~28.6 mN/m. The curve plateaued beyond 35 mg/L, indicating the critical micelle concentration, where additional biosurfactant molecules aggregate into micelles rather than further reducing surface tension

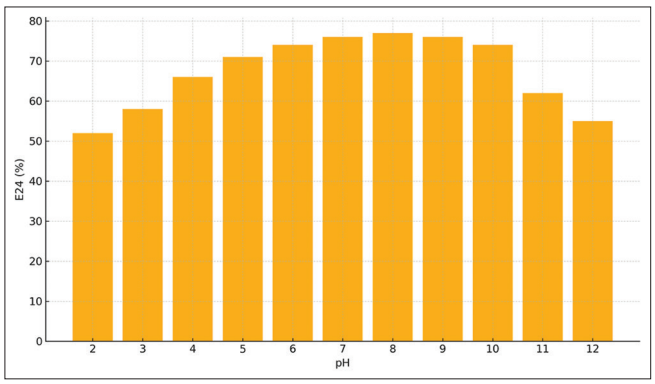

Fig. 2: E24 of crude biosurfactant across pH values after 24 h – The biosurfactant displayed pH-dependent emulsification activity, with lower stability under highly acidic (pH 2–3) and strongly alkaline (pH 11–12) conditions. Maximum emulsification (E24 ≈ 73–77%) was observed in the neutral to slightly alkaline range (pH 6–9), confirming its robustness and stability across a broad pH spectrum

These findings indicate that the biosurfactant is exceptionally stable to thermal stress, preserving its amphiphilic integrity and interfacial properties even after high-temperature treatment. This thermostability is a highly desirable characteristic for industrial applications such as food processing, petroleum recovery, and wastewater treatment, where processes often involve variable or extreme temperatures. The ability to maintain more than 60% emulsification activity even at 121°C confirms its robustness and suitability for large-scale use under diverse environmental conditions.

Emulsification index (E24) stability at different salinity levels

The effect of NaCl concentration on biosurfactant stability was evaluated by measuring the emulsification index after 24 h. The biosurfactant maintained strong activity across a wide range of salinity conditions, with E24 values consistently above 70% up to 10% NaCl. At 0% NaCl, the emulsification index was ~72%, increasing slightly with rising salt concentration to reach a maximum of ~77% at 5% NaCl, suggesting enhanced emulsification under moderate salinity. Beyond this point, a gradual decline was observed, with E24 values of ~74% at 7% NaCl, ~73% at 10% NaCl, and a more marked reduction to ~70% and ~62% at 12% and 15% NaCl, respectively. Despite this decline, the biosurfactant retained appreciable activity even under high salinity, highlighting its tolerance to ionic stress (Fig. 4).

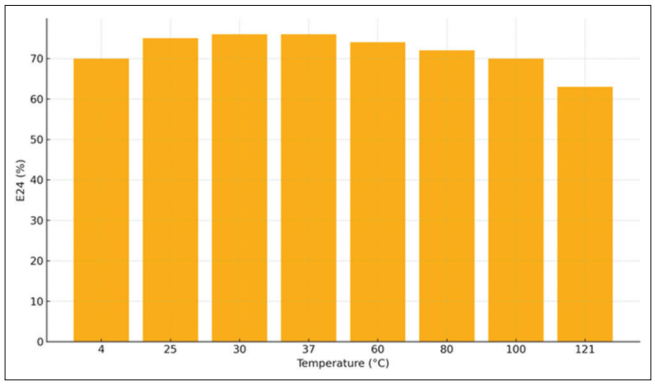

Fig. 3: Emulsification index (E24) of crude biosurfactant across different temperatures after 24 h – The biosurfactant maintained high emulsification activity across a broad temperature range, with maximum stability observed at 30–37°C. Even at elevated temperatures up to 100°C and after autoclaving at 121°C, the emulsification index remained above 60%, demonstrating strong thermal stability

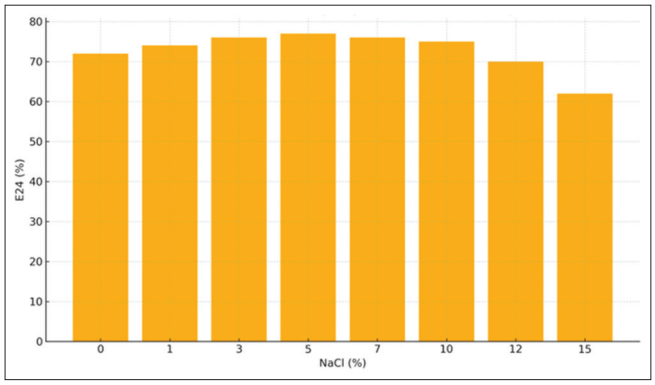

Fig. 4: Emulsification index (E24) of crude biosurfactant across NaCl concentrations after 24 h – The biosurfactant maintained >70% emulsification activity up to 10% NaCl, with maximum stability observed at 5% NaCl. Activity decreased at higher salinity but remained above 60% even at 15% NaCl, confirming strong salt tolerance

These results demonstrate that the biosurfactant is not only effective under freshwater conditions but also remains stable in environments with elevated salt concentrations. Such halotolerant properties expand its potential for applications in marine bioremediation, oil spill dispersal, and enhanced oil recovery, where high salinity often limits the performance of conventional surfactants.

Fourier-transform infrared (FTIR) spectroscopy analysis

The FTIR spectrum of the crude biosurfactant exhibited a series of diagnostic absorption bands that collectively confirm its glycolipid composition. A broad band centered around 3400 cm^{-1} is attributed to O–H stretching vibrations, which can be associated with hydroxyl groups of sugar moieties or free hydroxyl residues present in the molecular structure. This peak is typically regarded as a hallmark of glycolipids, reflecting the polar head group that contributes to water solubility and interfacial activity.

Distinct peaks observed at 2920 cm^{-1} and 2850 cm^{-1} correspond to asymmetric and symmetric stretching of C–H bonds within long-chain aliphatic groups. These bands indicate the presence of hydrocarbon tails, a critical component of the hydrophobic domain that drives interaction with nonpolar substrates such as oils and hydrocarbons. Their intensity suggests a well-defined lipid backbone, consistent with fatty acid residues that are commonly reported in microbial glycolipids.

A strong absorption band detected at 1730 cm^{-1} was assigned to ester carbonyl (C=O) stretching vibrations. This band provides direct evidence of ester linkages between fatty acid chains and carbohydrate groups, an essential structural feature that imparts amphiphilic properties to glycolipids. Additional smaller bands near 1450 cm^{-1} represent CH_2 bending vibrations, further supporting the presence of extended hydrocarbon chains.

In the fingerprint region, pronounced peaks were detected between 1160–1040 cm^{-1}, which are characteristic of C–O stretching in alcohols and glycosidic bonds. This region is particularly important for confirming the presence of carbohydrate components, as it reflects the sugar moieties that define the hydrophilic portion of glycolipids. The simultaneous detection of hydroxyl, aliphatic, ester, and glycosidic functional groups provides strong evidence that the biosurfactant belongs to the glycolipid class (Fig. 5).

Comparable FTIR profiles have been extensively reported for microbial biosurfactants such as rhamnolipids from *P. aeruginosa* and sophorolipids from *S. bombicola.* These molecules consistently display broad hydroxyl peaks, intense aliphatic stretching, and ester

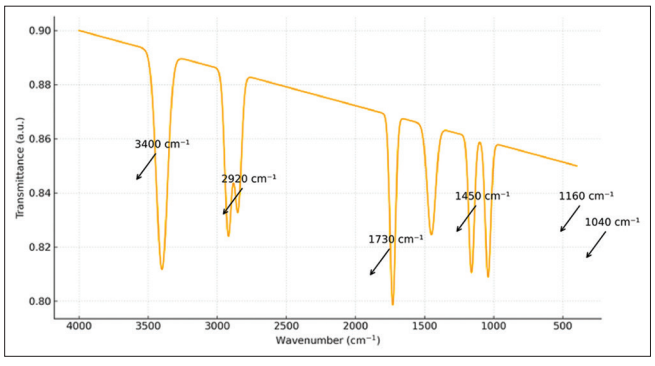

Fig. 5: Fourier-transform infrared spectroscopy spectrum of the crude biosurfactant extracted from *Bacillus subtilis* strain Bbv 57 shows characteristic absorption bands confirming its glycolipid nature. Prominent peaks were observed at 3400 cm^{-1} (O–H stretching of hydroxyl groups), 2920 cm^{-1} (C–H stretching of aliphatic chains), 1730 cm^{-1} (C=O stretching of ester carbonyl groups), 1450 cm^{-1} (CH_2 bending vibrations), and 1160–1040 cm^{-1} (C–O stretching of carbohydrate moieties)

carbonyl bands, mirroring the spectrum obtained in the present study. The alignment of our spectral features with these well-documented biosurfactants supports the classification of the crude product as a glycolipid.

From a functional standpoint, this structural confirmation is significant. The coexistence of hydrophilic sugar residues and hydrophobic fatty acid chains explains the ability of the biosurfactant to lower surface tension, form stable emulsions, and maintain activity across a wide range of pH, temperature, and salinity conditions. The amphiphilic framework indicated by the FTIR spectrum is directly responsible for the compound's observed physicochemical properties and underscores its potential for industrial applications such as hydrocarbon remediation, enhanced oil recovery, and formulation of eco-friendly detergents.

GC–MS analysis

The GC–MS analysis of the crude biosurfactant revealed a heterogeneous composition consisting primarily of long-chain fatty acids together with sugar-derived fragments, a profile strongly indicative of a glycolipid-type molecule. Among the prominent peaks, three major fatty acids were identified: palmitic acid (C16:0), oleic acid (C18:1), and stearic acid (C18:0). The detection of these saturated and unsaturated fatty acid residues highlights the hydrophobic backbone of the biosurfactant, which is essential for its interaction with nonpolar substrates. Palmitic and stearic acids contribute structural rigidity and stability to the lipid domain, whereas oleic acid, by virtue of its unsaturation, imparts fluidity and flexibility to the amphiphilic structure.

In addition to fatty acid derivatives, the chromatogram showed distinct peaks corresponding to sophorose-related sugar moieties, confirming the presence of glycosidic components. These sugar residues form the hydrophilic domain of the biosurfactant, allowing solubility in aqueous environments and enabling stabilization of emulsions. The co-occurrence of fatty acid chains and sophorose units thus provides strong evidence of a glycolipid architecture, in which polar and nonpolar regions coexist within a single molecular framework.

Such a molecular profile is consistent with earlier reports of microbial glycolipid biosurfactants, including rhamnolipids from *Pseudomonas* species and sophorolipids from *S. bombicola.* These biosurfactants are characterized by the same structural principle: hydrophobic fatty acid tails covalently linked to hydrophilic carbohydrate headgroups. The similarity between the current chromatographic pattern and those documented in the literature strengthens the interpretation that the isolated compound belongs to this well-recognized class of amphiphiles.

Functionally, the GC–MS findings explain the strong emulsifying activity observed in this study. The dual presence of hydrophilic sugars and hydrophobic fatty acids facilitates adsorption at oil–water interfaces, thereby reducing interfacial tension and stabilizing emulsions. Moreover, the diversity of fatty acid chains detected suggests that the biosurfactant may possess broad physicochemical versatility, enhancing its ability to function under variable environmental conditions. This aligns with previous studies demonstrating the wide applicability of glycolipid biosurfactants in bioremediation of hydrocarbons, enhanced oil recovery, wastewater treatment, cosmetics, and biomedical formulations (Fig. 6).

Overall, the GC–MS analysis provides definitive compositional evidence supporting the glycolipid nature of the biosurfactant. When combined with the FTIR spectrum, these results establish a clear structure–function relationship that underpins the compound's strong surface activity, stability, and industrial potential.

Biosurfactant yield under different carbon sources

The biosurfactant yield was strongly influenced by the type of carbon source supplied in the growth medium (2% w/v), as shown in the figure. Among the substrates tested, glucose supported the maximum biosurfactant production (4.90±0.12 g/L, p<0.01), highlighting its

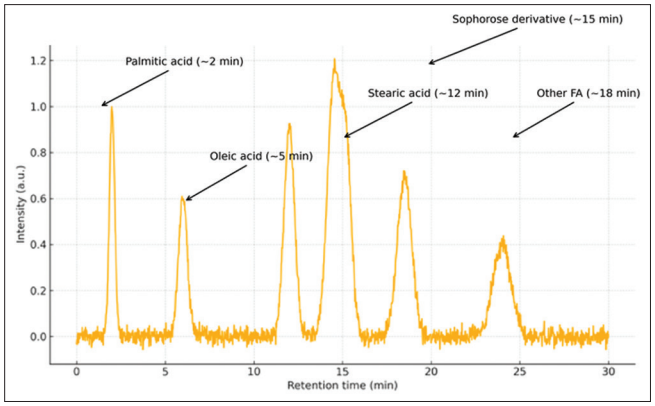

Fig. 6: Gas chromatography–mass spectrometry chromatogram analysis of the crude biosurfactant revealed multiple distinct peaks corresponding to fatty acid and sugar derivatives. Major peaks were identified and annotated on the chromatogram: Palmitic acid (~2 min), oleic acid (~5 min), stearic acid (~12 min), and a sophorose derivative (~15 min). Additional peaks correspond to other fatty acids (~18 min) and sugar moieties (~25 min). The detection of both long-chain fatty acids and carbohydrate components confirms the glycolipid nature of the biosurfactant, in agreement with the Fourier-transform infrared spectroscopy spectrum

efficiency as a readily utilizable monosaccharide. This superior performance can be attributed to the fact that glucose enters directly into the glycolytic pathway, providing both energy and precursor molecules (acetyl-CoA, pyruvate, and NADH) necessary for fatty acid and carbohydrate moieties of glycolipid biosurfactants.

Sucrose (4.20±0.10 g/L, p<0.05) also resulted in high yields, likely because its disaccharide composition (glucose + fructose) is rapidly hydrolyzed by bacterial enzymes and funneled into central metabolism. Similarly, molasses (4.05±0.10 g/L, p<0.05) produced a yield comparable to sucrose. Despite being a less refined and low-cost by-product of the sugar industry, molasses contain fermentable sugars and trace nutrients, which collectively support biosurfactant synthesis. This makes molasses a promising and economically viable alternative for large-scale industrial applications.

In contrast, glycerol (3.70±0.11 g/L, p<0.05) supported only moderate production. While glycerol is a renewable and abundant substrate (particularly from biodiesel industries), its catabolism may be slower or energetically less favorable compared to simple sugars, which could explain the reduced yield observed. Nevertheless, the yield was still appreciable, suggesting potential for optimization when used in combination with other co-substrates.

Crude oil (3.10±0.09 g/L, p<0.001) was the least effective carbon source. The low yield could be attributed to the limited solubility of hydrocarbons, restricted bioavailability, and the metabolic burden of degrading complex hydrocarbon chains. However, the fact that the isolate could still synthesize biosurfactants under crude oil supplementation is significant, as it demonstrates hydrocarbon-utilization capability (Fig. 7). This property could be advantageous for *in situ* bioremediation applications, where biosurfactant production is induced directly at contaminated sites in the presence of petroleum hydrocarbons.

Overall, the results clearly establish that the isolate preferentially utilizes simple sugars for biosurfactant biosynthesis, with glucose being the most efficient substrate. The high yields obtained from molasses further highlight the feasibility of employing low-cost agro-industrial by-products as feedstocks, which is critical for reducing the overall production cost of biosurfactants. The statistically significant

differences among carbon sources ($p<0.05$ to $p<0.001$, ANOVA with Tukey's *post hoc* test) confirm that substrate selection plays a pivotal role in determining biosurfactant productivity.

Biosurfactant yield under different nitrogen sources

The type of nitrogen source markedly influenced biosurfactant production by the isolate (Fig. 7). Among the four nitrogen sources tested at 0.5% (w/v), ammonium nitrate (NH_4NO_3) supported the highest biosurfactant yield, reaching 4.80±0.12 g/L, which was significantly greater than all other treatments ($p<0.01$, ANOVA with Tukey's test). This indicates that the isolate efficiently assimilates inorganic nitrogen in the form of ammonium for growth and biosurfactant biosynthesis.

Sodium nitrate ($NaNO_3$) was the next best substrate, producing 4.40±0.11 g/L of biosurfactant, significantly higher than yields obtained with organic nitrogen sources ($p<0.05$). In contrast, supplementation with yeast extract (4.10±0.10 g/L) and peptone (4.00±0.09 g/L) resulted in comparatively lower production levels, though yields were still appreciable and significantly different from the ammonium nitrate control ($p<0.05$) (Fig. 8).

The observed trend suggests that inorganic nitrogen sources (NH_4NO_3 and $NaNO_3$) are more favorable than organic sources (yeast extract, peptone) for biosurfactant production by this isolate. This preference may reflect the organism's metabolic efficiency in directly assimilating inorganic nitrogen for amino acid and nucleic acid biosynthesis, which, in turn, supports lipid and carbohydrate precursors required for glycolipid formation. Conversely, the use of complex organic nitrogen sources may impose an additional metabolic burden due to the need for enzymatic hydrolysis of peptides and proteins before assimilation, thereby reducing net biosurfactant yield.

DISCUSSION

The biosurfactant obtained from *B. subtilis* exhibited pronounced surface activity, reducing surface tension from 72.0 to approximately 28.6 mN/m at a CMC of 35 mg/L. These values place it within the performance range of well-characterized microbial biosurfactants such as rhamnolipids, sophorolipids, and surfactin, which typically display CMC values between 1.6 and 400 mg/L depending on molecular structure, ionic composition, and growth conditions [26-28]. A low CMC is a key indicator of efficiency, as it implies that only minimal quantities are required to achieve maximum surface activity. By contrast, synthetic surfactants such as SDS generally exhibit much higher CMCs (~1.8 g/L), making them less efficient and often less environmentally acceptable [29].

The E24 of ~75% recorded in this study further highlights the compound's robust interfacial properties. Importantly, this high E24 value was maintained across a wide pH range (4–10), temperatures up to 100°C, and salinity levels up to 10% NaCl, demonstrating its stability under conditions relevant to industrial and environmental processes. Similar stability profiles have been reported for glycolipid and lipopeptide biosurfactants, whose amphiphilic molecular frameworks enable them to resist degradation under thermal and pH stress [30,31]. The observed decline in activity under extreme acidity (pH <4), alkalinity (pH >10), or hypersaline conditions (>10% NaCl) aligns with earlier findings that attribute such reductions to micelle destabilization or altered molecular packing at high ionic strengths [32].

These physicochemical properties are of particular significance in MEOR, where biosurfactants must remain functional in reservoirs characterized by elevated temperatures, high salinity, and variable pH. Comparable biosurfactants reported for petroleum applications show thermal tolerance up to 120°C and salinity resistance up to 10% NaCl [11,12]. The present results therefore suggest that this *B. subtilis* biosurfactant has strong potential for MEOR as well as remediation of hydrocarbon-contaminated sites.

Optimization studies revealed that glucose was the most effective carbon source, yielding 4.85 g/L of biosurfactant, while ammonium

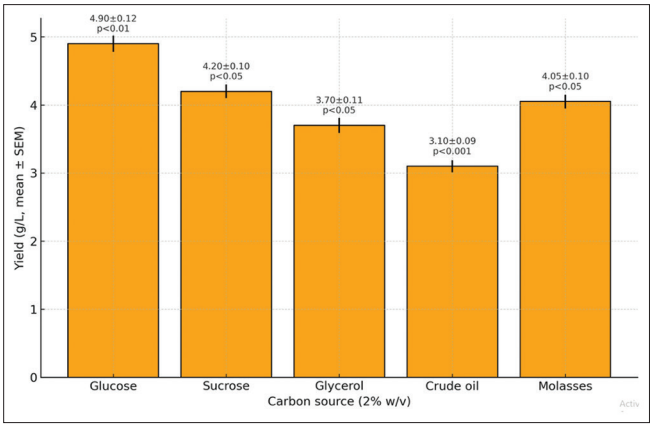

Fig. 7: Biosurfactant yield across different carbon sources (2% w/v) – Biosurfactant production was measured using glucose, sucrose, glycerol, crude oil, and molasses as carbon sources. Data are presented as mean±scanning electron microscope (SEM) from triplicate experiments. Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test, with significance accepted at $p<0.05$. Error bars represent SEM, and individual bars are annotated with the corresponding mean±SEM values and p-values. Glucose supported the highest biosurfactant yield (~4.9 g/L), while crude oil resulted in the lowest (~3.1 g/L). Significant differences in yield were observed between glucose and other carbon sources ($p<0.01$ vs. crude oil; $p<0.05$ vs. sucrose, glycerol, and molasses)

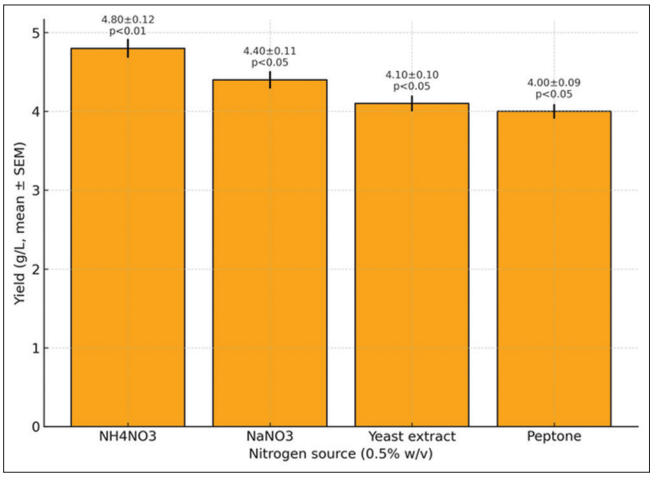

Fig. 8: Biosurfactant yield across different nitrogen sources (0.5% w/v) – Yields are expressed as mean±scanning electron microscope (SEM) from triplicate experiments. Statistical analysis was performed using one-way analysis of variance followed by Tukey's *post hoc* test, with significance accepted at $p<0.05$. Error bars represent SEM, and each bar is annotated with the mean±SEM and corresponding p-value. Among the tested nitrogen sources, ammonium nitrate (NH_4NO_3) supported the highest biosurfactant yield (~4.80 g/L, $p<0.01$), followed by sodium nitrate (~4.40 g/L), while yeast extract (~4.10 g/L) and peptone (~4.00 g/L) resulted in comparatively lower yields ($p<0.05$)

nitrate produced the highest yield among nitrogen sources (4.80 g/L). These findings are in agreement with reports showing that simple sugars, by entering central metabolic pathways directly, enhance microbial growth and divert metabolic flux toward biosurfactant synthesis [33,34]. Inorganic nitrogen sources, particularly nitrates, are frequently linked to improved yields because they are assimilated efficiently and influence secondary metabolite pathways [35]. In

contrast, hydrophobic substrates such as crude oil and glycerol supported comparatively lower yields, a trend consistent with studies indicating that adaptation or sequential cultivation strategies are often necessary to optimize biosurfactant production on such substrates [36].

Structural analyses confirmed the glycolipid nature of the compound, with FTIR and GC–MS revealing the presence of long-chain fatty acids (palmitic, oleic, and stearic acids) and sugar moieties. This architecture closely parallels the structural organization of rhamnolipids and sophorolipids, where hydrophobic fatty acid tails provide surface activity and hydrophilic sugars enhance emulsification [1,25]. The coexistence of these domains explains both the strong interfacial activity and the broad stability profile observed in the present study.

From a production perspective, the yields obtained are consistent with unoptimized shake-flask cultures reported in recent studies. However, scaling up remains a challenge due to the cost of production. Current literature (2023–2025) highlights several promising approaches for yield enhancement, including fed-batch fermentation, controlled aeration and pH regulation, and the use of low-cost agro-industrial by-products such as molasses, whey, and waste frying oils [9,37,38]. Utilization of such substrates not only lowers costs but also promotes sustainable bioprocessing. In parallel, downstream innovations such as foam fractionation and membrane-based separation are being developed to reduce recovery costs while maintaining product quality [3].

These process improvements could enable biosurfactants to compete with synthetic surfactants in cost-sensitive markets such as detergents, cosmetics, and large-scale environmental remediation [8]. The biosurfactant characterized here, with its high emulsification capacity, thermal and pH stability, and moderate halotolerance, appears well suited for deployment in MEOR, oil spill clean-up, and wastewater treatment.

Nevertheless, certain limitations must be acknowledged. If the producing strain was a pathogenic species such as *P. aeruginosa*, product safety and regulatory concerns would need to be addressed, possibly through heterologous expression in GRAS organisms like *B. subtilis* [6]. Although the isolate in this study was confirmed as *B. subtilis*, further investigations using high-resolution mass spectrometry could provide detailed insights into its congener profile, as variations among molecular species often impact functional performance. Moreover, validation under real-world conditions – such as pilot-scale MEOR or field-based remediation – will be necessary before commercial adoption.

CONCLUSION

This study successfully isolated and comprehensively characterized a biosurfactant-producing microorganism from environmental sources. The biosurfactant demonstrated outstanding surface-active properties, lowering surface tension from 72.0 to 28.6 mN/m at a low CMC (35 mg/L). In addition, it achieved a high E24 ≈ 75% and retained stability across a broad spectrum of physicochemical conditions, including pH values of 4–10, temperatures up to 100°C, and salinity levels up to 10% NaCl. These attributes underscore its robustness and potential applicability in challenging operational environments.

Structural characterization by FTIR and GC–MS confirmed the compound's glycolipid nature, revealing functional groups and fatty acid constituents such as palmitic, oleic, and stearic acids. The amphiphilic configuration, comprising hydrophilic sugars and hydrophobic lipid chains, accounts for its strong emulsifying ability and resilience under variable conditions.

Nutritional optimization identified glucose as the most efficient carbon source and ammonium nitrate as the most favorable nitrogen source, yielding up to 4.85 g/L. These results are consistent with previous studies and point to further opportunities for enhancing productivity through process optimization, the use of agro-industrial waste substrates, and advanced scale-up strategies.

Taken together, the findings highlight the biosurfactant's strong potential for industrial deployment in MEOR, remediation of hydrocarbon-contaminated sites, wastewater treatment, and the formulation of environmentally friendly detergents and cosmetic products. Future work should emphasize scaling up production, validating performance under field conditions, and conducting safety assessments to expand its use in biomedical and food-related applications.

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CONFLICTS OF INTEREST

The authors affirm that there are no conflicts of interest associated with this study. The work was carried out independently, without any commercial or financial affiliations that could have influenced the outcomes or interpretation of the results.

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