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Bioprospecting of biosurfactant-producing bacteria for hydrocarbon bioremediation: Optimization and characterization

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Abstract−Biosurfactants have been found capable of replacing synthetic surfactants which include ongoing bioprospecting of biosurfactant-producing bacteria as well as process optimization for maximum biosurfactant production. In this study, five morphologically distinct actinomycete strains isolated from hydrocarbon-polluted soil collected from an oil spill surface in Southeastern Algeria were tested for their ability to produce biosurfactants using preliminary biosurfactant screening assays. The 7SDS strain was selected as the most promising biosurfactant producer due to its greatest oil displacement diameter (7.83±0.15 cm), emulsification index (59.66±0.44%), and enhanced surface tension reduction (30.04±0.51 mN/m); it was identified as *Streptomyces thinghirensis* 7SDS using 16S rDNA sequence analysis. The 7SDS strain's biosurfactant production was optimized using the Face-centered central composite design (CCD) based on response surface methodology (RSM). To this end, five independent factors, i.e., residual frying oil, used engine oil, whey, CS filtrate, and incubation time, were assessed. The RSM's model predicted a surface tension of 27.48 mN/m using 2.44% (v/v) residual frying oil, 0.35% (v/v) used motor oil, 0.83% (v/v) whey, 0.39% (v/v) CS filtrate, and an incubation time of 219.3 h. The optimized medium produced 8.79 g/L of biosurfactant. The produced biosurfactant allows one to reduce the surface tension of distilled water from 70.86 mN/m to 27.96 mN/m at a critical micelle concentration of 350 mg/L, even over a wide range of pH (2.0-12.0), temperature (4-120 °C), and salinity (2-12%, W/V). Biochemical (Biuret, phenol-sulfuric acid, and phosphate tests) and compositional (FTIR and GC-MS) characterizations confirmed the phospholipid nature of the produced biosurfactant. Interestingly, the produced BS demonstrated significant antimicrobial activity as well as intriguing activity in removing hydrocarbons from polluted soil. Because of their appealing biological properties, strain 7SDS and its biosurfactant are attractive targets for a variety of applications such as biomedicine and environmental ones.

Keywords: Biosurfactant, Phospholipid, *Streptomyces*, Response Surface Methodology

INTRODUCTION

Scientific and biotechnological developments have gradually assisted researchers in exploiting natural resources [1]. According to the international energy agency (IEA), the global energy demand is growing rapidly and is projected to double by 2030 [2]. Alternative energy sources such as solar, hydroelectric, and geothermal energy are already in use, but petroleum has remained the most widely-used energy source [3]. The petroleum extraction and transportation and use of petroleum-based products cause various forms of environmental pollution [4], as most of these products are harmful to plants, animal, and human health. Spilled oil tanks as exam-

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ples of this pollution have harmed thousands of square kilometers of shielded areas around the world [5].

Petroleum-polluted environments can be remediated by using either physicochemical or biological methods. Usual physicochemical processes can remove most petroleum-derived compounds, but most of them are expensive and environmentally unfriendly [6]. However, biological approaches through bioremediation can be conceivable, which have the advantage of being both eco-friendly and economically productive [7]. Furthermore, microorganisms are not always able to completely degrade petroleum-derived compounds [7]. Therefore, adding bio-based products such as biological surfactants, so-called biosurfactants, is a promising approach to enhance the biodegradability of petroleum products [8].

Biosurfactants are amphipathic secondary metabolites with pronounced surface and interfacial activity [9]. These biomolecules outperform their chemical counterparts in many aspects, including biodegradability, low toxicity, and efficacy under critical conditions [10]. On the other hand, biosurfactants with these properties offer many applications in different fields, including medicine, food processing, agriculture, cosmetics, and textile manufacturing [11]. Recently, the global biosurfactants market has skyrocketed; it reached US$ 1.5 billion in 2019 and is expected to grow at a compound annual growth rate (CAGR) of 5.5% until 2026 [12]. In addition, biosurfactants can be produced from lipid- or carbohydrate-rich renewable wastes, allowing not only a significant reduction in production cost, but also a reduction in the amount of waste processed in various industries [13]. Several authors have advocated the use of industrial wastes including whey, frying oil, fats, and soap stock as inexpensive substrates for biosurfactant production [14]. Likewise, the optimization of media and growth conditions using statistical approaches can significantly improve yields and reduce the overall cost of biosurfactant production [1]. Traditional optimization techniques are time consuming, expensive, and difficult to perform for large numbers of variables [15]. Therefore, response surface methodology (RSM) is one of the alternatives that addresses the limitations of traditional approaches [16,17]. RSM is used to predict the relationship between responses and variables based on a mathematical model, while reducing the number of experiments [18].

Another essential part of increasing biosurfactant production is the use of efficient fermentation microorganisms. Microorganisms (e.g., yeasts, filamentous fungi, and bacteria) can produce biosurfactants [19] with different surface activity and chemical compositions, including lipopeptides, fatty acids, phospholipids, and glycolipids [20]. Numerous bacteria are used for biosurfactants production, including *Acinetobacter*, *Bacillus*, and *Pseudomonas* [19]. Apart from these bacteria, actinomycetes are abundant in soil and play a crucial role in the degradation of petroleum hydrocarbons [21], making them advantageous and promising candidates to produce biosurfactant [22]. These bacteria are considered capable of producing large quantities of secondary bioactive molecules with numerous applications in various fields such as the pharmaceutical industry and agriculture [23]. About 70,000 natural substances are of microbial origin, 29% of which are from actinomycetes [24]. From 2015 to 2020, 279 new bioactive molecules were produced from 121 *Streptomyces* species [25]. This genus, which is known for the industrial production of antibiotics [26], is also involved in the production of biosurfactants.

Based on the above-mentioned knowledge, this study focused on the isolation, screening for biosurfactant-producing actinomycetes, RSM modeling of BS production using low-cost wastes, partial purification, and characterization of the produced BS, and finally evaluation of biosurfactant's ability as a bioremediation and antimicrobial agent.

MATERIALS AND METHODS

1. Collected Samples

Highly hydrocarbon-contaminated soil samples were collected from crude oil spilled surfaces of the "24 February" production division of SONATRACH in Hassi-Messaoud, Ourgla-Algeria (32°10′19.6″ N 4°44′54.6″ E). Samples were collected according to the technique derived from the study of Pochon and Tardieux [27]. This technique involves taking 100-150 grams of soil (5-15 centimeters deep) with a sterile spatula and placing it on a sterile aluminum sheet after removing the first five centimeters of the surface layer of the soil and eliminating large debris (stones, roots, etc.). The soil samples were transferred to the laboratory in a sterile vial and stored at 4 °C for further analyses.

2. Pre-treatments of Soil Samples

As outlined below, four physicochemical pretreatments were applied to the preferential enrichment and isolation of actinomycetes from soil samples:

***Heat pre-treatment method*:** 1 g of soil was dried for 1 h in a Pasteur oven at 120 °C [28].

Phenol chemical pre-treatment method: 0.5 mL of soil suspension (1 g in 10 mL sterile physiological water) was added to 4.5 mL of sterile phosphate buffer (5 mM, pH 7.0) containing phenol at 1.5% (w/v). The mixture was kept at 30 °C for 30 min under periodic stirring [27].

***Sodium Dodecyl Sulfate (SDS) and Yeast Extract (YE) chemical pre-treatment method*:** 0.5 mL of soil suspension was added to 4.5 mL of sterile phosphate buffer (5 mM, pH 7.0) containing SDS at 0.05% (w/v) and YE at 6% (w/v). The mixture was kept at 40 °C for 20 min with occasional stirring [28].

***Calcium Carbonate ($CaCO_3$) enrichment method*:** 1 g of soil was mixed with 0.1 g of $CaCO_3$. The mixture was incubated for 7 days at 28 °C [29].

3. Enrichment of Actinomycetes

To promote the growth of actinomycetes, a pre-culture was carried out in Erlenmeyer flasks of 250 mL with the following compounds: 95 mL of humic acid-vitamin (HVA) broth (1 g humic acid, 0.5 g Na_2HPO_4, 1.71 g KCl, 0.05 g $MgSO_4{\cdot}7H_2O$, 0.01 g $FeSO_4{\cdot}7H_2O$, 1 g $CaCl_2$, B vitamins (0.5 mg each of thiamine-HCl, riboflavin, niacin, pyridoxine, Ca-pantothenate, inositol and p-aminobenzoic acid, and 0.25 mg of biotin), and 1 L water, pH 7.2). After sterilization by autoclaving at 120 °C for 20 min, 5 mL of pre-treated soil suspension was poured into each flask and then incubated at 30 °C at 180 rpm for 30 days.

4. Isolation and Purification of Hydrocarbonoclast Actinomycetes

Once the enrichment was complete, an aliquot (100 µl) of enrich-

ment in HVA broth was plated on international streptomyces project-9 (ISP9) medium (2.64 g $(NH_4)_2SO_4$, 2.38 g KH_2PO_4, 5.65 g K_2HPO_4, 1 g $MgSO_4 \cdot 7H_2O$, 1 mL of saline solution (2.64 g $CUSO_4 \cdot 5H_2O$, 2.38 g $FeSO_4 \cdot 7H_2O$, 5.65 g $MNCl_2 \cdot 4H_2O$, 1 g $ZNSO_4 \cdot 7H_2O$ and 100 mL water), 20 g agar, and 1 L water, pH 7.4) supplemented with 1% (v/v) of crude oil as the only source of carbon and energy. Petri dishes were incubated at 30 °C for 20 days. All colonies that show a macroscopic appearance similar to that of actinomycetes (powdery, hard colonies with fine filamentous borders) were enumerated, picked up, and then purified on Bennet's medium (1 g meat extract, 1 g yeast extract, 10 g glucose, 2 g casein hydrolysate, 15 g agar, and 1 L water, pH 7.3) until pure strains without contaminants were obtained. Pure strains were preserved as spore suspension in Bennet's broth with 20% (v/v) of glycerol at −20 °C.

5. Screening of Potential Biosurfactant (BS) Producing Actinomycetes

5-1. Pre-processing of the Substrate

Waste chicken skin (CS) collected from a restaurant in Constantine-Algeria was cut into small pieces, and then 30% (w/v) of CS was incubated at 75-80 °C in a shaking water bath for 45 min and filtered by using Whatman filter paper No. 1. The collected filtrate was stored at 4 °C until used.

5-2. Liquid State Fermentation for BS Production

To prepare a seed inoculum, morphologically different colonies were initially inoculated into 250-mL Erlenmeyer flasks containing 100 mL of Bennet's broth and then incubated at 30 °C in a rotary incubator shaker at 180 rpm until their optical density (O.D) reached 1.0 at 600 nm. An amount of 5 mL of inoculum was inoculated into 250-mL Erlenmeyer flasks containing 100 mL of Bushnell-Hass (BH) broth (1 g K_2HPO_4, 1 g KH_2PO_4, 0.02 g $CaCl_2 \cdot 2H_2O$, 1 g NH_4NO_3, 0.05 g $FeCl_3$, 0.05 g $MgSO_4 \cdot 7H_2O$, and 1 L water, pH 7.0) enriched with 1% (v/v) of CS filtrate as the sole carbon source and incubated at 30 °C under the agitation of 180 rpm for 10 days. After incubation, the fermentation medium was centrifuged at 10,000 rpm, at 4 °C for 20 min. The obtained cell-free culture (supernatant) was used to determine BS production during screening tests, namely, the oil displacement test, parafilm-M test, emulsification index, and surface tension measurement. As a result, the 7SDS strain was identified as the most promising bacterium for BS production.

5-3. Screening Methods for BS Production

In the relevant literature, different qualitative and quantitative methods have been recommended for selecting biosurfactants promoting microbes [30]. As described below:

Oil displacement test (ODT): 50 µL of crude oil was added to the surface of 20 mL of distilled water to form a thin oil layer (in a petri dish). Then, 20 µL of supernatant was gently placed in the middle of the oil layer. A negative control was produced with a non-inoculated sterile fermentation medium, and SDS (10%, w/v) was used as the positive control. If BS is present, the oil will be displaced, and a clear visible halo will be formed [2].

Parafilm-M test: 20 µL of supernatant was carefully transferred onto the hydrophobic surface of parafilm-M. The shape of the drop was observed after one minute. A negative control was produced with a non-inoculated sterile fermentation medium, and SDS (10%, w/v) was used as the positive control. The test is considered positive (presence of BS) if the drop spreads and becomes flat in comparison with the negative control [31].

Emulsification activity (E24): In the test tube, 2 mL of supernatant was homogenized with 2 mL of kerosene in a high-speed vortex for 2 min; to separate the aqueous and oil phases, the obtained mixture was kept at 25 °C for 1day. Emulsification activity was calculated using Eq. (1) [29].

$$\text{Emulsification index (EI) (\%)} = \frac{\text{Height of emulsion}}{\text{Total height of the mixture}} \times 100 \quad (1)$$

Surface tension (ST) measurement: Supernatants obtained after ten days of incubation were checked for ST reduction using a surface tensiometer, employing the principal of Du Nuoy ring detachment method [32]. The accuracy of ST measurements was verified with distilled water before each measurement.

6. Molecular Identification of Strain 7SDS

For molecular characterization, genomic DNA extraction, PCR amplification of 16S rRNA gene sequence, and bioinformatic sequences analysis were carried out in accordance with the protocol described by Mechouche et al. [33].

7. Statistical Optimization of BS Production

7-1. Selection of Fermentation Process Variables

Various low-cost industrial by-products and agricultural wastes are used as alternative substrates for biosurfactants production [34]. In this study, residual frying oil, used engine oil, whey, CS filtrate, and incubation time were selected as the five parameters for the optimization of BS production.

7-2. Optimization by Response Surface Methodology

BS production from 7SDS strain was optimized using RSM based on face-centered composite design (CCD), which was used to study the main effect and interactions of residual frying oil (A), CS filtrate (B), whey (C), and used engine oil (D) in the range between 0 and 3% (v/v), as well as incubation time (E) in the range between 120 and 360 h (Table 1); all variables were coded (−1, 0, +1) with 26 experimental runs and five repetitive central points (Table 4).

Table 1. The selected variables and their low/high levels

Code	Variables	Unit	Low level (−1)	Medium (0)	High level (+1)
A	Residual frying oil	% (v/v)	0	1.5	3
B	CS filtrate	% (v/v)	0	1.5	3
C	Whey	% (v/v)	0	1.5	3
D	Used engine oil	% (v/v)	0	1.5	3
E	Incubation time	H	120	240	360

7-3. Statistical Analysis and Modeling

Face-centered central composite experimental design was used to evaluate main effects, interaction effects, and quadratic effects, as well as to optimize parameter levels for maximum BS production. To this end, results were subjected to the analysis of variance to assess the effect of independent variables, check for errors, and determine the statistical significance of the model using the Fisher's F test. The empirical model that could relate the measured response (ST (mN/m)) to each parameter was represented by a polynomial equation of the second degree, as given in Eq. (2):

$$Y=\beta_0+\Sigma\beta_i X_i+\Sigma\Sigma\beta_{ij}X_iX_j+\Sigma\beta_{ii}X_i^2 \quad (2)$$

where Y stands for the predicted response, β_0 for the bias, β_i for the linear effect, β_{ii} for the quadratic effect, β_{ij} for the interaction effect, and X_i for the coded value of the independent variables.

The statistical software of Design-Expert (Stat-Ease, US, Version 13.0) was used to analyze the experimental data and plot the 3D response surface curves.

8. Extraction and Purification of BS

8-1. Extraction

The BS extraction was carried out according to suggestions given by Chebbi et al. [35]. BS produced during batch culture on an optimized medium according to the optimization prediction was extracted from the flasks after eight days of incubation. For the extraction, the supernatant obtained after centrifugation of microbial culture at 10,000 rpm for 20 min at 4 °C was subjected to acid precipitation at pH 2.0 using 6 N HCl. Then, BS was extracted from the supernatant with ethyl acetate (EA). A 1 : 1 (v/v) mixture of EA and supernatant was shaken vigorously in a separating funnel and left to settle for 15 min (until phase separation) at room temperature. The organic phase was recovered, concentrated, and dried at 40 °C using a rotary vacuum evaporator until a dark honey liquid residue (crude BS) was obtained, which was then incubated at 37 °C overnight to remove all traces of solvent, and stored at 4 °C for further studies.

8-2. Purification

Purification of the crude BS was carried out according to suggestions provided by Hentati et al. [36]. It was performed on a 200×15 mm chromatographic column containing 30 g of silica gel (60 Mesh). The column was pre-equilibrated with toluene: methanol (70 : 30, v/v) mixture; then, 1 g of the crude BS was loaded onto the column and eluted with the same solvent mixture at a constant flow rate of 1 mL/min. 1 mL fractions were collected and checked by thin layer chromatography and ODT for the presence of BS.

9. Characterization of BS

9-1. Biuret Test

The presence of amino acids in the crude BS was performed by adding a few drops of 1% (w/v) $CuSO_4$ to 2 mL of crude extract solution previously heated at 70 °C and mixed with 1 M NaOH. The test is considered positive if the color changes to violet due to the presence of peptide bonds [37].

9-2. Phenol-sulfuric Acid Test

For sugar detection, 1 mL of 5% (w/v) phenol was added to 2 mL of crude extract solution, followed by a few drops of concentrated sulfuric acid. A positive result was confirmed by the development of yellow to orange color [38].

9-3. Phosphate Test

The presence of phosphate ion in the crude BS was tested by adding 10 drops of 6 M nitric acid to 2 mL of crude extract solution; the mixture was heated at 70 °C. Then, a few drops of 5% (w/v) ammonium molybdate was added slowly to the mixture. A positive result was indicated by the formation of a bright yellow precipitate [39].

9-4. Thin Layer Chromatography

The crude BS was analyzed by thin layer chromatography (TLC) on silica gel 60 plates (ALUGRAM XTRA SIL G/UV254, 20×20 cm). 5 µL of crude BS was spotted on the plates and eluted with toluene/methanol (70 : 30, v/v) solvent mixture. Once the elution was completed, the plate was detached and left to air dry. The obtained chromatogram was analyzed by coloration with ninhydrin solution (0.5%, (w/v) ninhydrin in acetone) and iodine vapors to detect amino acids and lipids, respectively [40].

9-5. Fourier-transform Infrared Spectroscopy

Fourier-transform infrared (FTIR) spectroscopy was performed to determine the functional groups and the chemical bonds present in the crude BS. A 2-mg amount of BS was analyzed by IRAffinity-1 Shimadzu spectroscopy, and spectral measurements were taken in the 600-4,000 cm^{-1} range in transmittance mode.

9-6. Gas Chromatography-mass Spectrometry Analysis of BS

Gas chromatography-mass spectrometry (GC-MS) analysis was used to assess the fatty acid profile of the obtained BS. Fatty acids were esterified to their corresponding methyl ethers as described by Joy et al. [41]. 1 µL of Fatty acids methyl esters (FAMEs) was injected into an Agilent Hewlett Packard (6890 plus GC/5973 MS) GC-MS equipped with an HP-5MS capillary column (30 m×0.25 mm inner diameter; 0.25 µm film thickness). Helium was used as carrier gas at a flow rate of 0.5 mL/min. The injection port was operated at 250 °C and the column temperature was kept at 70 °C for 5 min followed by 3 °C/min to 220 °C for 4 min and then at 10 °C/min to 280 °C for 7 min. The electron impact ion source was kept at 230 °C. The electron impact mass spectra were recorded at 70 keV.

10. Measurement of the Critical Micelle Concentration

Critical micelle concentration (CMC) was determined by measuring the ST of successive dilutions of BS. Various concentrations of BS ranging from 0 to 800 mg/L were prepared in phosphate buffer (5 mM, pH 7.0). Then, the ST of each concentration was measured to determine the CMC value, which corresponds to the lowest BS concentration that allows the best ST reduction.

10-1. Effect of pH, Temperature, and Salinity on BS Stability

The stability of BS was tested at varying salinity, pH, and temperature. To do this, the crude BS prepared at its CMC was exposed at different pH (pH 2.0-12.0), at assorted concentrations of NaCl (2-12%, w/v) and at various temperatures (4-120 °C). Then, after 2 h of incubation for pH and salinity tests, and after overnight incubation at temperatures of 4-40 °C, two hours for temperatures 60-80 °C, and 30 min for temperatures 90-100 °C [42], and after autoclaving (20 min) at 120 °C, the ST was measured as described earlier in Section 2.5.3.

10-2. Emulsification Activity of BS Against Hydrocarbons

The emulsification activity of the crude BS was tested against

different hydrocarbons (diesel oil, hexane, toluene, and kerosene). The emulsification index (EI) was measured as described earlier in Section 2.5.3.

10-3. Application of BS as a Bioremediation Agent

To assess the effectiveness of BS in removing hydrocarbons from contaminated soils, 250 g of forest soil was ground in a mortar, impregnated with 50 mL of used engine oil, mixed well and incubated at 37 °C for 24 h. Fractions of 10 g of contaminated soil were placed in 250-mL Erlenmeyer flasks and underwent different treatments, adding 20 mL of a supernatant culture of the strain 7SDS, 20 mL of distilled water (control), 20 mL of crude BS at its CMC, 20 mL of chemical surfactants prepared at their CMCs: SDS (0.2304%, w/v), Tween 20 (0.05%, w/v), Tween 80 (0.0016%, w/v), and Triton X-100 (0.0155%, w/v) [43]. After incubation at 30 °C under the agitation of 180 rpm for 24 h, samples were centrifuged at 6,000 rpm for 20 min at 4 °C. Residual masses of used engine oil obtained by hexane extraction (v/v) from the supernatant were determined gravimetrically.

10-4. Antimicrobial Activity of BS

The antimicrobial activity of BS was tested quantitatively based on suggestions given in the study of Fariq and Yasmin [44], with slight modification. An inoculum of each pathogenic bacterium (*Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, and *Bacillus cereus*) was prepared from a 24 h culture in nutrient agar, suspended in sterile physiological water to reach an O.D between (0.8-1.0) at 600 nm. Bacterial strains were inoculated in test tubes containing 5 mL of nutrient broth, and approximately 7 mg/mL of crude BS was added to each tube, which was then incubated at 37 °C in a rotary incubator shaker at 180 rpm for 24 h. The percentage growth inhibition of strains was estimated by measuring O.D at 600 nm and calculated by Eq. (3) [45].

$$\text{Growth inhibition (\%)} = \frac{(\text{OD pathogenic strain} - \text{OD pathogenic strain} + \text{BS})}{\text{OD pathogenic strain}} \times 100 \quad (3)$$

RESULTS AND DISCUSSION

1. Isolation of Actinomycetes

Algeria has noteworthy climatic diversity, which influences an extraordinary biodiversity, wealthy and assorted in actinomycetes, that can effect great chemodiversity of metabolites in this country [46]. Numerous actinomycetes have been isolated from different Algerian ecosystems, including hypersaline regions [47] and Saharan soils [48]. In this work, actinomycetes were isolated from desert soil collected from the "24 February" production division of SONATRACH in Ourgla-Algeria. Soil sampling was limited to the top few centimeters of soil. According to [23], actinomycete population is most important in the surface soil layer and decreases significantly with depth. These soil samples were chosen because hydrocarbon-contaminated sites such as oil spill soils are known to be distinguished sources for isolating BS producing microorganisms [41]. In addition, microbial communities found in arid ecosystems are being studied and characterized to obtain strains with unusual properties [49]. Sarkar and Suthindhiran [50] reported that actinomycetes from extreme environments produce a broad set of secondary metabolites [50]. Johnsen et al. [51] also reported that actinomycetes were the dominant group among decomposers in some contaminated sites [51].

A total of five morphologically distinct actinomycete strains were isolated from crude oil-contaminated soil. This number of isolates may be due to the lack of water in arid regions, which limits plant growth and, consequently, microbial growth. Physical (heat), chemical (SDS-YE, phenol) treatment and one enrichment technique ($CaCO_3$ treatment) were used for the selective isolation of actinomycetes. Subramani and Sipkema [24] reported that actinomycetes spores are resistant to various chemicals, including phenol, benzethonium chloride, SDS, and chlorhexidine gluconate [24]. These chemicals can kill or inhibit the growth of unwanted Gram-negative bacteria, endospore-forming bacilli, and *Pseudomonas*, thereby increasing the potential for selective isolation of actinomycetes [52]. However, Enrichment on HVA medium followed by isolation on ISP9 medium supplemented with 1% of crude oil as a sole source of carbon and energy was found to be a suitable method for selective isolation of hydrocarbonoclast actinomycetes. Hayakawa and Nonomura [53] developed an HVA medium that is widely used for isolating rare actinomycetes and *Streptomyces* from natural samples while restricting the growth of non-filamentous bacteria [53]. Subramani and Aalbersberg [54] reported that an HVA medium with humic acid as a sole source of carbon and nitrogen provided considerable advantages for rapid morphological identification of actinomycetes by promoting good sporulation [54]. In general, isolated colonies appeared smooth at first, but later, developed aerial mycelia. The substrate and aerial mycelia were white to black in color.

2. Screening of BS Producing Actinomycetes

The five isolated strains were initially screened for extracellular BS production with four different methods: parafilm-M test, emulsification index (EI), ODT, and ST measurement. Bacterial strains were grown on a BH medium containing CS filtrate as a sole carbon source. CS was chosen as a suitable substrate for the selection of BS-producing actinomycetes due to its economic advantage and composition. The skin of a chicken, including fat, accounts for 8-20% of its total weight [55].

Among the five isolates, only three, 7SDS, 2SDS, and 3P, yielded promising preliminary BS screening results (Table 2). The Parafilm M test was used as the first criterion for detecting BS production. All three strains gave positive results, showing droplet spreading compared to the negative control (uninoculated medium). As shown in Table 2, oil displacement diameters were 7.83±0.15 cm, 5.23±0.31 cm, and 3.86±0.22 cm for 7SDS, 2SDS, and 3P strains, respectively, relative to uninoculated medium (0.0±0 cm). Moreover, ST measurements revealed a reduction ranging from 30 to 48 mN/m with a maximum reduction observed in 7SDS strain with a value of 30.04±0.51 mN/m compared to the uninoculated medium with an ST of 70.86±0.33 mN/m. Further confirmation of the BS production ability of the selected isolates was done by measuring EI. The three strains, 7SDS, 2SDS, and 3P, had emulsification indexes of 59.66±0.44%, 30±0%, and 35.33±0.44%, respectively, compared to uninoculated medium (0.0±0%). According to these findings, the 7SDS strain, which showed the highest emulsi-

Table 2. Screening tests of potential BS-producing strains

Strains	ODT (cm)	EI (%)	ST (mN/m)	Parafilm M
7SDS	7.83±0.15	59.66±0.44	30.04±0.51	+
3P	3.86±0.22	35.33±0.44	45.38±0.14	+
2SDS	5.23±0.31	30±0	48.93±0.18	+
C^m	0.0±0	0.0±0	70.86±0.33	–

Note: C^m: control (uninoculated medium); '+' positive; '–' negative.

fying activity, positive parafilm M test, excellent oil spread test, and greater ST reduction, was selected for further study.

Once again, these results have confirmed the importance of using different methods when screening BS-producing microorganisms. Sarafin et al. [56] stated that due to the heterogeneous nature of BS, various screening tests need to be performed to identify BS-producing microorganisms [56]. Arifiyanto et al. [22] noted that the presence of emulsifying activity can be indicative of the presence of BS [22]. Moreover, Zambry et al. [57] demonstrated the oil spreading method's sensitivity in detecting extremely-low amounts of BS [57]. Nayak et al. [58] also asserted that a bacterial strain capable of lowering the ST of the fermentation broth to ≤35 mN/m can be considered a promising BS producer [58].

Despite the fact that actinomycetes are not known as conventional biosurfactant producers [59], Chakraborty et al. [19] reported that out of thirteen actinomycete isolates, four strains gave positive results in all screening methods used to detect BS production [19]. Ferradji et al. [60] also highlighted the potential of *Streptomyces* strains (AB1, AH4, and AM2) in BS production with high emulsification indexes of 75.83%, 78.71%, and 86.66%, respectively [60]. In addition, Khopade et al. [61] reported that a marine isolates *Streptomyces* sp. B3 was able to produce BS with an ST reduction of up to 29 mN/m [61]. Shavandi et al. [62] also reported the isolation of a potent BS-producing bacterium *Rhodococcus* sp. strain TA6 with its ability to reduce the ST of medium to 29.8 mN/m [62].

However, some research aimed to lower BS production costs by using cheap substrates. Garnida et al. [63] reported on the importance of recycling food waste, including restaurant waste, for the production of novel metabolites [63]. Banat et al. [64] also reported on the potential role of industrial and agro-industrial waste for BS production instead of synthetic media [64]. In this regard, residual frying oil, used engine oil, whey, and CS may be interesting alternative feedstocks for BS production due to their lower cost. These substrates have been used in several studies for such purposes. Just to name a few examples, *Limosilactobacillus fermentum* ACA-DC 0183 was able to produce BS on a medium supplemented with cheese whey after 36 hours of incubation with a maximum ST reduction of up to 34.9 mN/m [65]. *Bacillus licheniformis* LRK1 produced lipopeptide BS in engine oil-supplemented mineral medium after 32 hours of incubation with a 33% reduction in surface tension [58]. Hentati et al. [43] highlighted the potential of the newly halotolerant *Pseudomonas* sp. strain to use waste frying oil to produce glycolipid biosurfactant [43].

3. Identification of the Selected Strain

The morphology of the 7SDS isolate revealed that the cells were initially round, but later developed white-grey aerial mycelium with a light brown substrate mycelium. Light microscopy revealed that 7SDS cells were immobile and sporulating. Based on macroscopic and microscopic characteristics, isolate 7SDS was putatively assigned to the genus *Streptomyces*. 7SDS strain's 16S rRNA sequence was aligned, and the resulting contig was subjected to blast analysis in two public 16S archaea and bacteria databases: BlastN from NCBI and Bibi from PBIL (Univ. Cl. Bernard Lyon1-UCBL1). Phylogenetic analysis indicated that strain 7SDS shared up to 99.31%

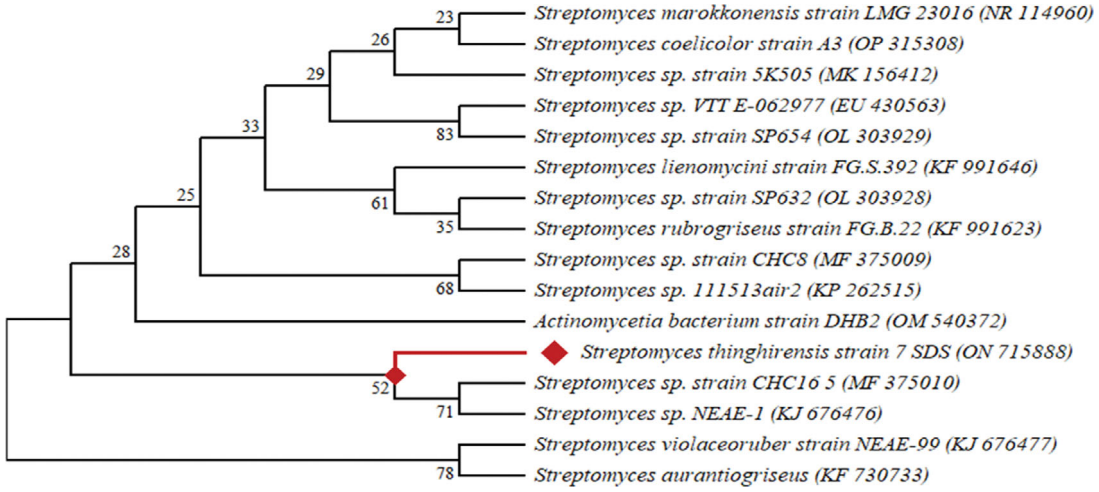

Fig. 1. Molecular phylogenetic tree of *Streptomyces thinghirensis* 7SDS and related *Streptomyces* sequences extracted from the NCBI database.

Table 3. Various biosurfactants produced by Streptomyces species

Strains	Isolation	BS	Application	Ref.
Streptomyces sp. SNJASM6	Seaweed	Glycolipid	Antimicrobial activity	[66]
Streptomyces griseoplanus NRRL-ISP5009	Soil	Mixture of carbohydrate, lipid, and protein	Antimicrobial activity	[67]
Streptomyces sp. B3	Marine sediment	Glycolipid	Antimicrobial activity	[61]
Streptomyces sp. ISP2-49E	Marine sediment	Rhamnolipid	NM	[68]
Streptomyces sp. S5	Egyptian desert	NM	NM	[69]
Streptomyces sp. MAB36	Marine sediment	Glycolipid	Antimicrobial activity	[70]
Streptomyces youssoufiensis SNSAA03	Marine sponge	Glycolipid	Antibiofilm	[15]

NM: not mentioned.

similarity to *Streptomyces thinghirensis* (Fig. 1). Multiple sequence alignments were performed using the ClustalW algorithm, and a phylogenetic tree was constructed by the neighbor-joining method using MEGA software (Version 11.0). 16S rRNA gene partial sequence of *Streptomyces thinghirensis* 7SDS was submitted to NCBI GenBank database under the accession number |ON715888|.

Streptomyces, the most prevalent group of actinomycetes, possesses numerous characteristics that make them excellent candidates for use in BS production. Table 3 provides some of *Streptomyces* BS-producing species.

4. Statistical Optimization of BS Production

4-1. Face-centered Central Composite Design-RSM Model Reliability Test

The cost of production in most bio-processes depends on 10-30% of raw materials [71]. In this regard, we sought to formulate a new economical fermentation medium based on low-cost lipid- or carbohydrate-rich wastes to maximize the BS production rate by the 7SDS strain. As stated, RSM is an excellent tool for studying process responses and finding the best correlations between process variables [72]. Face-centered CCD was applied in this study to investigating the effect of five tested variables (i.e., residual frying oil, used engine oil, whey, CS filtrate, and incubation time) at three levels (−1, 0, +1). The experimental and predicted response results are outlined in Table 4. The regression coefficient (R^2) is regarded as an overall measure of model quality as it enables the determination of the observed response's reliance on factors and their interactions [73]. The predicted data are in good agreement with the experimental data with a coefficient of regression R^2 and adjusted R^2 of 0.96 and 0.82, respectively, which means that the model can explain 96% of the variability of the response [74]. Experimental data were examined using multiple regressions to obtain the best-responding empirical model via the following quadratic polynomial equations:

$$ST\ (mN/m)=35.74+0*A+3.43*B+1.719*C+1.719*D+5.35*E+4.41*AB+6.13*AC+6.13*AD+2.50*AE+3.55*BC+3.55*BD-0.93*BE+1.73*CD-1.03*CE+2.59*DE+3.05*A^2-3.82*B^2+1.33*C^2+1.34*D^2+4.97*E^2$$

where A: residual frying oil; B: CS filtrate; C: whey; D: used engine oil, and E: incubation time.

4-2. Analysis of Variance

Analysis of variance (ANOVA) is a powerful mathematical tool commonly used to further test the validity of an empirical model [16]. ANOVA (Table 5) was performed to determine the significant effects of factors and interactions influencing the BS production. As shown in Table 5, the model's P-value (0.02) is relatively low (<0.05), indicating its significance, which is validated by the Fisher's value (F-value=6.99), which is higher than the critical F-value (F_{critic}=2.12) for a given number of degrees of freedom for the model at the significance level of 95% [75,76]. The Fisher's

Table 4. Face-centered central composite design matrix (Randomized)

Run	Variables					Response 1: ST (mN/m)	
	A	B	C	D	E	Measured	Predicted
1	+1	+1	−1	−1	+1	34.84	34.77
2	0	0	0	0	0	33.00	35.65
3	+1	+1	+1	−1	−1	45.54	45.47
4	+1	−1	+1	+1	−1	34.84	34.77
5	0	0	0	0	0	35.03	35.65
6	−1	+1	+1	−1	+1	38.28	38.21
7	−1	−1	+1	+1	+1	34.84	34.77
8	+1	−1	−1	+1	+1	45.54	45.47
9	−1	0	0	0	0	38.28	38.73
10	0	0	0	−1	0	34.84	35.29
11	0	0	0	0	0	38.28	35.65
12	+1	−1	+1	−1	+1	38.28	38.21
13	0	0	−1	0	0	34.84	35.29
14	+1	+1	−1	+1	−1	38.28	38.21
15	0	−1	0	0	0	27.96	28.41
16	+1	0	0	0	0	38.28	38.73
17	−1	+1	+1	+1	−1	38.28	38.21
18	0	0	+1	0	0	38.28	38.73
19	0	0	0	0	+1	45.54	46.00
20	0	+1	0	0	0	34.84	35.29
21	−1	−1	−1	−1	−1	59.24	59.09
22	0	0	0	0	−1	34.84	35.29
23	0	0	0	0	0	38.28	35.65
24	−1	+1	−1	+1	+1	45.54	45.47
25	0	0	0	+1	0	38.28	38.73
26	0	0	0	0	0	38.28	35.65

Table 5. ANOVA for ST response

Source	SS	DF	MS	F-value	P-value
Model	846.06	20	42.30	6.99	0.0201
A: residual frying oil	0.0000	1	0.0000	0.0000	1.0000
B: CS filtrate	23.67	1	23.67	3.91	0.1048
C: whey	5.92	1	5.92	0.9782	0.3681
D: used engine oil	5.92	1	5.92	0.9782	0.3681
E: incubation time	57.26	1	57.26	9.47	0.0276
Interactions					
AB	25.51	1	25.51	4.22	0.0952
AC	49.27	1	49.27	8.15	0.0357
AD	49.27	1	49.27	8.15	0.0357
AE	8.21	1	8.21	1.36	0.2966
BC	16.54	1	16.54	2.74	0.1591
BD	16.54	1	16.54	2.74	0.1591
BE	1.15	1	1.15	0.1897	0.6813
CD	3.96	1	3.96	0.6548	0.4552
CE	1.39	1	1.39	0.2304	0.6515
DE	8.84	1	8.84	1.46	0.2806
Quadratic					
A^2	22.92	1	22.92	3.79	0.1091
B^2	35.72	1	35.72	5.91	0.0594
C^2	4.39	1	4.39	0.7265	0.4329
D^2	4.39	1	4.39	0.7265	0.4329
E^2	60.49	1	60.49	10.00	0.0250
Residual	30.24	5	6.05		
Lack of fit	6.21	1	6.21	1.03	0.3666
Pure error	24.03	4	6.01		
Total	876.30	25			

Note: SS: sum of squares, MS: mean square, DF: degree of freedom.

value is defined as the ratio of the model's mean square to the residual error [77], and it properly indicates how well the factors explain the data's variation around its mean [16]. P-values were used to confirm the significance of each term; P-values less than 0.05 indicate significant regression coefficients [78]. Thus, among the model terms, linear and quadratic terms of incubation time with p-values of 0.027 and 0.025, respectively, as well as interactive terms between residual frying oil*whey and residual frying oil*used engine oil with a p-value of 0.035 appear to have a more significant effect than the other terms on BS production, with at least a 95% probability. In addition, the error due to the lack of fit is found to be non-significant ($F_{0(0.05;\ 1\text{-}4)}$=1.03≤F_{critic}=7.71) indicating that the generated mathematical model correlated very well with the observed responses versus the predicted ones.

4-3. Response Surfaces

To better understand the effects of the studied variables on the BS production by the 7SDS strain, the Design Expert software was used to analyze our data and generate response surface graphs, which are very useful to localize the optimum zone. These graphs aid in comprehending results, forecasting optimum conditions, and recognizing the interaction effects of variables on system response [79]. Response surfaces were charted by plotting the interaction of two variables while keeping the other three independent variables at their fixed level (i.e., level 0). Thereby, the interactions among the variables may seem like an antagonistic or synergistic effect [80]. Referring to Fig. 2(A), a decrease in whey concentration and an increase in residual frying oil concentration resulted in a reduction of ST, which led to maximum BS production. Similarly, Fig. 2(B) shows that the lowest ST was obtained when residual frying oil was increased from the middle to the high level while used engine oil remained at its low level. The results showed that the lowest concentration of whey and used engine oil privileged the BS production. Note that high substrate concentrations do not always result in increased BS production [81]. This can be linked to the fact that substrates present in high concentrations are directed towards the production of cellular biomass [81]. The RSM model predicted the optimal values of the five variables to be: 2.44% (v/v) residual frying oil, 0.35% (v/v) used engine oil, 0.83% (v/v) whey, 0.39% (v/v) CS filtrate, and a 219.36 h incubation time.

5. Extraction and Purification of BS

In the current study, BS was produced during batch culture on an optimized medium using residual frying oil, used engine oil, whey, and CS filtrate as carbon sources according to the RSM prediction. Acid precipitation followed by solvent extraction was used

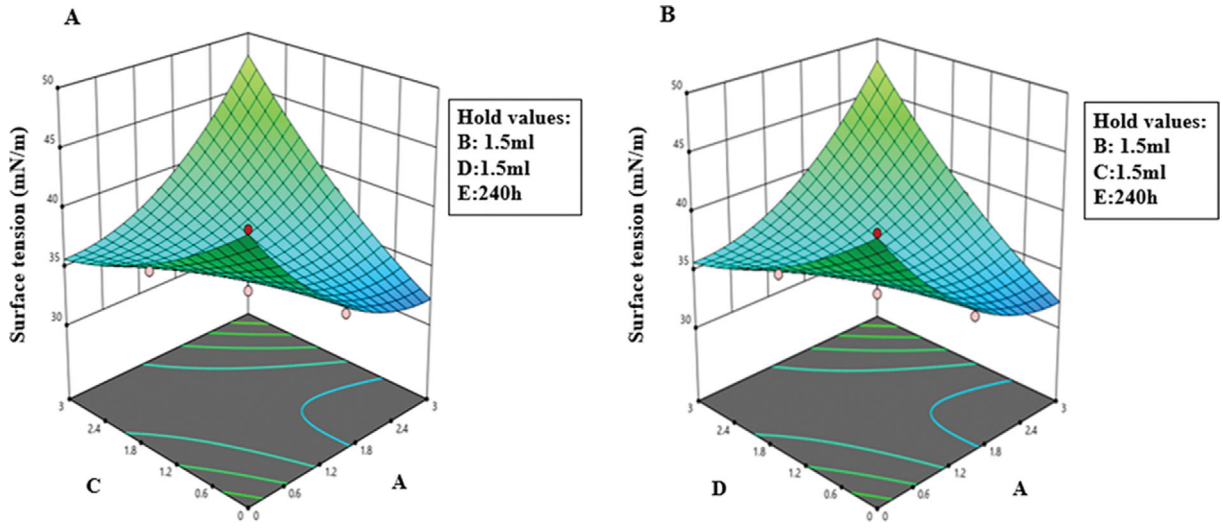

Fig. 2. 3D response surfaces graphs illustrating the interaction between: A: residual frying oil and whey, and B: residual frying oil and used engine oil.

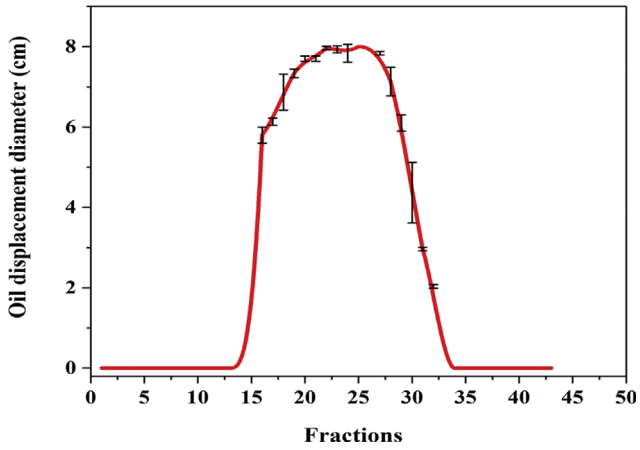

Fig. 3. Chromatographic profile of *Streptomyces thinghirensis* 7SDS BS on the silica gel column.

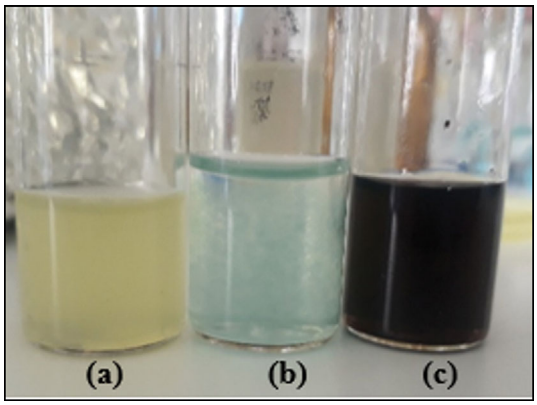

Fig. 4. Biochemical characterization of BS: (a) phosphate test, (b) biuret test, and (c) phenol-sulfuric acid test.

to recover the produced BS. Many researchers have already used this technique to extract biosurfactants from culture broth due to its effectiveness [82] and the economic advantage of solvent recovery [83]. The yield of the crude BS obtained from the 7SDS strain was 8.79 g/L. The same result was reported by Zambry et al. [57] where *Streptomyces* sp. R1 strain was found capable of yielding 7.19 g/L of lipopeptide in starch casein broth containing 3% olive oil[57]. While, in another study, Bhuyan-Pawar et al. [84] reported that strain V2 of *Streptomyces* sp. produced around 56.7 mg/L of proteoglycan BS using molasses as substrate [84]. Furthermore, Kumari et al. [85] reported that *Rhodococcus* sp. NJ2 strain produced only 10 μg/mL of glycolipid in a nutrient medium [85].

However, fixed bed chromatography using silica gel 60 columns was utilized to purify the crude BS. Among the 40 collected fractions, only four (26,27,28,29) were observed as a single spot on the TLC plate, and only one fraction (26) presented the highest surface activity with the oil spreading assay (Fig. 3).

6. Biochemical and Compositional Characterization of the Produced BS

6-1. Biochemical Characterization

Characterization of biosurfactants is a crucial part of this study, as it enables detailed structural clarification to discover new chemical structures and determine potential applications [83]. In this context, the produced BS was biochemically characterized by the biuret, phenol-sulfuric acid, and phosphate tests (Fig. 4). In the biuret test, no purple coloration (due to the reaction of copper sulfate with peptide bonds in an alkaline medium) appeared, showing the absence of amino acids or proteins in the tested BS. Then, the phenol-sulfuric acid test gave a negative result, implying the absence of sugars in the produced BS. Finally, in the phosphate test, there was a noticeable appearance of yellow color, which occurred due to the formation of a yellow precipitated layer of ammonium phosphomolybdate, indicating the presence of phosphate in the crude BS. These findings are similar to those reported for the phospholipid BS produced by *Klebsiella pneumoniae* strain IVN51 [86].

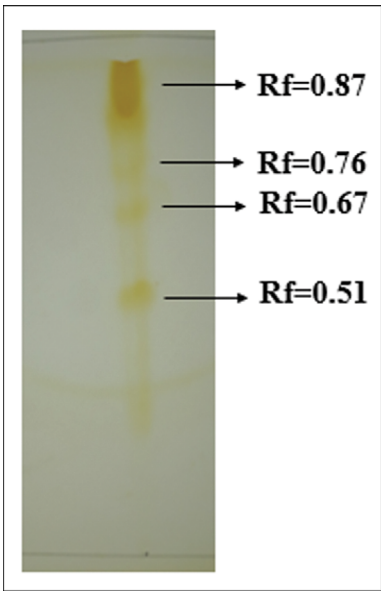

Fig. 5. TLC chromatogram of phospholipid BS from *Streptomyces thinghirensis* 7SDS.

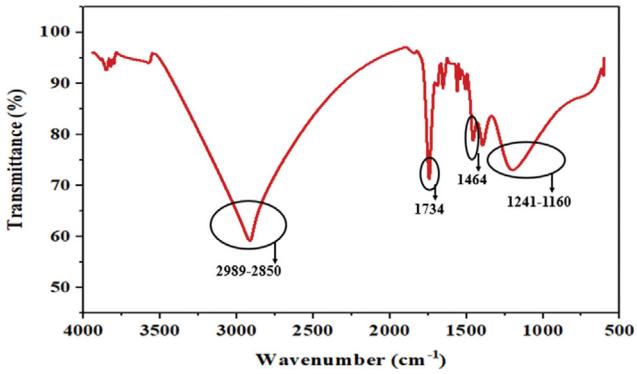

Fig. 6. FTIR spectrum of the 7SDS strain BS.

6-2. Thin Layer Chromatography (TLC)

This technique is the most effective and broadly used approach for the chemical analysis of biosurfactants [87]. The TLC analysis of the crude BS was developed using toluene : methanol (70 : 30) as the best mobile phase. Staining the TLC plate with ninhydrin revealed no red spot, which indicated the absence of protein or amino acids in 7SDS BS. However, iodine vapors revealed four yellow spots with different retention factor values of: 0.51, 0.67, 0.76, and 0.87, indicating the presence of lipids in the crude BS (Fig. 5).

6-3. Fourier-transform Infrared Spectroscopy

Fourier-transform infrared (FTIR) spectroscopy is an essential tool when studying different forms of biosurfactants [88]. In this study, the functional groups present in crude BS were examined using FTIR spectroscopy. The FTIR spectrum (Fig. 6) showed absorption peaks at 2,854.69 cm^{-1}, 2,923.17 cm^{-1}, 2,989.17 cm^{-1}, and 1,464.96 cm^{-1} corresponding to symmetric CH_2, antisymmetric CH_2, antisymmetric CH_3 stretching, and CH_2 scissoring, respectively. The carbonyl group (C=O) was observed from the bands at 1,734.99 cm^{-1}. This group is found between the phospholipid molecule's hydrophobic tails and hydrophilic head group [89]. The peak at 1,161.16 cm^{-1} occurred due to C-O stretching. The absorption peaks located at 1,241.21 cm^{-1} were attributed to PO_2 stretching. The FTIR profile of the produced BS was found to be similar to those of phospholipids previously reported by [90,91]. Therefore, by observing the results of biochemical tests, TLC, and FTIR described above, it can be concluded that the BS produced by the 7SDS strain is phospholipid. To date, this is the first report on the production of phospholipid BS from *Streptomyces thinghirensis*.

Despite the fact that there is a surprising lack of information on the production of phospholipid biosurfactants by actinomycetes, Kretschmer et al. [92] reported that the phosphatidylethanolamine produced by *Rhodococcus erythropolis* was strong enough to reduce the interfacial tension between water and hexadecane above 1 mN/m. In addition, Yagüe et al. [93] reported that *Corynebacterium insidiosum* isolated from clinical samples was capable of producing phospholipid BS.

7. GC-MS Analysis

GC-MS analysis is another reliable method for determining the side chain composition of fatty acids present in biosurfactants [94]. The fatty acid content of the BS produced by *Streptomyces thinghirensis* 7SDS was analyzed after the GC-MS characterization (Table 6 and Fig. 7). Major peaks obtained at the retention times of 33.95 min, 39.17 min, 39.34 min, and 40.07 min revealed ester derivatives of hexadecanoic acid, 9,12-octadecadienoic acid, 12-octadecenoic acid, and isooctadecanoic acid, respectively. According to previous studies, palmitic acid and oleic acid were observed in the GC-MS chromatogram of the phospholipid BS produced by *Klebsiella pneumoniae* strain IVN5 [86]. The GC-MS analysis of BS produced by *Streptomyces* sp. SNJASM6 revealed the following major peaks: octadecanoic acid, pentadecanoic methyl ester, hexadecanoic acid, oleic acid, and pentadecane [66]. Seghal Kiran et al. [95] reported that the hydrophobic moiety of the lipopeptide BS from *Brevibacterium aureum* MSA13 was an octadecanoic acid methyl ester [95]. The newly characterized phospholipide BS from the 7SDS strain was dubbed "Streptingfactin" since it was first pro-

Table 6. GC-MS analysis of the fatty acid composition of 7SDS strain BS

Compounds	Retention time (min)	Molecular formula	Molar mass (g/mol)
Hexadecanoic acid	33.95	$C_{16}H_{32}O_2$	256.42
9,12-Octadecadienoic acid	39.17	$C_{18}H_{32}O_2$	280.4
12-Octadecenoic acid	39.34	$C_{18}H_{34}O_2$	282.5
Isooctadecanoic acid	40.07	$C_{18}H_{36}O_2$	284.5

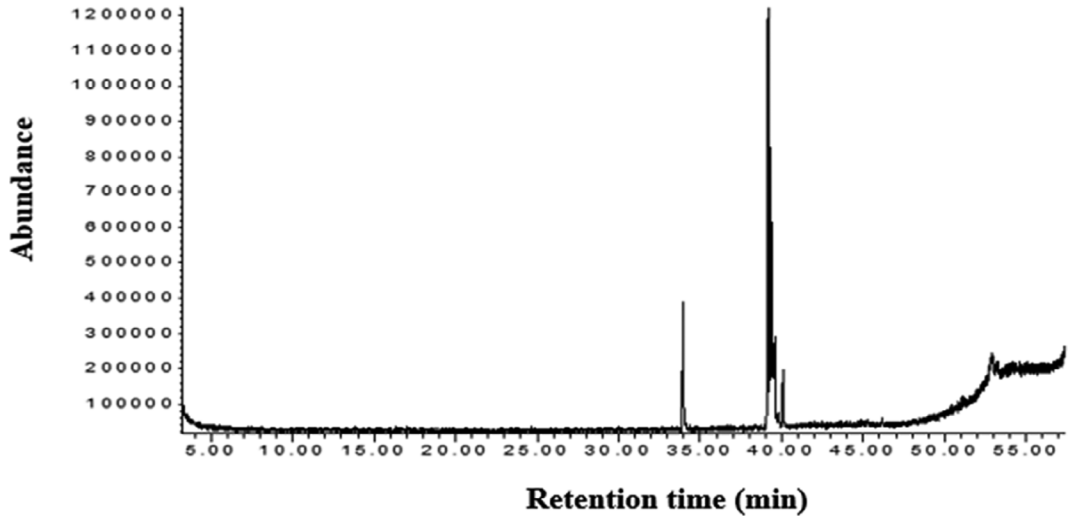

Fig. 7. GC-MS analysis of the partially purified BS from *Streptomyces thinghirensis* 7SDS.

duced by *Streptomyces thinghirensis* strain 7SDS.

8. Surface Tension and CMC Determination

A key parameter in assessing surfactant activity is the critical micelle concentration (CMC), which is the concentration of surfactant; it allows the micelles to form spontaneously without any change in the surface activity. A low CMC and a reduction in ST of solutions are regarded as important properties of a potent surfactant [96]. In this sense, the CMC value of the produced BS was evaluated by measuring the ST of various BS concentrations. The plot of ST against crude BS concentration (Fig. 8) showed a rapid decrease in ST as the concentration of BS was increased with a minimum ST reading of 27.96 mN/m obtained at 350 mg/L concentration. Even after increasing the BS concentration beyond 350 mg/L, no decrease was observed in ST. The ability of a BS to reduce the ST of a solution to less than 40 mN/m was deemed to be a crucial property of a promoting tensioactive agent [97]. The CMC value of certain chemical surfactants such as tetradecyl trimethyl ammonium bromide and SDS (CMC ranging between

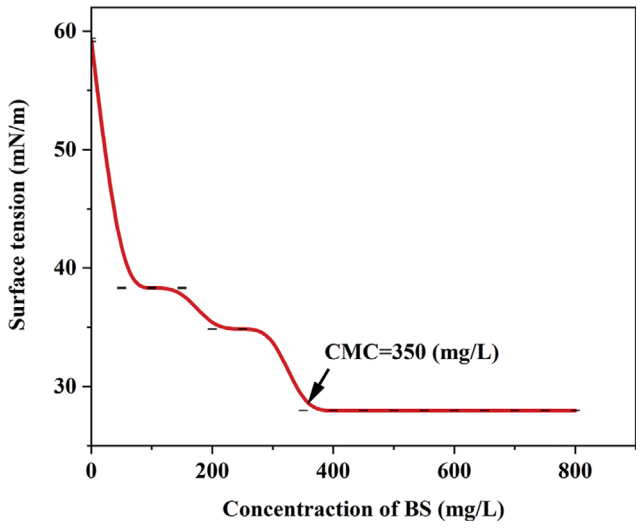

Fig. 8. CMC of BS from *Streptomyces thinghirensis* 7SDS.

1,000-2,300 mg/L) was found to be superior to CMC's 7SDS [98]. The CMC value indicated that the produced BS was relatively consistent with other microbial surfactants already presented in the literature. In this context, Javee et al. [66] reported that strain SNJASM6 of *Streptomyces* sp. showed its capacity to decrease ST to 32.8 mN/m at a CMC value of 36 mg/L. Elkhawaga [67] also reported that the BS produced by *Streptomyces griseoplanus* NRRL-ISP5009 was able to reduce ST of water from 68 to 40 Dynes cm^{-1} with a CMC value of 110 mg/L [67].

9. Stability of the Obtained BS

The stability of bioactive molecules is critical and serves as a deciding factor for their use as antimicrobial, bioemulsifier, or therapeutic agents over wide salinity, pH, and temperature ranges [99]. In this study, the stability of the produced BS in the presence of different temperatures, wide pH ranges, and different NaCl concentrations was investigated in terms of ST (Fig. 9). Changing the temperature of BS from 4 to 120 °C did not significantly affect its performance. ST of the BS solution at the highest incubation temperature was observed to be 27.96±0.03 mN/m. The stability of BS against different pH (2.0-12.0) showed that the ST of the phospholipid BS (27.96±0.03 mN/m) remained relatively stable over the wide pH range tested. In addition, despite varying NaCl concentrations from 2 to 12% (w/v), the ST of BS solution remained constant at around 27.96±0.03 mN/m. These findings are similar to those in the literature by Khopade et al. [61], who indicated that the ST of a glycolipid BS produced by *Streptomyces* sp. B3 remained stable after heating to 100 °C. Santos et al. [100] stated that the BS from *Streptomyces* sp. DPUA 1559 had high salt tolerance as well as thermal and pH stability. Moreover, Hayder et al. [101] stated that the bioemulsifier from *Streptomyces* sp. SS 20 was potent over a wide temperature (30-100 °C) and pH (3-7) [101].

The stability of biosurfactants under various environmental conditions, including temperature, pH, and salinity, shows their broad applicability [102]. The high thermal stability of biosurfactants is a hallmark of their use in the food, pharmaceutical, environmental protection, and cosmetic industries [103]. Likewise, biosurfactant stability over a range of salt concentrations demonstrates their appli-

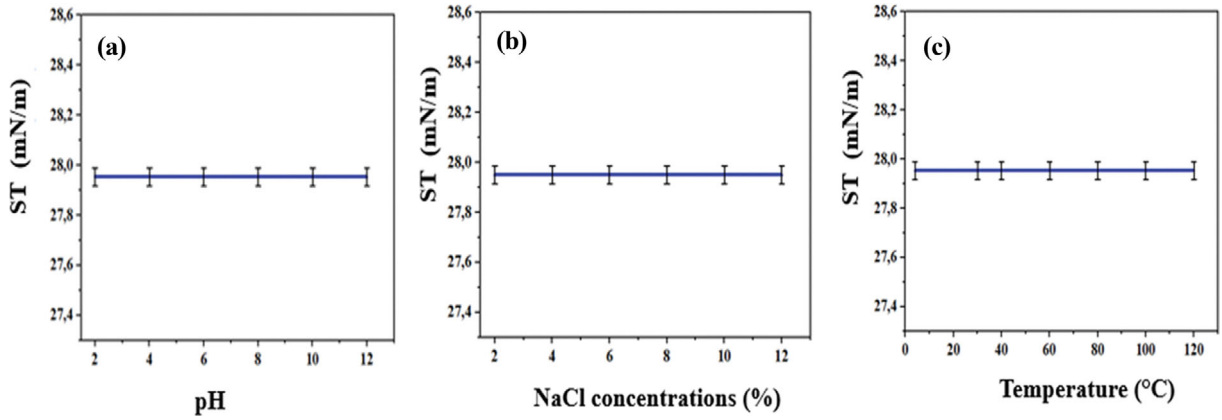

Fig. 9. Stability of 7SDS strain BS towards: (a) pH, (b) salinity, and (c) temperature.

cability to the removal of oil spills in the marine environment [104]. Similar to temperature and salinity, microbial biosurfactants show promising pH stability under alkaline conditions, offering opportunities for use in the detergent industry [105].

10. Emulsification Activity of the Produced BS

A biosurfactant's emulsifying activity is its ability to maintain a hydrocarbon or oil emulsion in water [106]. The ability of 7SDS biosurfactant to emulsify hydrocarbons was tested against diesel oil, kerosene, toluene, and hexane (Fig. 10). The maximum emulsification was observed with diesel oil (49.33±1.11%), followed by hexane (39.33±1.11%), whereas kerosene (0±0%) and toluene (0±0%) were not emulsified. The results indicated that the produced BS was able to effectively emulsify long and short chain hydrocarbons. Goswami and Deka [104] emphasized that the degree of emulsification index is influenced by the chain length of the interacting hydrocarbon and that the emulsification index increases as the hydrocarbon chain lengthens. Our finding is similar to that of Shavandi et al. [62] who found that the produced BS by *Rhodococcus* sp. strain TA6 exhibited specific emulsification properties for long-chain hydrocarbons. Furthermore, Santos et al. [107] reported that the isolated BS from *Streptomyces* sp. DPUA1566 was able to form a stable emulsion using several oils such as *Cariocar brasiliense* oil, motor oil, and soybean oil.

11. BS as a Bioremediation Agent

Most recent research has centered on the environmental uses of biosurfactants due to their eco-friendly, enhanced physicochemical properties and various structures [108]. In this context, an experiment was carried out using the crude BS, 7SDS strain's cell-free supernatant, and chemical surfactants (Tween 20, SDS, Triton X-100, and Tween 80) to confirm the removal of used engine oil contaminant from soil samples (Fig. 11). Results showed that the chemical surfactants, SDS, Tween 20, Tween 80, and Triton X-100, increased the solubilization of used engine oil by 19.7%, 18.93%, 30.76%, and 22.43%, respectively, compared to the control (water). On the other hand, the 7SDS supernatant and BS improved used engine oil solubility by 39.36% and 80.43%, respectively. Moreover, it can be concluded that crude BS was more efficient in remobilizing hydrocarbons than synthetic surfactants, which were widely characterized as dangerous to ecosystem functioning because of their toxicity and low biodegradability [36]. Biosurfactants can

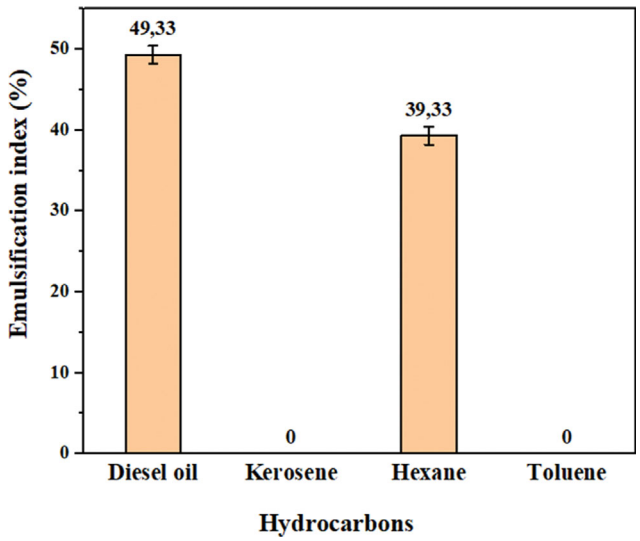

Fig. 10. The 7SDS strain biosurfactant's emulsification activity against various hydrocarbons.

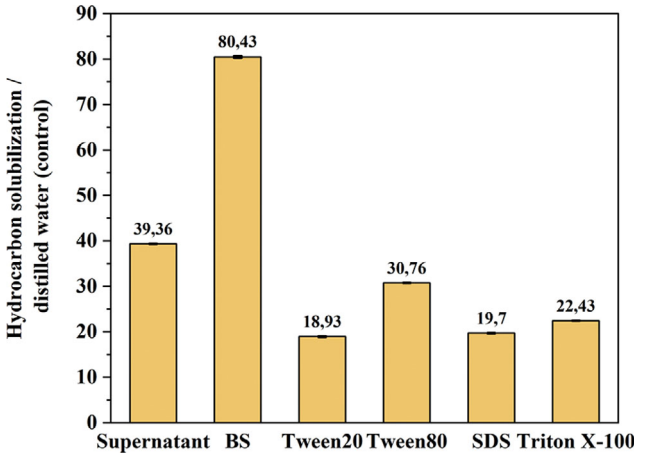

Fig. 11. 7SDS biosurfactant's effect versus synthetic surfactants on hydrocarbons removal from used engine oil-contaminated soil.

help the degradation of organic pollutants, such as hydrocarbons, by mobilizing, solubilizing, and emulsifying them [109]. Mobilization occurs at a concentration lower than the BS's CMC, resulting in a decrease in ST; solubilization with micelle formation occurs at a concentration greater than the BS's CMC, resulting in an increase of pollutant's solubility; and emulsification produces an emulsion in which an oil droplet is suspended in a liquid [109]. These mechanisms promote the bioremediation of the environment's pollutants. In this respect, Ivshina et al. [110] showed that biosurfactants produced by *Rhodococcus ruber* strain IEGM 231 demonstrated a high polyaromatic hydrocarbons removal compared to synthetic surfactants. In addition, Peng et al. [111] stated that biosurfactants produced by *Rhodococcus erythropolis* strain 3C-9 could be used to clean up oil spills. In addition, Kiran et al. [112] found that the bioremediation process in marine contaminated water is more effective using biosurfactant produced by the marine actinobacterium *Nocardiopsis lucentensis* MSA04 than chemical surfactants.

12. Antibacterial Activity of 7SDS Strain BS

The emergence of microbial antibiotic resistance is an urgent problem due to the overuse of conventional antibiotics. Besides the dire situation, the number of new antimicrobial compounds approved for use has been declining in recent years [113]. In this regard, BS (approximately 7 mg/mL) derived from *Streptomyces thinghirensis* strain 7SDS exhibited excellent antibacterial activity, which was measured by the growth inhibition percentage of the pathogenic strains (Fig. 12). The partially-purified BS showed the highest growth inhibition of 69±0.13% against *B. cereus*. The *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were susceptible to the BS with percentage growth inhibition of 60±0.12% and 54.34±0.33%, respectively, whereas *P. aeruginosa* ATCC 27853 was less sensitive with a percentage growth inhibition of 19.36±0.44%.

Many biosurfactants have been described in numerous studies as bioactive compounds with antifungal, antibacterial, and antiviral activities. *Streptomyces* sp. MAB36-derived glycolipid BS has shown the highest effectiveness against many pathogenic microbes,

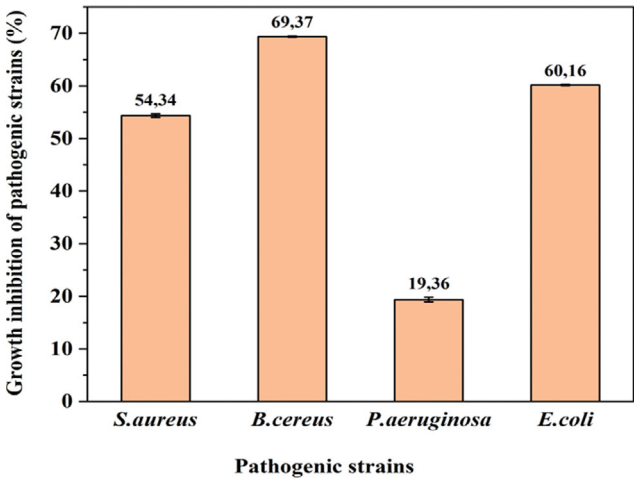

Fig. 12. Antimicrobial activity of the produced BS by *Streptomyces thinghirensis* 7SDS.

including fungi, and Gram-positive and Gram-negative bacteria [70]. Bioactive surfactant from *Streptomyces* sp. SNJASM6 exhibited high antimicrobial activity against bacterial pathogens (*P. aeruginosa*, *E. coli*, *K. pneumoniae* and *B. cereus*) and fungi (*Candida albicans*) [66]. The purified lipopeptide from *Nocardiopsis alba* MSA10 showed good antibacterial activity against *Enterococcus faecalis* and *B. subtilis*, but no activity was observed for *P. aeruginosa*, hemolytic *Streptococcus*, and *E. coli* [114]. The antimicrobial effectiveness of biosurfactants is determined by their concentration, structure, and class of bacteria tested [115]. The antimicrobial potency of biosurfactants can be attributed to their adhesion properties to the microbial cell surface (Fig. 13). Incorporation of biosurfactants into cell membranes leads to membrane expansion and significant ultrastructural degradation of cells [67], resulting in a disruption of microbes' nutrition cycle due to the defeat of membrane proteins, hence initiating cellular lysis.

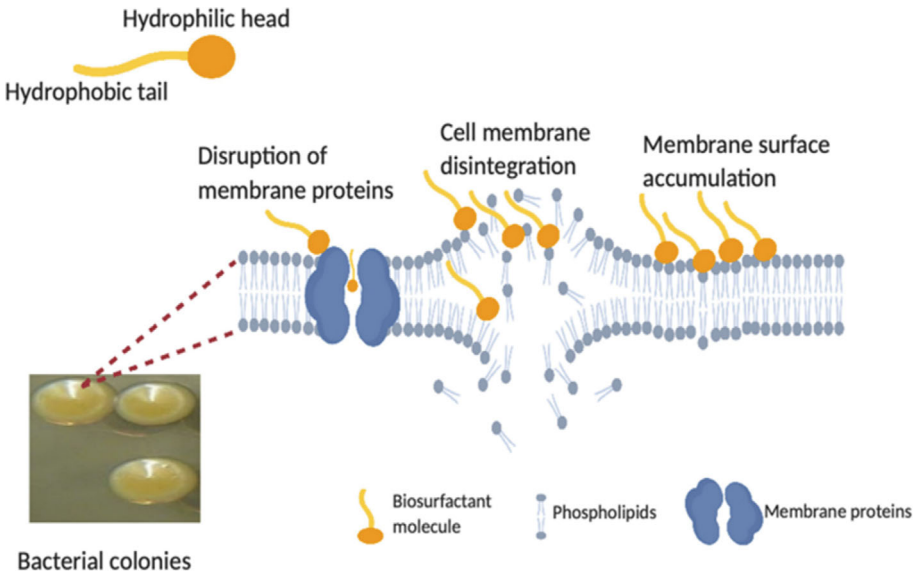

Fig. 13. Possible interactions between BS molecules and bacterial cells [113].

CONCLUSION

Nowadays, the development of low-cost and eco-friendly methods to produce bioactive molecules for biotechnological applications has become very important. In this regards, the present study is the first to report BS production by a novel strain 7SDS of the *Streptomyces thinghirensis* species. This strain demonstrates a noteworthy capacity to produce BS, which is reinforced by statistical optimization with RSM-CCD using an optimized medium enriched with cheap waste substrates. 7SDS's phospholipid BS, named "Streptingfactin", displayed remarkable ST reduction with excellent stability under critical conditions, including high concentrations of NaCl, temperatures, and pH. Interestingly, the produced phospholipid BS by *Streptomyces thinghirensis* 7SDS showed its effectiveness as an antimicrobial and bioremediation agent. These encouraging findings suggest that Streptingfactin from *Streptomyces thinghirensis* 7SDS could be used to synthesize surfactants in a wide variety of biotechnological applications.

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DECLARATION OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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