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Isolation and characterization of novel hydrocarbon-degrading bacteria from oil polluted soil near Nacharam, Hyderabad, India

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Petroleum is a vital and strategic energy resource for boosting a country's GDP. Despite its high economic value, it is considered a primary factor in environmental deterioration. Bioremediation strategies employ indigenous microbial strains to propose an economical and sustainable alternative to conventional remediation practices. The current study investigates the isolation, identification, and characterization of five novel biosurfactant-producing and petroleum hydrocarbon-degrading bacterial species: *Rhodococcus indonesiensis* strain SARSHI1, *Pseudomonas aeruginosa* strain SARSHI2, *Pseudomonas argentinensis* strain SARSHI3, *Acinetobacter baumannii* strain SARSHI4, *and Rhodococcus qingshengii* strain SARSHI5. Molecular identification was determined via 16S rRNA sequencing, and their taxonomic identities were validated through biochemical assessments. Their partial sequences were deposited in NCBI with accession numbers: 'PV034287', 'OP597529', 'OP584476', 'OQ711779', and 'OQ711775' respectively. Amongst them, *R. indonesiensis* exhibited the highest biosurfactant and hydrocarbon-degrading potential with a critical micelle concentration of 70 mg/L, reduced surface tension of 27 mN/m, an emulsification index (E_{24}) of 85.34%, and hydrocarbon-degrading potency of up to 90%. Gravimetric analysis revealed up to 84% hydrocarbon degradation when supplemented with glycerol, and GC-MS analysis confirmed the selective degradation of n-alkanes (C18–C24). Structural studies employing NMR established the biosurfactant as a lipopeptide. Statistical optimization utilizing RSM - Box-Behnken design obtained the optimized conditions for enhanced biosurfactant and biodegradation activity. Microcosm studies further assessed SARSHI1's bioremediation potential under field-simulated treatments, achieving up to 95% degradation rates under the combined treatment of Bioaugmentation + Biostimulation + Biosurfactant (BA + BS + B), signifying the amplified bioavailability of hydrocarbons. Phytotoxicity tests confirmed the environmental impact of the bacterial strain. The results govern a robust framework for advancing microbial applications in environmental remediation and further support *R. indonesiensis* SARSHI1 for large-scale biotechnological paradigms.

Keywords Petroleum hydrocarbon-degrading bacteria, Bioremediation, Biosurfactant production, Biostimulation, 16S rRNA sequencing, Box-Behnken experimental design

Petroleum, a heterogeneous mixture of hydrocarbons, includes aliphatic (n-alkanes), alicyclic, and aromatic polycyclic hydrocarbons (PAH)[1]. Petroleum and its by-products play a paramount role as a strategic energy resource. It governs a country's GDP and economic development. Petroleum constitutes different compositions and physical properties depending on its reservoir's origin[2–5]. Various hydrocarbons work as raw materials in myriad industries: petrochemicals, refineries, pharmaceuticals, paints, etc. However, Petroleum Hydrocarbon Pollution (PHP) is mainly caused due to accidental spillage and untreated discharges from these industries into water bodies and the atmosphere[6,7]. The primary reason for PHP is the incomplete combustion of a variety of volatile and organic molecules that produce lethal toxins and are arduous to break down. It prevents the growth of flora and fauna, causes genetic mutations, and augments chemical oxygen demand, thus imposing very high health risks to humankind (Fig. 1)[8].

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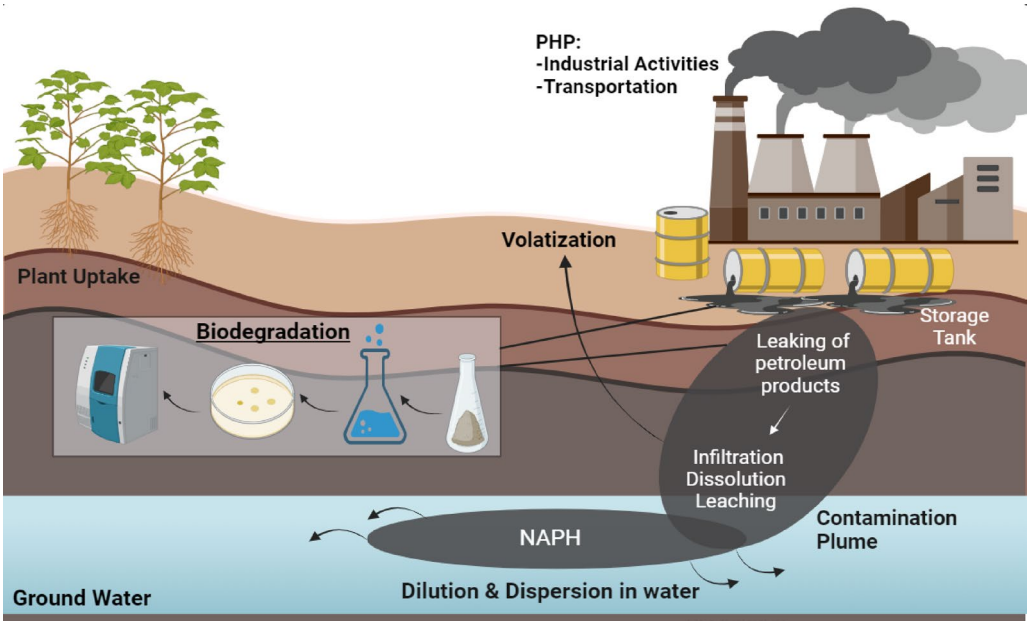


Fig. 1. Schematic representation of petroleum hydrocarbon pollution.

Driven by technological advancements, many physical remediation methods (adsorption and membrane filtration, etc.), and chemical remediation methods (wet oxidation, ozone catalytic oxidation, etc.), are highly implicated. Despite these methods being a quick solution for treating oily effluents, they are expensive and can easily produce secondary contamination[9,10]. Biological methods, such as bioremediation, biostimulation, and bioaugmentation, have become increasingly prevalent for scouring up petroleum hydrocarbons. Biological methods are efficient, economical, and favourable to the environment[11,12]. Recent studies have demonstrated that numerous indigenous microorganisms from different bacterial genera govern oil-degrading potential, such as *Pseudomonas*, *Bacillus*, *Sphingobacterium*, *Streptomyces*, *Acinetobacter*, *Rhodococcus*, *Burkholderia*, etc[13,14]. These bacterial species exhibit the potential to utilize crude oil as the sole carbon and energy source. Additionally, they allow oil-rich toxins to be converted to non-toxic metabolites by altering their key environmental parameters in the biogeochemical cycles[1].

The efficiency of biodegradation varies among different microbial genera, generating mitigated efficiency rates due to the complex composition of hydrocarbons. Different microorganisms under distinct environmental conditions degrade different components of petroleum[15]. The enzymatic apparatus allows sequential metabolism under both aerobic and anaerobic conditions. One of the proposed and most studied alkane degradation pathways is by *Pseudomonas putida* Gpo1, encoded by the OCT plasmid[2,16,17]. It exhibits various gene products, such as alk, nah, etc. They are present in different locations and perform diverse functions. They allow the conversion of the alkane to alcohol by membrane monooxygenases, rubredoxins, and rubredoxin reductases[18,19].

Despite the augmented biodegradation efficiency of the bacterial strains, hydrophobicity is regarded as a significant constraint for degrading the hydrocarbons. The degradation of hydrocarbons is facilitated by a class of amphiphilic surface-active substances produced by bacteria that break down petroleum.[10]. The biological compounds displaying surfactant activities are divided into higher and lower-molecular-weight compounds, determined as emulsifiers and biosurfactants, respectively. The biosurfactants exhibit numerous advantages over the chemically tailored surfactants due to their high biodegrading efficiency, lower critical micelle concentrations (CMC), augmented stability, and ability to procure renewable raw materials. As a result, petroleum-degrading bacteria that may yield biosurfactants are commonly employed to accelerate the decomposition of crude oil or petroleum by-products. Additionally, biosurfactants also mitigate the toxicity levels of crude oil and may facilitate the growth of additional bacterial strains that break down hydrocarbons, hastening the breakdown of petroleum altogether[20].

In the current era of modern civilization and anthropogenic stirs, petroleum has encompassed a different outlook. It has been proven to be a bane rather than a boon due to its unquantifiable environmental deterioration properties. Since bacteria demonstrate the potential to break down petroleum hydrocarbons as an energy source, their usage in bioremediation studies is an inevitable outcome. Although biodegradation has been marked as a feasible approach to remediate polluted areas and an augmented amount of research has already been carried out, primarily focusing on this application, there is still scope for further examination. The current research aims to isolate, identify, and characterize five biosurfactant-producing, and petroleum-degrading novel bacterial strains: *Rhodococcus indonesiensis*, *Pseudomonas aeruginosa*, *Pseudomonas argentinensis*, *Acinetobacter baumannii*, and *Rhodococcus qingshengii*. The study highlights their emulsification and biodegradation activity under optimized environmental conditions, using the statistically determined regression model. The study further establishes a link between cell-hydrophobicity and biosurfactant production with the biodegradation of petroleum hydrocarbons.

Methodology

Sample collection

Samples of industrial effluents were collected from Nacharam district, Hyderabad, India ("17.430800 N 78.559500 E"). The selected site encompasses many services and gas stations containing high levels of oil contamination. A soil probe or trowel was used for collecting the soil samples at varying depths (10 cm to 1 m). Many sub-samples were collected, added to a large container, and mixed thoroughly, making it a homogenized mixture. After thorough mixing, a handful of soil (300 mg) was put in 50 mL of pre-sterilized, labelled, and self-sealed Falcon tubes. These sterilized Falcon tubes were then kept at 4 °C in the laboratory and maintained at the same temperature until the next step of analysis[21].

Isolation of the microbial consortia

After field sampling, the petroleum-degrading bacteria isolation was carried out according to the method specified in Drifa Yalaoui-Guellal et al. (2017)[22]. 10 g of the homogenized soil sample was added to a 500 mL flat bottle containing 100 mL of physiological saline (1% NaCl). The mixture was vigorously shaken for 1–2 hours and then allowed to stand for 1 hour. From this mixture 5 mL of supernatant was taken, and subsequently added into the flask containing 45 mL of Mineral Salt Medium (MSM); 1.0 g/L of NH4NO3, 0.7 g/L of MgSO4·7H2O, 3.0 g/L of Na2HPO4, 2.0 g/L of KH2PO4, 3.0 g/L of Na2HPO4, and 1.0 ml of trace element solution. The trace element solution consisted; 0.50 mg/L of CuSO4·5H2O, 30 mg/L of FeCl3, 10 mg/L of ZnSO4·7H2O, 0.50 mg/L of MnSO4·H2O, and 20 mg/L of CaCl2. To promote the growth of the subject bacterial species, 2% (v/v) of crude oil was added to this culture medium and incubated in an orbital shaker at 150 rpm at 30 °C for 7 days. Following incubation, 5 mL of culture was transferred to a fresh medium containing the mixture of MSM medium and crude oil 2% (v/v), and re-incubated at 150 rpm at 30 °C for 7 days.

The experiment was conducted in triplicate. Pure bacterial strains were procured by spreading onto the petroleum-coated MSM agar plates and incubated at 37 °C for 3–4 days. Morphologically distinct bacterial colonies were streaked onto fresh nutrient agar plates and kept at 4 °C for further analysis. The growth curve for the isolated bacteria was obtained by determining the Optical Density (OD) at 610 nm (A_{610}) (Fisher Scientific) for 10–12 days[4,23].

Estimation of growth

A 2 mL sample broth was collected after every 24h cycle for 10–12 days. The samples were centrifuged at 4 °C for 15 min at 5000 g. The crude oil extracts were removed by washing the cell pellet with 2 mL of *n*-hexane and dried at 100 °C in a hot air oven for 24 hours. The bacterial samples were weighed and biomass was determined on a dry weight basis (g/L)[24].

Biosurfactant screening of the petroleum degrading bacteria

The selected petroleum-degrading bacteria culture was tested for its potential to produce biosurfactants by undergoing many biosurfactant screening analysis.

Oil – spread method

The oil - spread assay was used to detect the presence of surface-active compounds as described by Rodrigues et al.[25], with slight modifications. Briefly, 20 mL of distilled water was added to the petri plates, followed by adding 20 µl of crude oil on the surface of the water. Additionally, 10 µl of cell-free culture broth was introduced to the oil-presented petri plate. After 30 sec–1 min, a clear zone was observed under the visible light. The experiment was conducted in triplicate, and the results were measured in mean ± standard error mean (SEM)[26–29].

Drop collapse test

The drop collapse test was conducted as described by Bodour and Maier[30], with slight modifications. The test was employed for detecting the destabilized liquid droplets produced by the biosurfactant. Briefly, the 96-well microplate was coated with 2 µl of crude oil and left to dry for 24 hours. 5 µl of the culture broth was transferred to the oil-coated well plate. The results were determined after a few minutes by calculating the drop sizes. The presence of biosurfactants was observed by a flat drop, whereas the drop remains stable in the absence of biosurfactants. The experiment was conducted in triplicate, and the results were measured in mean ± standard error mean (SEM)[31,32].

Hydrocarbon overlay test

This test was primarily employed for identifying hydrocarbon-degrading bacteria and to screen them for biosurfactant production. MSM plates were used containing 40 µl of kerosene, hexadecane, benzene, or toluene as carbon sources. The designated plates were spot-inoculated with the bacterial culture and incubated for 3–7 days at 28 °C. The zones of clearance or halos were observed around the bacterial colonies, which indicated the bacteria's hydrocarbon degrading potential. The experiment was conducted in triplicate, and the experimental results were represented in mean ± standard error mean (SEM)[22,33].

Bacterial adhesion to hydrocarbon (BATH) assay

This method was employed for evaluating the hydrophobic surface characteristics, cell surface hydrophobicity, and measuring the number of bacteria adhering to the hydrocarbon phase. The bacterial cell pellets were collected, washed, and suspended in a buffer salt solution (g/L): 7.3 KH_2PO_4, 16.9 K_2HPO_4, and then diluted in the corresponding buffer solution with an optical density of 610 nm (A_{610}). Subsequently, 100 µl of crude oil was added to 2 mL of cell suspension medium and vortexed for 3 mins. After vortexing, the solution was allowed to stand for 1 hour, separating the oil and aqueous layer. OD for the aqueous layer was measured using

a spectrophotometer (Fisher Scientific), which calculated the percentage of cells that were adhered to the oil surface[34,35]. The following formula was used for calculating the percentage adherence:

$$Bacterial\ Cell\ Adherence\ (\%) = \frac{OD\ initial - OD\ shaken\ with\ oil}{ODinitial} \times\ 100 \quad (1)$$

In which, $OD_{initial}$ indicated the OD pre-mix value of the cell suspension in the buffer solution, and $OD_{shaken\ with\ oil}$ indicated the OD value of vortexed cells using crude oil. In the BATH assay, a few drops of the INT solution (2-(4-iodophenyl)−3-(4-nitrophenyl)−5- phenyl tetrazolium chloride) were added and observed under the light microscope. The viability of the cells attached to the crude oil droplets was indicated by the reduction reaction, which turned the INT solution red. The experiment was conducted in triplicate, and the results were in mean ± standard error mean (SEM)[36].

Surface Tension Measurement

The surface tension (ST) reduction of the cell-free culture broth was calibrated using a tensiometer and the Nouy ring method (K20, KRUSS Scientific). The bacterial strains were grown in MSM broth, and 2% (v/v) crude oil was added as a sole carbon source, and the culture was incubated for 7 days at 200 rpm. After incubation, 5 mL of broth supernatant was transferred to a submerged glass tube in a water bath at a constant temperature (28 °C). The height of the ascended liquid in the capillary tube determined the surface tension. The test was performed by taking Triton X-100 solution, 1 mg/mL concentration as a standard control. The experiment was conducted in triplicate, and the results were represented in mean ± standard error mean (SEM)[37–39].

Determination of critical micelle concentration (CMC)

Different biosurfactant concentrations were prepared in the range 0–100 mg/L using an appropriate buffer solution (Sodium dihydrogen phosphate – disodium hydrogen phosphate of pH 7, 37 °C). The tube that lacked the biosurfactant sample was marked as a negative control. The CMC was demonstrated from the threshold value of the ST versus the logarithm of the biosurfactant concentration curve. The Nouy ring tensiometer (K20, KRUSS Scientific) was employed for determining the ST. The experiment was conducted in triplicate, and the results were represented in mean ± standard error mean (SEM)[40,41].

Biochemical characterization

The enumerated bacteria were isolated and stored in a nutrient agar medium at 4 °C for biochemical identification. The primary identification was based on colony formation, cell morphology, and gram staining. The secondary identification was achieved by executing a series of biochemical tests. The observed bacterial structures were compared with Bergey's Manual of Determinative Bacteriology. All the biochemical tests were performed at 30 °C[42–44].

Stability test calculation for emulsification index (E_{24})

The stability test for enunciating the total emulsification index values was conducted according to[45–49]. For this experiment, 6 mL of crude oil was taken and added to the 4 mL cell-free culture broth, shaken using a vortex for 2 mins, and allowed to stand for 24 hours. The emulsion index (E_{24}) was calculated as a percent ratio of the emulsified zone layer (mm) to the total height (mm), as described in the following equations[50–52].

$$Emulsification\ index\ =\ \frac{Height\ of\ the\ emulsification\ layer}{Total\ height} \times\ 100\ (\%) \quad (2)$$

Media optimization for biosurfactant production

The bacterial culture isolates were grown on MSM medium containing 2% crude oil as the sole carbon source under different physical and nutritional conditions to ascertain suitable parameters for significant hydrocarbon degradation, biosurfactant production, and emulsification index (E_{24}). All the experiments were conducted in triplicate, and results were represented in the form of mean ± standard error mean (SEM).

Effect of pH

To optimize the pH of the solution, 9 pH values (pH: 4–9) were selected. The MSM medium was prepared, and 2% (v/v) crude oil was added as a sole carbon source. The pH of the medium was optimized by adjusting the pH using 0.1 N HCl and 0.1 N NaOH solutions in adequate quantities. After pH adjustment, the media were subjected to sterilization for 15 mins at 121 °C. The activated culture of the isolate was inoculated and incubated at 37 °C in an orbital shaker for 7 days at 150 rpm[51,52].

Effect of temperature

To optimize the temperature of the solution, 7 temperature values (10–70 °C) were chosen. The MSM medium was prepared, and 2% (v/v) crude oil was added as a sole carbon source; pH was regulated to 7, and the media were subjected to sterilization for 15 mins at 121 °C. The activated culture of the isolate was inoculated and incubated at chosen temperatures in an orbital shaker for 7 days at 150 rpm[51,52].

Effect of carbon

The effect of carbon was determined by taking 8 different carbon sources: diesel, coconut oil, crude oil, maltose, sucrose, starch, glycerol, and mannitol. In the MSM medium, 1% of each carbon source was added, the pH was

kept at 7, and then the entire solution was sterilized at 121 °C for 15 mins. The activated culture of the isolate was inoculated and incubated at 37 °C in an orbital shaker for 7 days at 150 rpm[51,52].

Effect of nitrogen
The effect of nitrogen was indoctrinated by taking 8 different nitrogen sources: ammonium sulfate, ammonium phosphate, ammonium nitrate, peptone, ammonium chloride, yeast extract, potassium nitrate, and urea. The MSM medium was prepared, and 2% (v/v) crude oil was added as a sole carbon source, and a 1 g/L concentration of each nitrogen source. The pH was adjusted to 7 and the solution was sterilized at 121 °C for 15 mins. The activated culture of the isolate was inoculated and incubated at 37 °C in an orbital shaker for 7 days at 150 rpm[51,52].

Effect of carbon and nitrogen concentration
The best-optimized carbon and nitrogen sources were regulated for the best concentrations that were required for maximum yield. The carbon and nitrogen sources were added separately in the MSM medium at different concentrations: 1–7%. The pH of the medium was also adjusted to 7, and then the solution was subjected to sterilization at 121 °C for 15 mins. The activated culture of the isolate was inoculated and incubated at 37 °C in an orbital shaker for 7 days at 150 rpm[51,52].

Analysis of the optimized conditions
The bacterial isolates were streaked on fresh agar plates and incubated at 37 °C for 48 hours. The production process was conducted in a shake flask consisting of 250 mL of MSM medium and 2% (v/v) crude oil as the carbon source. 2 mL of pre-cultured bacteria was inoculated into each flask and incubated for 12 days at 28 °C at 200 rpm. Culture media samples were taken and checked at regular intervals to assess the emulsification activity and biomass growth. The proliferation of cells was done by using the dry cell weight technique[51–53].

Biosurfactant extraction and dry weight calculation

The biosurfactant production was executed in the MSM medium, constituting crude oil as a sole carbon and energy source. The extraction of the biosurfactant was done by centrifuging the fermented broth via a method elaborated in[54], with slight modification. Briefly, the subsequent culture of the bacterial isolate was grown on MSM broth overnight. 2% of the pre-bacterial culture was taken and put into the medium exhibiting the biosurfactant production with crude oil, and incubated for 7 days at 25 °C in a shaking incubator at 120 rpm. The solution was subjected to centrifugation for cell removal at 5000 rpm for 20 min at 4 °C. The supernatant was taken, and pH was regulated to 2 by employing 1M H_2SO_4. Then an equal amount of chloroform: methanol (2:1) was added for biosurfactant extraction, followed by separation of the organic phase and solvent evaporation to concentrate the biosurfactant. The biosurfactant was purified by concentrating the extract in a silica gel (60–120 mesh) glass column and containing chilled acetone as an eluent. The fraction was eluted, and the solvent was evaporated by drying to remove the acetone. Lastly, the purified fraction was dissolved in sterile water, and dry weight was calculated by keeping the dry residues in a sterile petri-plate in a hot air oven for 25 min at 115 °C. After drying, the weight of the plate was determined[55]. The resulting dry weight of the biosurfactant was calculated by the following formulae:

$$Dry\ weight\ of\ biosurfactant = (Weight\ of\ \ plate\ after\ drying - Weight\ of\ \ the\ empty\ plate) \quad (3)$$

All the data were estimated as mean standard error mean (SEM). The experimental data were analyzed by employing the two-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison tests. The statistical analysis determined that $p < 0.05$ was considered significant. All the analyses were performed using R programming version 4.3.1.

Hydrocarbon biodegradation by gravimetric method

The biodegradation profile of the bacterial species was tested by following the methodology by Hossain et al.[56], with slight modifications. Briefly, the selected bacterial isolates were inoculated in 100 mL of MSM medium containing 2% crude oil in 250 mL conical flasks on an orbital shaker and incubated at 30 °C at 150 rpm for 21 days. To evaluate the impact of additional carbon sources, a parallel set of experiments was conducted by employing 0.5% glycerol as a supplement to boost biosurfactant production. Control was kept under the same conditions without seeding the bacterial strain.

After incubation, residual crude oil was extracted by using a mixture in a ratio of 1:1 of acetone, and petroleum ether, in which 1 N HCl was added to cease the bacterial activity, and then transferred to a selecting funnel and kept at incubation for 15–20 mins at 120 rpm in an orbital shaker. After incubation, the organic phase was meticulously collected in a pre-weighed beaker, and anhydrous sodium sulfate (Na_2SO_4) was added to remove the moisture content. Subsequently, the solvent was evaporated by employing a rotary evaporator at 50 °C to prevent further thermal degradation of hydrocarbons. The final weight of the beaker exhibiting the residual crude oil was noted, followed by determining the weight of the extracted crude oil by subtracting the initial weight of the empty beaker. The percentage of hydrocarbon degradation was calculated using the following formula[57]:

$$\text{Hydrocarbon Degradation } (\%) = \frac{\begin{matrix} Weight\ of\ residual \\ oil\ in\ control \end{matrix} - \begin{matrix} Weight\ of\ residual \\ oil\ in\ isolate\ sample \end{matrix}}{Original\ weight\ of\ the\ residual\ oil} \times 100 \quad (4)$$

The experiment was conducted in triplicate, and the results were in mean ± standard error mean (SEM). To ensure experimental precision, the pre-weighed beakers were employed in which the complete solvent evaporation was verified before weighing. An abiotic control sample was also included for assessing the non-biological degradation of the crude oil.

Analytical approach

Preliminary characterization of the crude biosurfactant

The preliminary characterization was performed by employing two dyes; Sudan Red III (lipid soluble dye), and Methyl orange (water soluble dye) for determining the emulsion by the produced biosurfactants. Further, the total protein and carbohydrate content was estimated using the Folin lowry, and Phenol Sulfuric Acid methods respectively, of the biosurfactant extract[58,59].

Nuclear magnetic resonance (^1H NMR) analysis

The ^{1}H-NMR spectra were recorded on a Bruker 400 MHz NMR spectrometer (Rheinstetten, Karlsruhe, Germany) equipped with a 5 mm PA BBI 400SI H-BB-D-05 Z probe and using Bruker SampleXpress (BrukerBiospin, Rheinstetten, Germany) for automatic sample change. The sample was dissolved by deuterated chloroform ($CDCl_3$) (99.9%) solvent in a Schotte economic NMR sample tube (5 mm o.d./178 mm length). NMR acquisition and processing were performed as described in Saito et al., 2009[60] at 25 °C with 5000–5200 accumulations. For each sample, eight scans were recorded with a 90° pulse and a 60-sec relaxation delay with an acquisition time of 2.56 sec to obtain the maximum sensitivity. The start and end points of the integration of the peak area were selected manually. Chemical shifts are given in δ values (ppm) relative to $CDCl_3$ as an internal standard[61,62].

Gas chromatographic (GC-MS) analysis

The extracted crude oil samples, both the biodegraded, and abiotic control were tested for fingerprinting, i.e. assessing the molecular, and gross composition by using the gas chromatographic method, as described in Wang et al.[63], with slight modifications. The residual alkane fractions were procured at regular time intervals of 10–12 days followed by analysis on a GC-MS gas chromatograph – mass spectrometer (Agilent 7890 A). The extracted alkane fractions were reduced to 200 µL by using a vacuum evaporator. A DMPS capillary column (30 m x 0.25 mm x 0.25 mm) was employed for separating the crude oil components. Helium was used as a carrier gas flowing at a rate of 1.2 mL/min. The column temperature was raised from 80 °C to 240 °C at 5 °C/min and kept at 240 °C for a brief period of 30 mins. The detector and injector temperatures were adjusted to 300 °C and 260 °C respectively. The biodegradation efficiency of the chief hydrocarbons was enunciated by studying the area under the peak in degraded, and abiotic control samples, by following the previously established studies, and the equation below[64]:

$$Biodegradation\ Efficiency\,(\%) = \frac{A_c - A_s}{A_c} \times 100 \tag{5}$$

Where A_c, and A_s are the hydrocarbon degradation efficiency by the abiotic control, and stipulated bacterial sample.

Statistical optimization utilizing experimental design models

Medium components were statistically optimized for procuring the maximum yield, emulsification index of the biosurfactant, and hydrocarbon-degrading potential. It was performed by executing the Response Surface Methodology (RSM) with a Box-Behnken Design (BBD) model. All the experiments were conducted by utilizing the statistical and subsequent experimental design packages in R-programming version 4.2.3.

Box- Behnken experimental design (BBD)

The optimization of the E_{24} index and hydrocarbon degradation potency was carried out by employing a Box-Behnken experimental design (BBD), a three-level full factorial design (TFFD), and response surface methodology (RSM). These applications were chiefly used to analyze the relationship between the three independent variables and the dependent variable (E_{24} index and hydrocarbon degradation (%)), and identify the optimal levels of the independent variables for the dependent variable (response). The three independent variables were selected at three different levels: pH (x1), temperature (x2), and salinity (x3) (Table 1). The levels of each variable were determined via a thorough literature survey[65–68]. The generalized second-order polynomial model was employed to fit the response surface analysis:

Independent Variable	Symbols	Levels		
		−1	0	+ 1
pH	x1	4	7	12
Temperature	x2	10	40	70
Salinity	x3	1	3	5

Table 1. Levels of independent variables for the experimental design.

$$Y = {}_0 + \sum {}_{i=1ii}X_i^2 + \sum {}_{i=1}^{n} \sum {}_{j=i+1ij}^{n} X_i X_j \quad (6)$$

Where Y is the response variable (E_{24} index and hydrocarbon degradation (%)), b_0, b_i, b_{ii}, *and* b_{ij} are the regression coefficients for intercept, linear, quadratic, and interaction terms, respectively, and X_i and *Xj designate* the independent variables (*i & j*). The models were employed to evaluate the effect of each independent variable on the response[69–72].

Validation of the experimental design

The experiments were set up to validate the factors at optimum conditions of pH, temperature, and salinity suggested by RSM-BBD. The emulsification activity and hydrocarbon degradation potency for the bacterial isolate were determined, followed by some additional tests[70–72].

Statistical analysis

The effect of independent variables, pH, temperature, and salinity, on the response variables was assessed in triplicate. Analysis of variance (ANOVA) was employed to assess the effects of the designated variables, their interactions, and the statistical significance of the model. The goodness and lack of fit of the 2nd -grade polynomial model equation were expressed by the coefficients of determination R^2. The statistical significance and the significance of regression coefficients were also evaluated by F-test at a probability (p) ≤ 0.05[70–72]. The optimal extraction conditions were determined via regression analysis, 2D contour, and 3D response surface plots by using R-programming version 4.3.1.

Isolation and amplification of DNA

After following a series of screening procedures, the genomic DNA of each isolate was obtained from the pure cultures employing the phenol/chloroform extraction method. The purity and integrity of the isolated DNA were analyzed on agarose gel electrophoresis, and the procured bands were amplified using PCR. PCR amplification was carried out for each isolate in a PCR thermocycler exhibiting 50 µL master-mix; 10X Polymerase assay buffer, 1µL of genomic DNA template, 1µL of Taq DNA polymerase enzyme (3U/mL), 4 µL of deoxy-nucleoside triphosphates dNTPs (2.5 mM each), 2µL of universal forward and reverse primers (each), 1X of gel loading buffer, and 30µL of water. Universal primers; 5'- GGATGAGCCCGCGGCCTA – 3' (forward), and 5'- CGGT GTGTACAAGGCCCGG – 3' (reverse) were used for the study. Thirty cycles were run to obtain the amplified product, which was further stored at 4 °C. DNA detection was rendered by taking 3µL of each PCR product, which was subsequently loaded onto 2% w/v agarose gel. The gel was stained with 3µL ethidium bromide (10 mg/mL), and subjected to electrophoresis in 1X Tris EDTA Buffer (TEB Buffer (pH 8.3); 89 mM Tris base, 89 mM Boric Acid, 2 mM EDTA dissolved in 1 L of water) for 90 mins at 10 V/cm and 50 mA against the 500 bp ladder as a molecular size standard. The gel components were visualized under a UV transilluminator (Fisher Scientific) or GelDocXR software (Bio-Rad)[73–77].

16S rRNA sequencing

The amplified products were eluted from the gel and purified using a GeneJET Gel Extraction PCR purification kit. Ribotyping was adopted for sequencing the PCR amplified products and performing taxonomic analysis. Owing to the limitations of sequencing, most studies can only use the partial sequences of the 16S rRNA gene product[78–82]. After attaining the required partial sequences, raw data files of each sample (trace files) were procured and aligned using DNA Baser software v5.15 version. After alignment, the assembled sequences were saved in fasta format and further analyzed using myriad bioinformatics tools and algorithms[83–87]. The saved fasta files were compared and cross-checked using NCBI – BLASTn, and similar nucleotide sequences were determined[88,89].

Evolutionary analysis

The topological similarities between phylogenetic trees were estimated by specific algorithms that took the node structure of a tree into a multi-dimensional model[90–92]. Different algorithms were used to quantify the discrepancies between the sub-trees. The quantification analysis also permitted the exploration of correlative relationships amongst distinct sub-regions of 16S rRNA in terms of phylogenetic resolution. For phylogenetic tree construction, briefly, a taxonomic list was constructed consisting of the obtained sequences from the 16S rRNA sequencing method and the compared sequences from the cross-checked method using BLASTn. The highly similar sequences were aligned using the Multiple Sequence Alignment (MSA) format by utilizing the Muscle algorithm. The Neighbor-Joining (NJ) method from the database set was used for constructing the phylogenetic tree using the Unweighted Pair-Group Method with Arithmetic Averaging (UPGMA), and the Maximum-Likelihood model[93,94]. The bootstrap test's relationship with all the taxa was aggregated after 1000 replicates of the tree formation. Molecular Evolutionary Genetics Analysis version XI (MEGA-XI)[95,96] software was assigned to conduct the evolutionary analysis.

16S rRNA structure prediction

The secondary structures of 16S rRNA were mainly constructed by employing free energy minimization techniques or algorithms. These free energies were procured from experimental measurements of RNA structural element stability. Since the three-dimensional structure of RNA exhibits a plethora of potential configurations, predicting the equilibrium native structure was an arduous task. Moreover, studies have proven that RNA structures exhibit myriad diacritic motifs, and each motif bears a colossal amount of probable sequence combinations[97–99]. Few of the algorithms, such as the neighbor model, predicted the stabilities of the

RNA duplex with only Watson-Crick pair algorithms, proposed by Borer et al., an alternate algorithm proposed by Gray et al.. also explored the thermodynamic parameters for the concerned structure. With the advancement in technology and phylogenetic information, the thermodynamic principles were being articulated for near and good prediction of RNA structure and its properties in duplex formation, along with the determination of spectroscopic properties of RNA[100–102].

Thus, for determining the secondary structure of the subsequent 16 S rRNA sequences, the Unified Nucleic Acid Folding and Hybridization Package (UNAfold) was employed. The MFold server was utilized by employing the Zuker-Stiegler algorithm for assessing the minimum free-energy structure based on the nearest neighbor model. It further utilized the empirical estimates for determining the thermodynamic properties to demonstrate the secondary RNA structure. Moreover, myriad suboptimal structures were produced, which exhibited a certain percentage of the free-energy ranges. The algorithm further calculated the mole fractions of varied chemical species as a function of temperature, UV absorbance (260 nm), complete melting profiles for melting simulations, heat capacity (Cp), etc.[103,104]

Microcosm study

Preparation of crude oil polluted soil samples

The microcosms were prepared for determining the bioremediation analysis by the stipulated bacterial isolates, in manually polluted agricultural soil. The composition of the agricultural soil was prepared by following Varjani et al.[105], with few modifications. Briefly, 1 kg of pulverized air-dried soil samples was prepared and mixed with 2% crude oil concentration in a large container, followed by five treatment processes, as described in Table 2. Biodegradation was studied under five soil treatment samples; Treatment 1: This lacked all the nutrients, bacteria, or biosurfactant to assess the abiotic loss of crude oil [Abiotic control (AC)], Treatment 2: the nutrients were added but lacked bacteria to assess the biotic loss of crude oil by stimulated microorganisms [Biostimulation (BS)]. Treatment 3: The subsequent bacterial strain SARSHI1 was added but lacked nutrients to assess the effectiveness of the subsequent strains to utilize hydrocarbons [Bioaugmentation (BA)], Treatment 4: The bacterial strains were added along with nutrients for simultaneously assessing the effect [BA + BS]. Treatment 5: nutrients were added along with the biosurfactants, but it lacked the bacterial strain SARSHI1 to assess the role of biosurfactant in enhancing the degradation of indigenous microorganisms [Biostimulation + Biosurfactant (BS + B)]. Treatment 6: In addition to BA + BS, an additional biosurfactant was exogenously added to enhance the bioremediation efficiency [BA + BS + B]. The nutrients were added in a 100:10:1 ratio - Carbon: Nitrogen: Phosphorus (C: N: P), in which glycerol was consumed as a carbon source, NH_4NO_3 as nitrogen, and Na_2HPO_4 as a phosphorus source. All this while, the moisture content was maintained at 20% along with the addition of distilled water. Tilling and aeration were done once a week to break the lumps and enhance the homogenization of the soil, which augments the contact between microbes and crude oil[106]. The experiment was conducted in triplicate, and the results were measured in mean ± standard error mean (SEM).

Phytotoxicity study

The Phytotoxicity Assay was used to determine the biological activity of the stipulated bacterial isolates, following the OECD 2006 guidelines (Organisation for Economic Co-operation and Development OECD, 2006). Two vegetable seeds, *Triticum aestivum* (wheat) and *Vigna radiata* (Mung Bean), were selected for the subsequent analysis and primarily treated with sodium hypochlorite. They were then washed with distilled water to remove excess biocide and incubated in the dark for 5–7 days at 20 °C. Petri plates were utilized to evaluate the effect of different treatments (Treatment 1–6) on soil samples. The non-contaminated soil was taken as a positive control. The surface sterilized 10 seeds each were taken in different Petri plates and grown at 25 ± 1 °C with a photoperiod of 16/8 h day/night. Each Petri plate was sprinkled with 30 mL of distilled water daily till the 12thday. Lastly, the germination index was calibrated using Eq. vi[107,108]. The experiment was conducted in triplicate, and the results were measured in mean ± standard error mean (SEM).

$$\text{Germination Index (GI)}\,\% = \frac{\boldsymbol{SGe/SGc}}{\boldsymbol{RLe/RLc}} \times 100 \tag{7}$$

Where SGe and SGc designate seeds germinated in extract and control, while RLe and RLc designate mean root length in extract and control, respectively.

Treatment type	2% Crude oil	Nutrients	Bacterial strain	Biosurfactant	Sample code
Treatment 1	⁂	–	–	–	Abiotic control (AC)
Treatment 2	⁂	⁂	–	–	Biostimulation (BS)
Treatment 3	⁂	–	⁂	–	Bioaugmentation (BA)
Treatment 4	⁂	⁂	⁂	–	BA + BS
Treatment 5	⁂	⁂	–	⁂	BS + B
Treatment 6	⁂	⁂	⁂	⁂	BA + BS + B

Table 2. Treatment types for microcosm study. Where, ⁂ - added, - not added.

Results

Isolation and biochemical characterization of the petroleum degrading bacteria

Petroleum degrading bacteria were enriched and isolated using MSM medium containing crude oil (2%, v/v) as the sole carbon and energy source. The enrichment cultures were subjected to dilution and then transferred to solid MSM media for colony formation. Nutrient agar plates were used to obtain the pure colonies. The bacterial samples were grown in MSM media containing crude oil as a soil carbon and energy source. Out of seventy two samples, only five gave positive results of foam-forming biosurfactants. The selected five strains were labelled as SARSHI1 – SARSHI5, and subjected to classic morphological and biochemical tests by following Bergey's manual as a reference. The tests determined that the selected bacteria belonged to different species; *Rhodococcus* species 1 (SARSHI1–5.42 10^8 CFU/mL), *Pseudomonas* species 1 (SARSHI2–5.05 10^8 CFU/mL), *Pseudomonas* species 2 (SARSHI3–5.32 10^8 CFU/mL), *Acinetobacter* species (SARSHI4–5.6 10^8 CFU/mL), and *Rhodococcus* species 2 (SARSHI5–5.37 10^8 CFU/mL). The meticulous biochemical characterization analysis has been mentioned in [Supplementary Material 1, Table S1, and Figure S1-S6(b)].

Kinetic growth analysis

The biosurfactant production was studied by using 2% (v/v) crude oil as a sole carbon and energy source. This yielded an augmented amount of biosurfactant production than the concentrations of other sources like glucose or glycerol. The kinetic growth curve of SARSHI1- SARSHI5 tends to follow an exponential growth relationship between the biosurfactant production and the cell biomass (Fig. 2). The results revealed that the highest biomass concentration for isolates SARSHI1 – SARSHI5 was 3.35 g/L, 2.83 g/L, 2.33 g/L, 2.51 g/L, and 3.1 g/L, respectively, after a cultivation time between 120 and 170 h, thus giving an exponential growth phase, followed by gradual declination in the death phase. The mitigated growth reveals the probability of cell growth cessation due to exhaustion of nutritional stress. A parallel relationship with the biosurfactant production was observed, which gave the values of 2.93 g/L, 2.53 g/L, 1.84 g/L, 2.32 g/L, and 2.78 g/L, respectively, for the subsequent isolates. The production embarked with the cultivation onset between 42 and 74 hours, during the early exponential phase, and attained the maximum summit between 120 and 170 hours. This implies that the biosurfactants could also be employed as a substrate by the bacterial cultures when there is a scarcity of the original substrate. Thus, marking the reduction in biosurfactant production in the MSM medium during the onset of the stationary phase. Furthermore, it can be inferred that biosurfactant production mainly occurs in the exponential phase of the curve, suggesting the biosurfactant to be a primary metabolite that has a parallel relationship with biomass growth. It was proved by the production of biosurfactants when a particular hydrocarbon was utilized as a sole carbon source, signifying its petroleum degradation potential.

Screening for biosurfactant production

Oil** – spread **assay

The oil spread assay was employed to characterize the biosurfactant potential of bacterial cultures. This test was rapid and easy to carry out. According to the study of Morikawa et al., the oil displacement test generally demonstrates a directly proportional relationship with the active compound formed on the surface of the

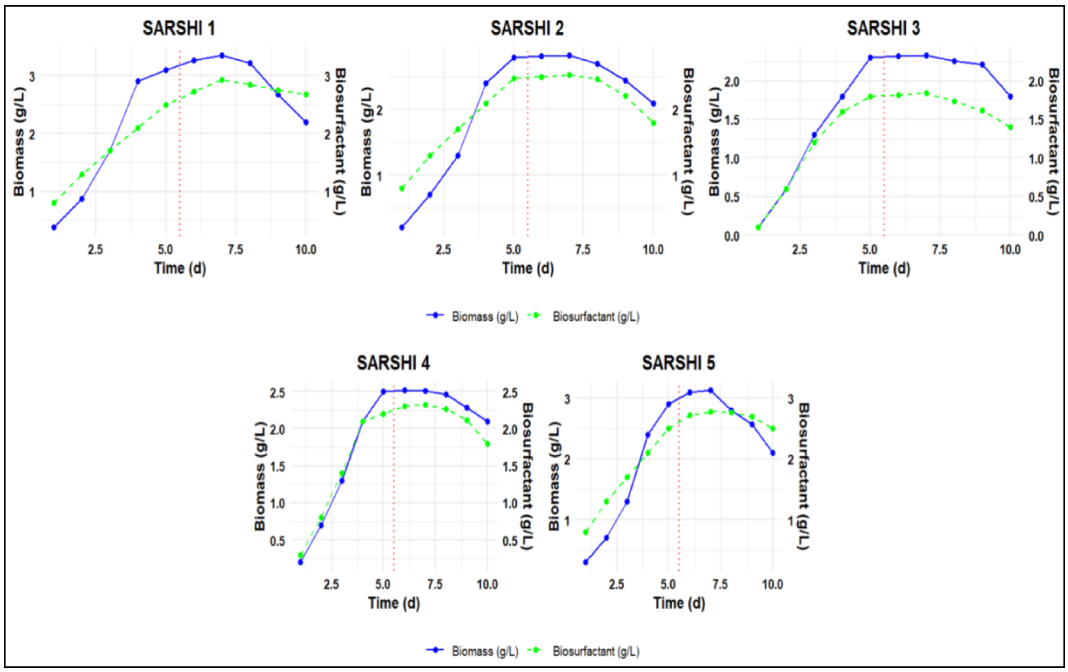


Fig. 2. Time course of growth and biosurfactant production by SARSHI1 – SARSHI5 in MSM medium with 2% (v/v) crude – oil as sole carbon source.

Microorganism	Drop Collapse Assay	Oil Spreading Assay	Surface tension measurement	E_{24} Index (%)
SARSHI1	+++	Nil	+++	85.34
SARSHI2	+++	++	++	65
SARSHI3	Nil	+	++	20
SARSHI4	+++	++	++	55
SARSHI5	+++	Nil	+++	78

Table 3. Drop collapse assay, oil spreading assay, and surface tension measurement. Drop Collapse Assay: "+++" indicates drop collapses within 1 min, "++" – drop collapses after 1 min, "+" – drop collapses after 2 mins. Oil Spread Assay: "+++" – clear zone of oil spread with a diameter > 2.5 cm, "++" clear zone of oil spread with a diameter between 1.5–2.4 cm, "+" clear zone of oil spread with a diameter < 1.4 cm. Surface Tension: "+++" - surface tension≤ 30 mN/m, "++" - surface tension between 31 mN/m – 55 mN/m, "+" - surface tension between 56 mN/m – 75 mN/m.

Microorganism	BATH Assay	Hydrocarbon overlay assay			
		Kerosene	Hexadecane	Benzene	Toluene
SARSHI1	+++	+++	Nil	++	Nil
SARSHI2	++	++	++	++	++
SARSHI3	+	Nil	++	Nil	+
SARSHI4	++	++	Nil	++	++
SARSHI5	++	+++	Nil	++	Nil

Table 4. BATH and hydrocarbon overlay assay results. BATH assay: "+++" signifies cell adhesion > 90%, "++" signifies cell adhesion 60–89%, "+" signifies cell adhesion 40–59%. Hydrocarbon Overlay Assay: "+++" – clearance zone 2.1–3.5 mm, "++" – clearance zone 1.1–2 mm, "+" clearance zone < 1 mm.

solution. However, in the present study, only the qualitative characteristic was enunciated. The results are represented in Table 3 and [Supplementary Material 2 (Figure S7)] for SARSHI1.

Drop collapse test

The drop collapse test was primarily employed for screening the surfactant-producing bacterial cultures. In the experiment, drops of the cell suspension, or culture supernatant, were placed on 2 µl crude oil-coated 96-well plates and equilibrated for 24 hours. The drops were then subjected to observation with the aid of a magnifying glass for a couple of minutes. The test relied on the destabilization of liquid droplets, which were produced by the surfactants. The surfactants decreased the surface tension of the liquid, which caused the polar water molecules to be repelled from the hydrophobic surface. The stability of the drops was directly dependent on the concentration of the surfactants and interfacial tension. The bacterial isolates demonstrated a positive result by the formation of a flat drop in the petri dish that collapsed in the time frame of 50 sec, 1 min 10 sec, 0 s, 1 min 30 sec, and 58 sec for samples SARSHI1 – SARSHI5, respectively. The results are represented in Table 3 and [Supplementary Material 2 (Figure S8)] for SARSHI1.

Hydrocarbon overlay assay

The Hydrocarbon Overlay Test was employed for determining the hydrocarbon-degrading potential of a bacterium. The chief purpose of the test was to demonstrate the ability of the bacteria to utilize different hydrocarbons as a sole source of carbon and energy. The test further estimated the ability of the bacteria to generate surface-active agents that can degrade hydrocarbons. The test estimated the quantitative trait of bio-emulsifiers that demonstrated the zone of clearance for each bacterium: SARSHI1 and SARSHI5 gave 2.11 mm, 1.54 mm, and 1.89 mm, 1.22 mm diameters for kerosene and benzene, but gave a negative result for Hexadecane and Toluene, respectively. SARSHI2 gave 1.73 mm, 1.63 mm, 1.3 mm, and 1.15 mm diameters for kerosene, hexadecane, benzene, and toluene, respectively. SARSHI3 gave negative outcomes for kerosene and benzene, 1.45 mm and 0.7 mm for hexadecane and toluene, respectively. SARSHI4 gave 1.76 mm, 1.34 mm, and 1.12 mm for kerosene, benzene, and toluene, respectively, with a negative result for hexadecane. The results are represented in Table 4.

BATH assay

The Bacterial Adhesion to Hydrocarbon (BATH) Assay was employed for evaluating the hydrophobic surface characteristics and the adhesion behavior of bacterial cells. The major principle behind the test involved mixing a bacterial culture with a hydrocarbon suspension that evaluated the decreased optical density of the cultured phase, indicating the ability of the bacteria to utilize the subsequent hydrocarbon as a carbon and energy source. The results revealed that the sample SARSHI1 displayed the highest hydrophobicity, while SARSHI3 displayed the least hydrophobicity. The results are represented in Table 4.

Surface tension measurement

The surface tension was measured in the cell-free culture broth, which demonstrated alleviated surface tension levels. The results revealed a direct correlation between the drop collapse assay and surface tension measurements. The control sample's surface tension was measured as 70.65 ± 0.02 mN/m at the onset of the experiment. According to the literature study, the primary criteria of a good biosurfactant are to reduce the ST below or equal to 35 mN/m, and the current isolates (SARSHI1 – SARSHI5) do fall in the acceptable cadre. The bacterial isolates gave a positive result by reducing the surface tension; 27 mN/m, 32 mN/m, 39 mN/m, 35 mN/m, and 30 mN/m for samples SARSHI1 – SARSHI5, respectively. The results are represented in Table 3. The profile indicating the relationship between the ST and cell growth has been illustrated in [Supplementary Material 2 (Figure S9)] for all the isolates.

Determination of critical micelle concentration

CMC has been proven to be a chief parameter of a biosurfactant to form micelles. Triton X-100 solution – 1 mg/mL concentration was employed as a standard control, which demonstrated an ST value of 70.65 ± 0.02 mN/m at the onset of the experiment. The results revealed that the surface tension was reduced at varied biosurfactant concentrations (0–100 mg/L). It became constant after it reached the threshold biosurfactant concentration. The CMC was determined at specific concentrations: 70 mg/L, 80 mg/L, 95 mg/L, 89 mg/L, and 75 mg/L for sample SARSHI1 – SARSHI5, respectively [Supplementary Material 2 (Figure S10)].

Optimization of biosurfactant production for *Rhodococcus indonesiensis* strain SARSHI1

The emulsification index is rendered to be an indirect mechanism to screen the efficacy of subsequent bacterial isolates. Studies have revealed that the presence of biosurfactants in the solution allows emulsification of hydrocarbons. In the current research, crude oil was employed as a hydrophobic substrate, and the E_{24} value of all five isolates was determined (Table 3). The maximum E_{24} activity was noted by SARSHI1- 85.34%, while the minimum activity was noted by SARSHI3–20%.

The results of optimized E_{24} index values for SARSHI1 were revealed at pH 7–85.34%, with optimum temperature between 35 and 40 °C at 72–81%, indicating a moderate thermostable capability (Fig. 3a,b). Among the 8 different carbon sources, the maximum activity was at 60% by crude oil, followed by sucrose at 51%, while mannitol exhibited the least activity at 16%, suggesting its hydrocarbon degrading potential (Fig. 3c). Consequently, among the 8 different nitrogen sources, yeast extract obtained the highest activity – 60%, followed by urea – 58%, and potassium nitrate exhibited the least activity – 18% (Fig. 3d). Since crude oil procured the highest activity, further experiments were conducted in which E_{24} activity was examined at different crude oil concentrations, the highest was at 4% concentration – 75% (Fig. 3e). Similarly, E_{24} was examined at different yeast concentrations, and maximum activity was obtained at 4% concentration – 74% (Fig. 3f). Salinity concentrations were also examined, and maximum activity was obtained at 3% concentration – 76% (Fig. 3g). The above results state that the subsequent bacterial isolate SARSHI1 can be an important tool that can be applied in microbial–enhanced oil recovery (MEOR), and it signifies its petroleum degrading potential in various optimized conditions.

Biosurfactant extraction and dry weight

The supernatant was collected by inoculating the culture in the MSM medium with crude oil as the sole energy and carbon source. The collected supernatant was then mixed with an equal amount of chloroform: methanol (2:1). The experiment resulted in white-colored sediments, indicating biosurfactant production. Moreover, the maximum amount of biosurfactant produced by SARSHI1 was 0.846 per 100 mL of the medium. The dry weight analysis has been represented in [Supplementary Material 2, Table S2].

Hydrocarbon degradation by gravimetric method for *Rhodococcus indonesiensis* strain SARSHI1

The biodegradation efficiency of SARSHI1 was assessed using the gravimetric method under both glycerol-supplemented and non-glycerol-supplemented conditions. At 0 days of incubation, the weight of the crude oil was 2 g, which decreased progressively over time. In the absence of the additional carbon source, the SARSHI1 exhibited moderate biodegradation efficiency by achieving 65% degradation after 21 days. However, it achieved 84% crude oil utilization when supplemented with glycerol, thus gaining a fold change of 1.29 in its degradation efficiency. This corroborates the role of high biosurfactant production, which enhanced the hydrocarbon bioavailability and solubility, facilitating microbial metabolism and uptake.

Analytical approach analysis for *Rhodococcus indonesiensis* strain SARSHI1

Primary characterization

The type of emulsion was demonstrated by taking the mixture of the biosurfactant and dye under a light microscope. The two dyes, Sudan Red III and Methyl orange, were employed and observed under a microscope revealing colorless droplets against an orange background. This suggests that it is a form of o/w emulsion, which explains the colorless yield of the water-soluble dye against an external aqueous phase where the oil droplets stayed colorless. The nature of the o/w emulsion was further tested by employing Sudan red III, in which the droplets emerged as red against the colorless background. The biosurfactant was further analyzed for protein and carbohydrate content, and it was revealed that it contained 30.26% and 15.62% content, respectively.

Nuclear magnetic resonance results

The 1H-NMR spectrum of the lipopeptide biosurfactant, recorded at 400 MHz, presents a rich and intricate profile that underscores the compound's structural diversity. The spectrum features a prominent triplet at δ

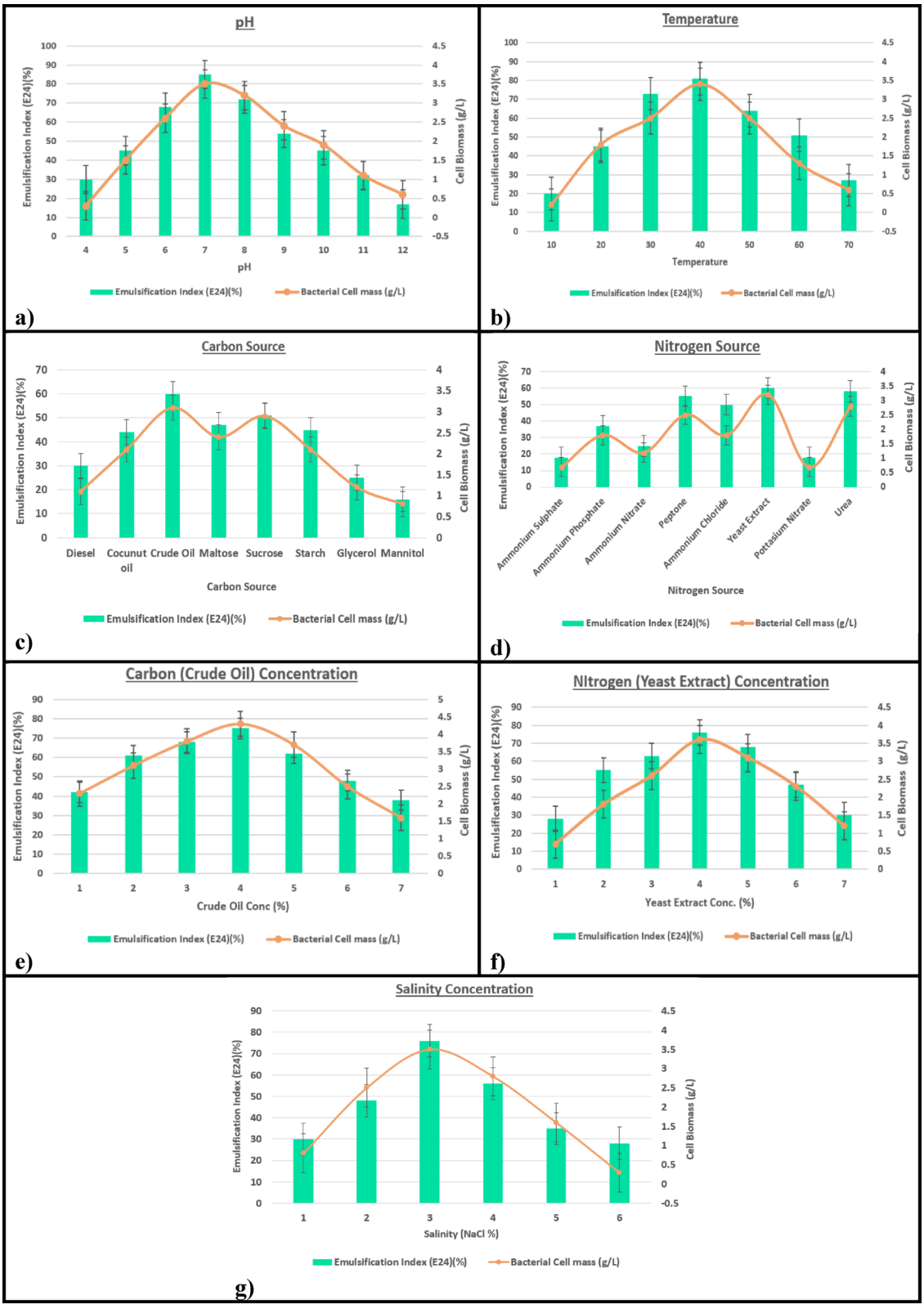


Fig. 3. (**a**) pH, (**b**) temperature, (**c**) carbon source, (**d**) nitrogen source, (**e**) carbon concentration, (**f**) nitrogen source concentration, (**g**) salinity concentration optimization to maximize the yield of biosurfactant. Graph determines the E_{24} index values and cell biomass corresponding to varying physical and chemical parameters for *Rhodococcus indonesiensis* (SARSHI1).

0.87 (2 H, J = 6.84 Hz), which is characteristic of terminal methyl groups, a common feature in lipid-derived molecules. This is complemented by a series of overlapping signals in the aliphatic region (δ 1.16–2.18 ppm), where methylene protons exhibit a variety of coupling patterns, suggesting the presence of multiple distinct environments and potentially branched structures. Further downfield, the region between δ 2.40 and 3.70 ppm reveals protons that are likely near electronegative atoms, resulting in chemical shifts that are indicative of functionalities such as amines or esters. This is significant as it points to the possible presence of polar groups within the biosurfactant structure, which may contribute to its surface-active properties.

In the aromatic region (δ 6.87–7.95 ppm), the spectrum displays complex splitting patterns that are typical of substituted aromatic rings. These patterns indicate a sophisticated arrangement of protons, reflecting the structural complexity often associated with lipopeptides.

A total of 43 distinct peaks were identified in the spectrum, highlighting the intricate molecular architecture of the lipopeptide biosurfactant. This complexity not only emphasizes the rich chemical environment of the molecule but also suggests the presence of various functional groups that could be responsible for its biological activity and surfactant properties. Further characterization and analysis will be essential to fully elucidate the molecular structure and its implications for potential applications. The results are illustrated in Fig. 4.

Gas chromatographic analysis

The GC-MS analysis of the residual crude oil fraction revealed the presence of n-alkanes ranging from C14 to C36, with distinct variations in peak intensities. The chromatographic profile indicated that C18 and C24 exhibited the highest relative intensities, suggesting their dominance in the sample composition. A trend of fluctuating abundance was observed across the carbon chain lengths, with C16, C22, and C30 also showing notable intensities, while C34 and C36 exhibited comparatively lower peaks. However, following biodegradation analysis, it was revealed that C24 was completely degraded (100%), while C18 had a significant reduction (97%), thus, suggesting an effective microbial consumption. Consequently, other carbons atoms, such as C16, C22, and C30 had 99%, 95%, and 93% hydrocarbon utilization respectively. On the other hand, C34, and C36 comparatively showed lesser degradation, suggesting partial presence in the residual fraction. The varied distribution pattern of hydrocarbon degradation indicates a potential preferential utilization of longer chained carbons, which could be due to microbial diacritic solubility, evaporation, and degradation. The observed pattern of degradation in the mid-chain carbons (C18 – C24) indicates the optimal solubility and little resistance to enzymatic degradation. Thus, the results determine that SARSHI1 can utilize crude oil as a sole carbon, and energy source, and can attain degradation efficiency up to 100% for specific alkanes. The results for the same have been illustrated in Fig. 5.

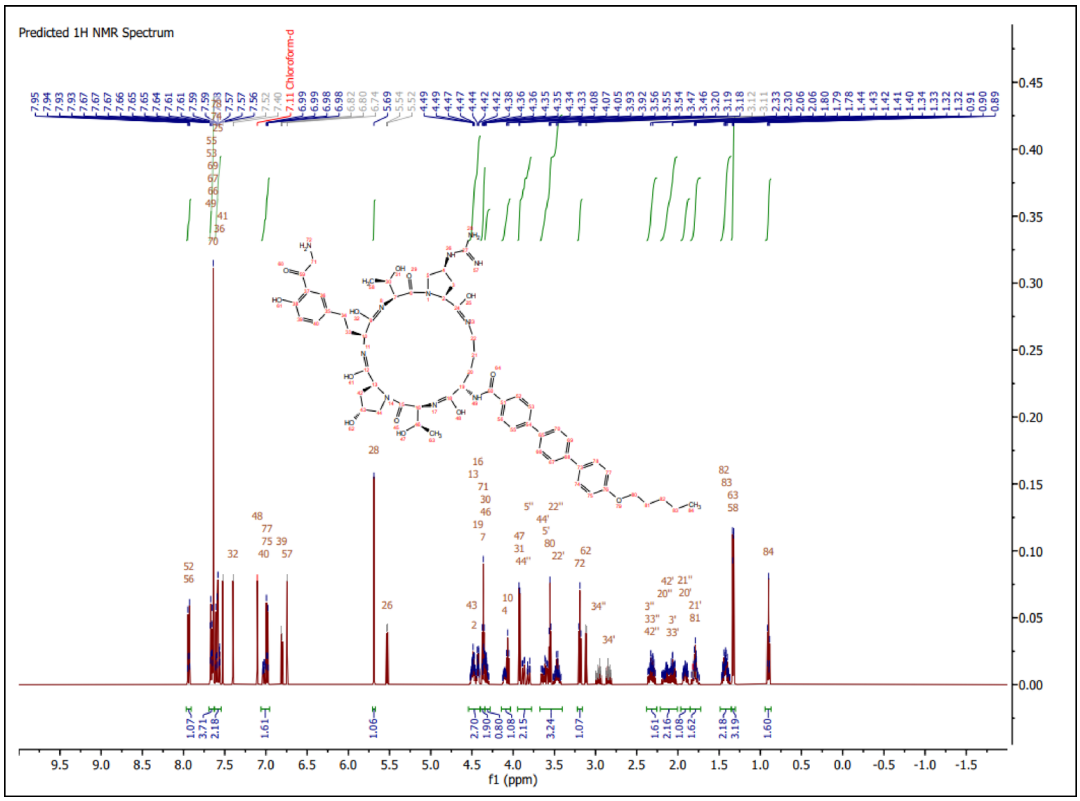
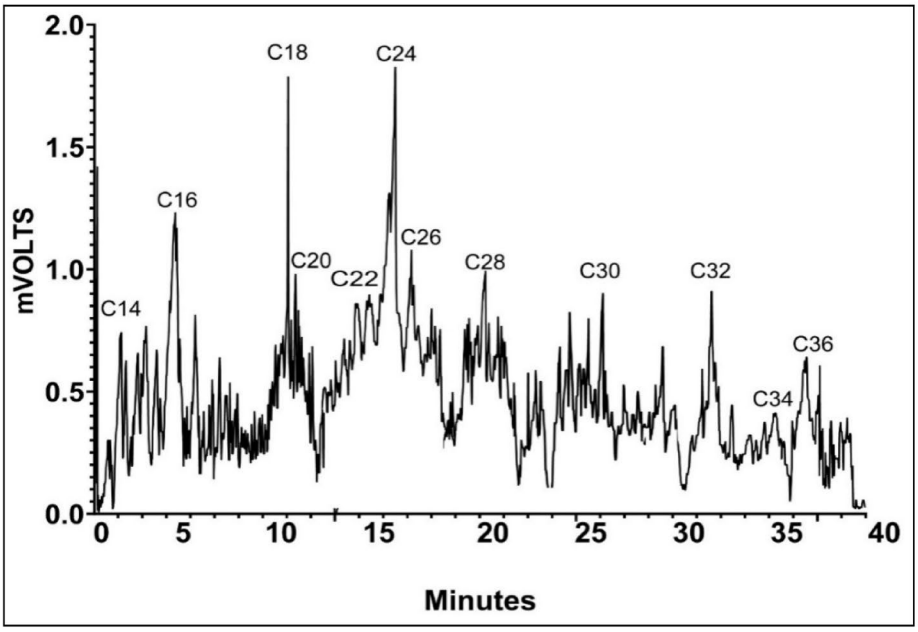


Fig. 4. NMR Spectrum of the biosurfactant obtained for *Rhodococcus indonesiensis* (SARSHI1).

a)



b)

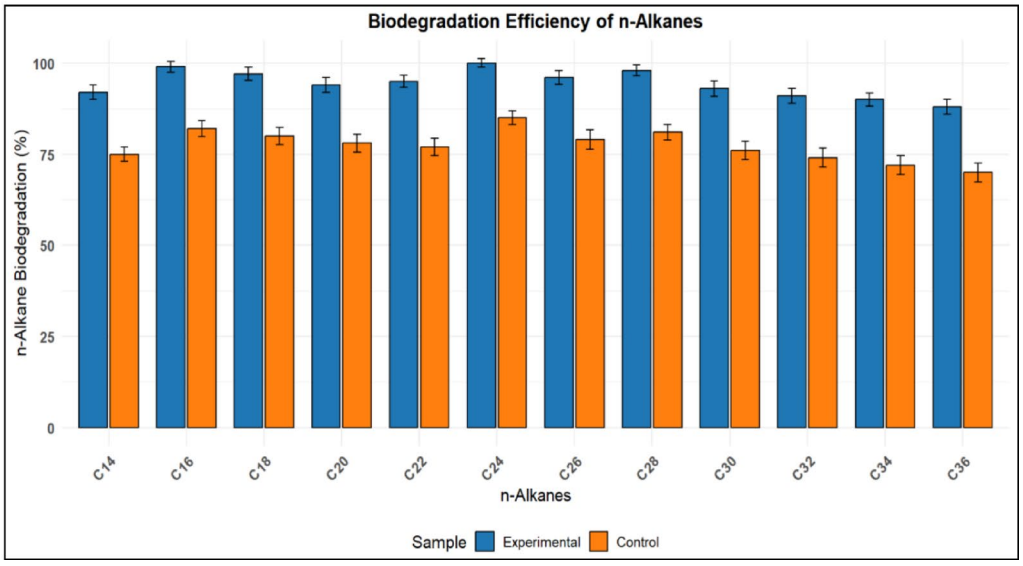


Fig. 5. GC analysis and hydrocarbon efficiency for *Rhodococcus indonesiensis* (SARSHI1).

Experimental design and statistical analysis

RSM–BBD

The emulsification index (E_{24}) and hydrocarbon degradation potential are designated to be the chief factors for evaluating the synergistic efficacy of bacterial isolates in bioremediation studies. Biosurfactants boost the degradation of hydrocarbons by augmenting the bioavailability of hydrophobic substances via dispersion and emulsification processes. Therefore, optimization analysis for biosurfactant production was undertaken as a strategic step for improving the bacterial isolates' overall hydrocarbon degrading potential.

Response surface methodology (RSM) was further employed to scout the optimal mode of interaction between factors and to determine the optimal conditions for the emulsification and hydrocarbon degradation with a minimum number of experimental runs. The RSM application governed the effects of independent variables on the processes, either individually or in a set of factors. The Box-Behnken design constituted 16 experimental runs with 3 replicates of the central point on which the statistical analysis was applied. Table 5 demonstrates the design matrix and outcomes of the BBD experimental design for both processes. The procured

Experiment Runs	x1	x2	x3	Y1	Y1°	Y2	Y2°
1	−1	−1	0	70.57851	73.47136	73.8218	77.23087
2	0	0	0	42.74642	39.81457	46.70406	50.94424
3	0	−1	1	30.67765	32.59617	49.43481	50.64123
4	0	0	1	52.53853	46.93749	63.84704	60.31419
5	1	0	−1	26.51142	31.05745	42.10231	46.30956
6	−1	1	−1	71.58054	72.6419	84.20782	85.43152
7	0	0	0	41.08208	39.81457	56.25718	50.94424
8	0	1	−1	47.32132	45.4028	57.54186	56.33544
9	1	−1	0	26.66056	25.53806	46.98124	46.6926
10	0	0	1	44.52052	46.93749	60.45244	60.31419
11	−1	1	0	45.40641	44.93688	60.83303	59.28612
12	0	0	1	85.34536	81.6565	89.51423	84.67315
13	−1	0	1	41.13432	37.64965	56.29473	53.20887
14	0	0	0	33.49249	39.81457	47.42409	50.94424
15	1	1	0	56.68769	53.26417	64.39713	60.37621
16	0	1	1	63.29129	68.04151	69.16725	74.7178

Table 5. Results for Box-Behnken experimental design. x1 = pH, x2 = Temperature(°C), x3 = Salinity, Y1 = E_{24} (%), Y1° = Predicted E_{24}(%), Y2 = Hydrocarbon Degradation (%), Y2° = Predicted Hydrocarbon Degradation (%).

Coefficients	Estimate	Std. Error	Student T-test	*P*-value	
Emulsification index (%)					
(Intercept)	39.81457	3.0473	13.06552	1.24E-05	***
ph	−9.9015	2.316154	−4.27498	0.005234	**
temp	−0.20209	1.961167	−0.10305	0.921284	
sal	−6.6054	2.057957	−3.20969	0.018374	*
ph*temp	14.06515	2.759902	5.096248	0.00223	**
ph*sal	25.29604	4.875165	5.188756	0.002037	**
temp*sal	17.92476	2.759902	6.494708	0.000634	***
ph^2	6.106697	3.707708	1.647028	0.150651	
temp^2	3.38135	3.647999	0.926905	0.389738	
sal^2	13.72833	3.214631	4.270576	0.00526	**
Hydrocarbon degradation (%)					
(Intercept)	50.94424	3.022466	16.85519	2.79E-06	***
ph	−7.36205	2.297278	−3.20468	0.018491	*
temp	−1.06529	1.945184	−0.54765	0.60369	
sal	−3.91239	2.041185	−1.91673	0.103736	
ph*temp	7.90709	2.73741	2.88853	0.027747	*
ph*sal	22.05424	4.835435	4.560964	0.003847	**
temp*sal	13.10357	2.73741	4.78685	0.003041	**
ph^2	7.58688	3.677492	2.063058	0.084697	.
temp^2	2.365325	3.618269	0.653717	0.537526	
sal^2	13.28234	3.188433	4.165788	0.005906	**

Table 6. Statistical significance of model parameters. The p-value marks the significant nature; '***' denotes p-value < 0.001, '**' denotes p-value < 0.01, '*' denotes p-value < 0.05, '.' denotes p-value < 0.1.

and predicted E_{24} index varied from 26.51 − 85.34%, and 31.05 − 81.65%, respectively, whilst the procured and predicted hydrocarbon degradation varied from 42.10 − 89.51%, and 46.30 − 84.67%, respectively. In both processes, the lowest value was attributed to the 5th run, and the highest value was attributed to the 12^{th} run, suggesting the synergistic behavior between the two.

The experimental data demonstrated in Table 6 was further used to generate a 2^{nd} grade -quadratic polynomial equation model based on three-factor interaction. The model numerically explained the substantial correlation between the experimental and anticipated values for both the E_{24} index and hydrocarbon-degrading potential.

$$E_{24}\,(\%) = 39.8145 - -9.9015ph - 0.20209temp - 6.6054sal + 14.0651\ ph * temp + 17.9247\ temp * sal + 25.29604\ ph * sal + 6.1067\ ph^2 + 3.3813\ temp^2 + 13.7283\ sal^2$$

$$Hyd\ Deg\ (\%) = 50.94424 - -7.36205ph - 1.06529\ temp - 3.91239sal + 7.90709\ ph * temp + 22.05424temp * sal + 13.10357ph * sal + 7.58688\ ph^2 + 2.365325\ temp^2 + 13.28234sal^2$$

For procuring the RSM results, the regression model was further analyzed by ANOVA to test the significance and fitness of the equation (Table 7). Following this statistical study, the model was highly significant, which was confirmed by the Fisher test of F-model value = 14.89, and 9.172 on 9 and 6 DF, and p-value = 0.001881, and 0.00695 for E_{24}, and hydrocarbon degradation, respectively. Consequently, obtaining a 99.81% and 99.304% confidence value of the Fisher F-test, the regression model explained a significant amount of variation in the response values. The coefficient of determination (R^2) was also calculated to determine the outputs of the RSM model. An augmented score implies a more favorable match between the experimental and predicted values. To validate the regression function, the value of the adjusted R^2(adj-R^2) was also evaluated. The adj-R^2 value ought to be high and relatively close to the normal R^2 value. The R^2 and adj-R^2 values corresponded to 95.72% and 89.29%, 93%, and 83%, respectively. The regression model depicts a high degree of accuracy for E_{24}, and hydrocarbon degradation potential. The student's t-test was also used to establish the significance of each coefficient.

The stability test for the bacterial isolate *Rhodococcus indonesiensis* against dependent variables (pH, temperature, and salinity) yielded the E_{24} index values and hydrocarbon-degrading potential values (dependent variables). The results have been displayed in the two-dimensional contour plots and three-dimensional response plots, as illustrated in Fig. 6. One variable is set at a constant level, while the other two variables fluctuate within their defined ranges. Residual plots (Figure S11-S12) and normal plots (Figure S13–S14) determine the significant capability of the model for calculating the emulsification activity, and hydrocarbon degradation, respectively [Supplementary Material 3].

The results of the statistical optimization study suggest the interdependent relationship between hydrocarbon degradation and emulsification activity. The results demonstrate a mutual contribution to effective bioremediation. The dual optimization analysis dictates the bacteria's potential on the industrial scale by mitigating petroleum pollution and adding value on the ecological front.

Validation of experiment

According to the second-order polynomial equation of the RSM-BBD model, experimental validation was conducted under the optimized conditions. Based on the RSM analysis, the parameters for maximum emulsification activity were procured at pH 7, temperature between 20 and 40 °C, and salinity between 3 and 5%. The validations were carried out in triplicate. The modified emulsification activity came out to be E_{24} – 88%, while hydrocarbon degradation came out to be 91%. The obtained biosurfactant production was found to be 4.2 g/L, with biomass 5.08 g/L, suggesting a 1.51- and 1.62-fold increase when compared with the un-optimized conditions. The results suggest an augmented accuracy of the developed model and its validation results. An enhanced response can also be generated by amending the optimization conditions by integrating other chief process parameters. Furthermore, additional tests like CMC determination were also enunciated by taking the optimum conditions, which decreased the CMC value to 55 mg/L and surface tension to 25 mN/m [Supplementary Material 3, Figure S15–S16].

Therefore, the statistically validated model in this study examined the optimized parameters for maximum growth of biosurfactant production and degradation of hydrocarbons. Additionally, it also underlined the

Terms	Df	Sum Sq	Mean Sq	F-value	*P*- value	
Emulsification Index (%)						
First Order	3	1077.846	359.2819	11.56341	0.006613	**
Two Way Interaction	3	2369.991	789.997	25.42589	0.000825	***
Pure Quadratic	3	717.3226	239.1075	7.695626	0.017648	*
Residuals	6	186.4235	31.07058	Nil	Nil	
Lack of fit	3	105.6102	35.20339	1.306842	0.415571	
Pure error	3	80.81328	26.93776	Nil	Nil	
Hydrocarbon degradation (%)						
First Order	3	745.1356	248.3785	8.125917	0.015553	*
Two Way Interaction	3	1111.678	370.5592	12.12316	0.005872	**
Pure Quadratic	3	666.3759	222.1253	7.26702	0.020125	*
Residuals	6	183.3973	30.56622	Nil	Nil	
Lack of fit	3	121.0343	40.34477	1.940803	0.299866	
Pure error	3	62.363	20.78767	Nil	Nil	

Table 7. ANOVA results and lack of fit of the regression model. The p-value marks the significant nature; '***' denotes p-value < 0.001, '**' denotes p-value < 0.01, '*' denotes p-value < 0.05, '.' denotes p-value < 0.1.

a)

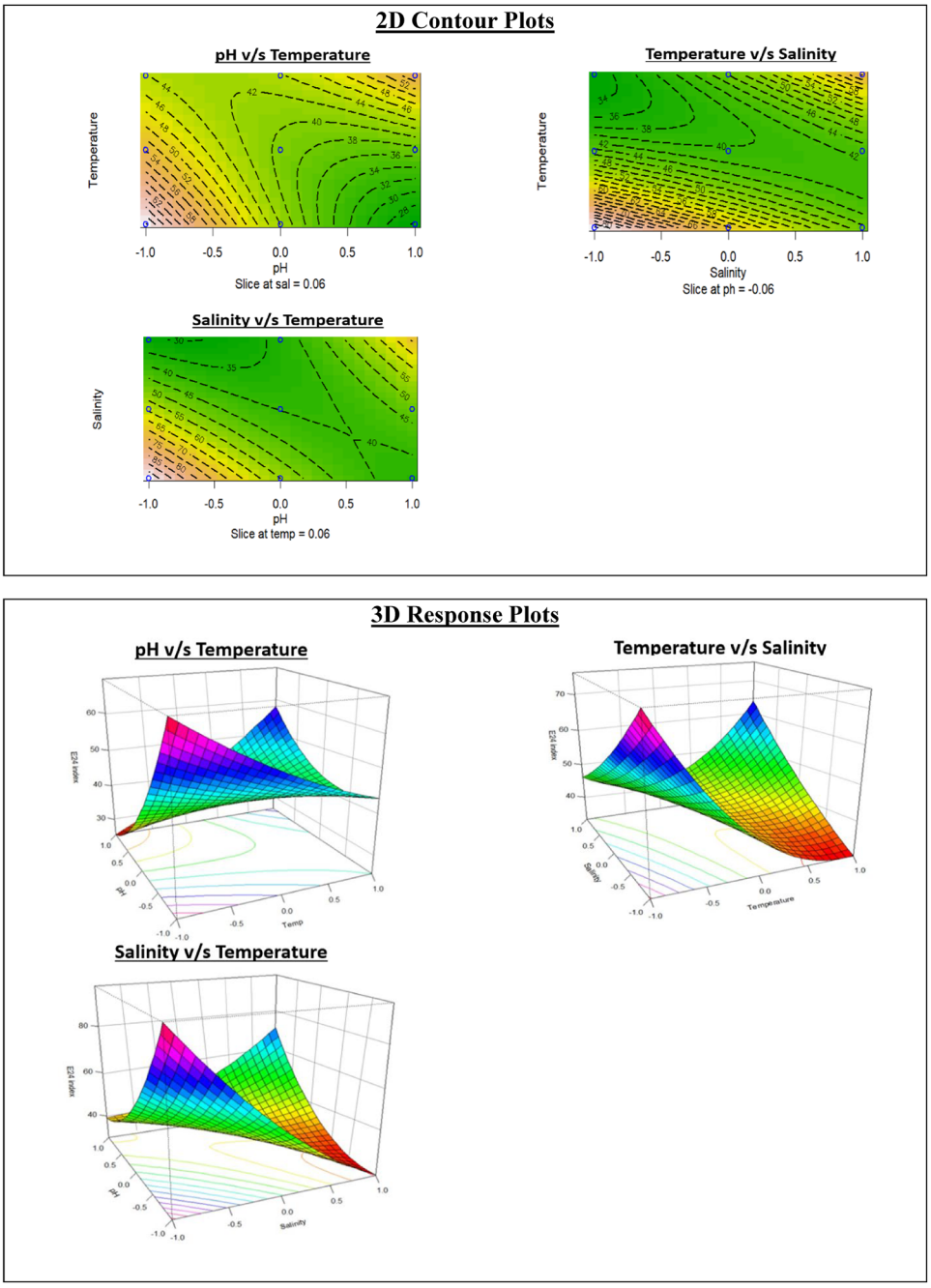


Fig. 6. (**a**) Contour Plots, and 3D response plots for E_{24} index. (**b**) 2D Contour Plots, and 3D response plots for hydrocarbon degradation (%).

reliability of the approach in predicting the outcomes with augmented accuracy. The results indicate that the biosurfactant indeed plays a chief role in emulsifying the hydrocarbons and enhances their bioavailability potential for bacterial hydrocarbon degradation.

DNA isolation, amplification, and 16S rRNA sequencing

After examining the biochemical and biosurfactant-producing characteristics of the five collected samples, they were subjected to DNA isolation and amplification. The phenol/chloroform extracted DNA samples were subjected to thermal cycler conditions for amplifying their gene products, followed by quantification analysis, using the nanodrop and qubit fluorometer. The Qubit fluorometer gave a DNA yield ranging between 1000 μgL^{-1} to 1372.5 $\mu gL^{-1,}$ which was of suboptimal quality and purity for all the samples.

After attaining the quality and purity of the subsequent DNA samples, they were subjected to 16S rRNA Sanger sequencing. The trace files (reverse and forward AB1 files) obtained from the sequencer were assembled

b)

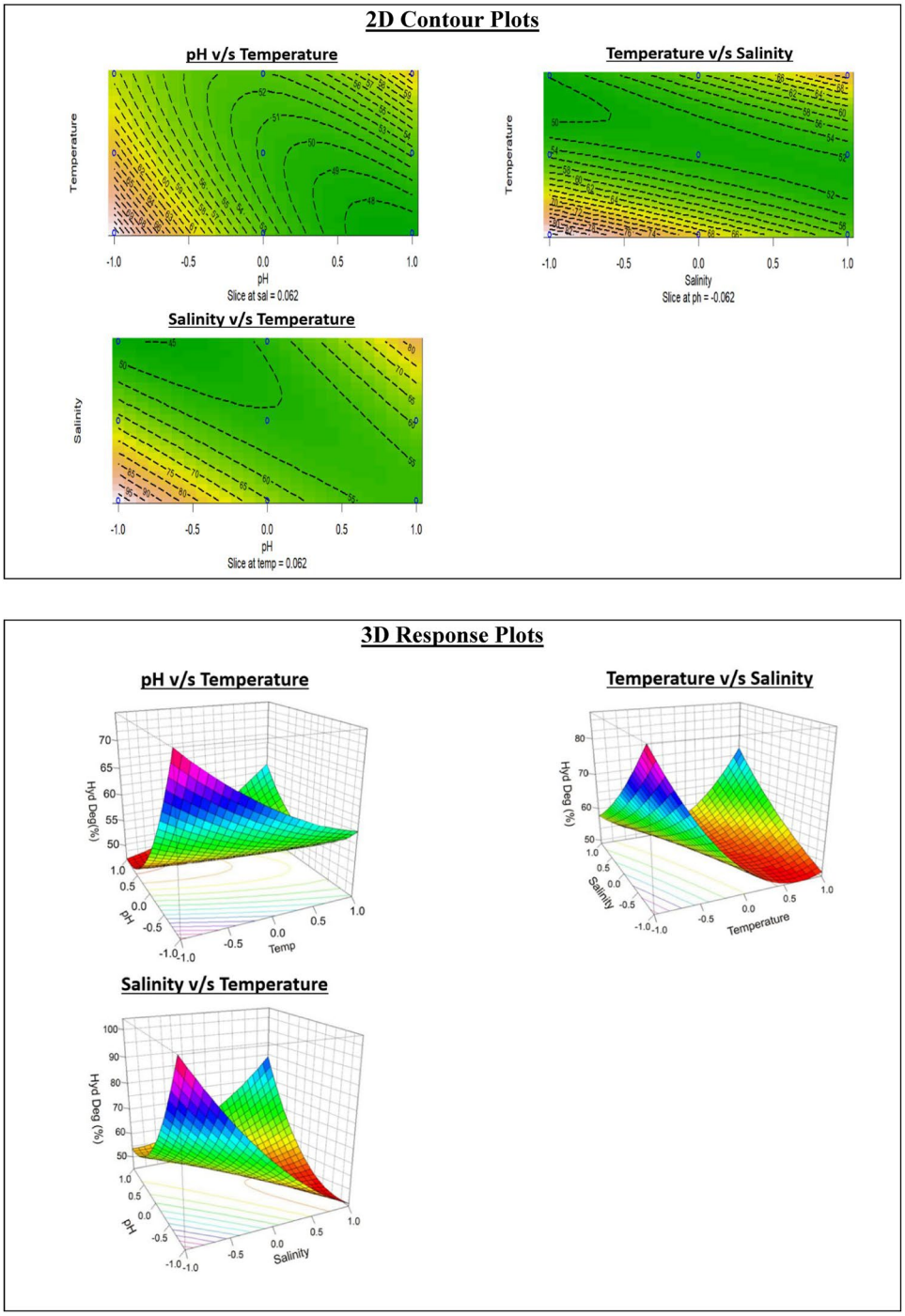


Figure 6. (continued)

and created using DNA Baser software. To facilitate subsequent bioinformatics investigation, the contig file was saved in the FASTA format. The procured sequences were further aligned against the Genbank database using NCBI - BLASTn. The outputs generated a table of several taxonomic sequences with some degree of identity or homology, but none created a 100% absolute similarity.

Consequently, conclusions were reached that the partial sequences were of novel strata. These novel strains were named, *Rhodococcus indonesiensis* strain SARSHI1, *Pseudomonas aeruginosa* strain SARSHI2, *Pseudomonas argentinensis* strain SARSHI3, *Acinetobacter baumannii* strain SARSHI4, and *Rhodococcus qingshengii* strain SARSHI5. The sequences were deposited in the Genbank database with accession numbers: 'PV034287', 'OP597529', 'OP584476', 'OQ711779', and 'OQ711775' with sequence lengths of 720bps, 1372bps, 1336bps, 1215bps, and 1081bps for SARSHI1 to SARSHI5, respectively.

Evolutionary analysis

The BLASTn search generated a taxonomic list that constituted only those sequences that were ~ 97% homologous with the selected sampled sequences, thus governing their novel aspect. The generated hits had query coverage, e-value, percentage identity score, and other chief properties. The phylogenetic assessment was calibrated by utilizing the Muscle algorithm in MEGA-XI software. The trees were constructed using the NJ method. The evolutionary distance was computed by applying the Maximum Composite Likelihood. The UPGMA model delivered the result in terms of the number of base substitutions per site. The matrix generated through the muscle program was competently and manually aligned. Furthermore, the missing data and the coding gaps in the binary format did not affect the phylogenetic branch support and its subsequent topology. The evolutionary lineage had a sum of branch length (SBL) – 0.16, 0.135, 0.129, 0.1414, and 0.0691 for bacterial samples SARSHI1 to SARSHI5, respectively.

The bootstrap consensus trees were inferred from 1000 replicates. The branches corresponding to reproduced partitions were in < 50% bootstrap replicates. The analyses were conducted by taking 20 nucleotide sequences and removing all the ambiguous positions from every sequence pair, using the pairwise deletion method. A total of 720, 1376, 1538, 1226, and 1084 positions were calibrated for the final dataset entry for evolutionary analysis for SARSHI1- SARSHI5 respectively. The phylogenetic tree for *Rhodococcus indonesiensis* strain SARSHI1 has been illustrated in Fig. 7. The respective phylogenetic trees, and heatmaps for all the samples have been represented in [Supplementary Material 4, Figure S17- S21(b)].

Further, the phylogenetic models were devised for determining the alignment results, including the total number of sites, conserved regions, variable regions, singleton regions, and parsim-info sites [Supplementary Material 4, Table S3]. Additionally, a discrete gamma distribution (0.0500) was used to enunciate the difference in the evolutionary rates between these sites. Mean evolutionary rates in these categories were 0.00, 0.00, 0.00, 0.03, and 4.97 substitutions per site. For computing the Maximum Likelihood Estimate (MLE) of the substitution

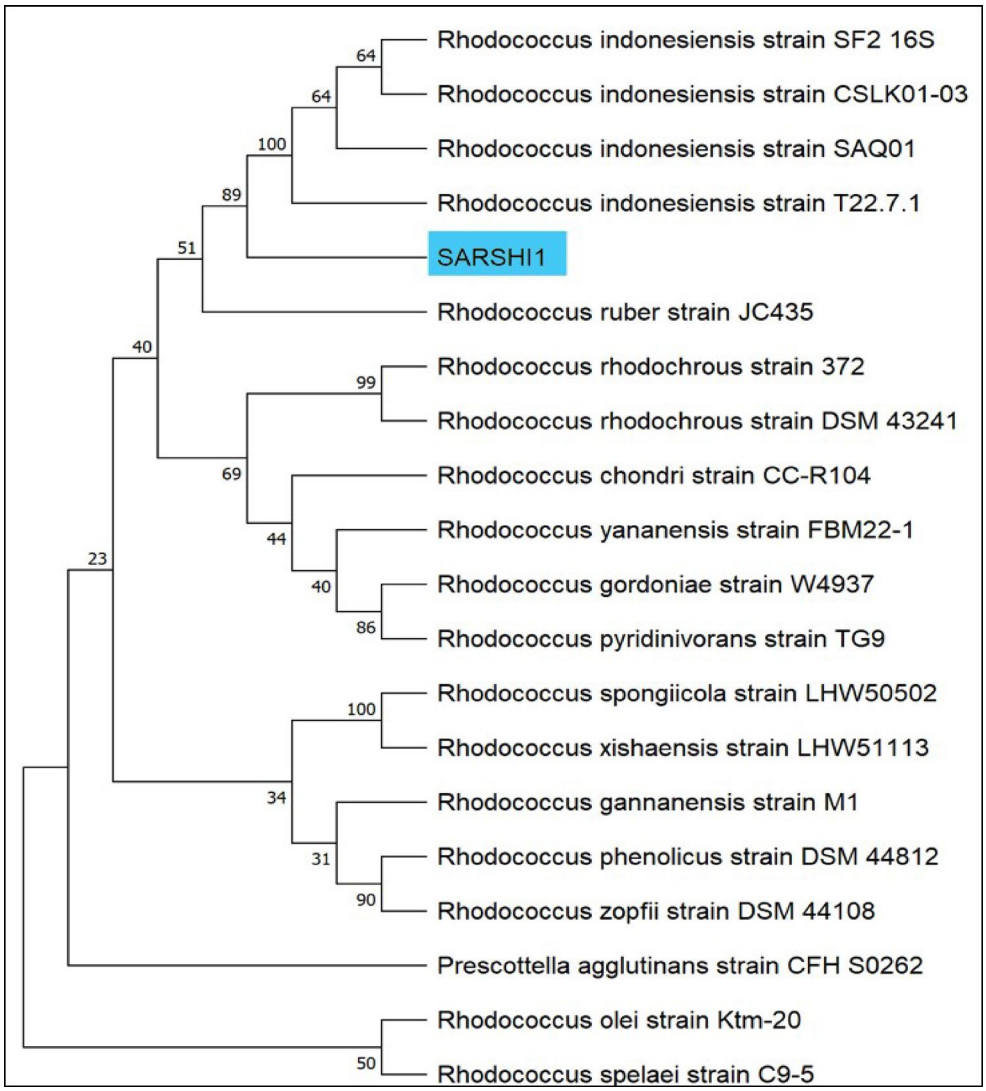


Fig. 7. Phylogenetic Tree for *Rhodococcus indonesiensis* strain SARSHI1.

Strain	Structural Element	ΔG (kcal/mol)	Information
Rhodococcus indonesiensis strain SARSHI1	External loop	−1.70	11 ss bases & 3 closing helices.
	Stack	−3.30	External closing pair is G ^{396}-C ^{717}
	Multi-loop	0.10	External closing pair is C ^{398}-G ^{715} 18 ss bases & 4 closing helices.
	Bulge loop	0.50	External closing pair is G ^{648}-C ^{713}
	Interior loop	−4.20	External closing pair is C ^{393}-G ^{720}
Pseudomonas aeruginosa strain SARSHI2	External loop	−0.50	6 ss bases & 1 closing helices
	Stack	−2.10	External closing pair is A ^{4}-U ^{1369}
	Multi-loop	2.40	External closing pair is C ^{27}-G ^{1347} 3 ss bases & 3 closing helices
	Bulge loop	1.7	External closing pair is C ^{10}-G ^{1362}
	Interior loop	2.30	External closing pair is G ^{6}-U ^{1367}
Pseudomonas argentinensis strain SARSHI3	External loop	−1.80	11 ss bases & 2 closing helices
	Stack	−2.40	External closing pair is C ^{52}-G ^{1332}
	Multi-loop	2.70	External closing pair is G ^{61}-U ^{1322} 22 ss bases & 3 closing helices
	Bulge loop	4.20	External closing pair is A ^{1220}-U ^{1247}
	Interior loop	1.20	External closing pair is U ^{57}-A ^{1327}
Acinetobacter baumannii strain SARSHI4	External loop	−1.00	5 ss bases & 3 closing helices.
	Stack	−3.30	External closing pair is G ^{1196}-C ^{1215}
	Multi-loop	1.70	External closing pair is A ^{126}-U ^{1185} 10 ss bases & 4 closing helices
	Bulge loop	1.70	External closing pair is U ^{1144}-A ^{1166}
	Interior loop	4.50	External closing pair is U ^{1134}-G ^{1177}
	Hairpin loop	4.20	Closing pair is C ^{1201}-G ^{1210}
Rhodococcus qingshengii strain SARSHI5	External loop	−1.00	5 ss bases & 1 closing helices
	Stack	−3.40	External closing pair is G ^{2}-C ^{1077}
	Multi-loop	1.70	External closing pair is C ^{4}-G ^{1075} 10 ss bases & 3 closing helices
	Bulge loop	0.50	External closing pair is G ^{46}-C ^{1064}
	Interior loop	1.40	External closing pair is U ^{52}-G ^{1056}

Table 8. Thermodynamic dataset of folding of 16S rRNA structures.

matrix, each entry was used as the probability of substitution (r) from one base (row) to another (column). The MLE for Transition/Transversion (R): 1.07, 2.62, 1.93,1.25, and 1.34 for SARSHI1- SARSHI5, respectively, was calculated, and the tree topology was enunciated by applying the Kimura2–parameter model [Supplementary Material 4, Table S4]. The results for the Maximum Composite Likelihood (MCL) were also calculated, in which the sum of substitution (r) was made equal to 100, and MCL transition/transversion bias (R) was also calculated [Supplementary Material 4, Table S5]. The distance metric analysis and an average score for the evolutionary distances for all the novel isolates were also determined [Supplementary Material 4, Table S6].

16S rRNA structure prediction

The secondary structure of the 16S rRNA of all the bacterial strains was elucidated using the RNA UNAFold software. It portrayed the helical regions with an interior hairpin, bulge, and multi-branched loops that may have the capacity to bind with 23S rRNA. The bacterial strains SARSHI1- SARSHI5 exhibited free energy (ΔG): −328.70 kcal/mol, −537.20 kcal/mol, −506.70 kcal/mol, −450.30 kcal/mol, and − 454.40 kcal/mol, respectively. The thermodynamic properties of the structures were also determined (Table 8). Moreover, the entropy of RNA structures was estimated by utilizing the RNA fold server that depicted the mountain/hill plot. The 16S rRNA structures and their respective hill plots for SARSHI1 – SARSHI5 have been demonstrated in [Supplementary Material 5, Figure S22(a)-S26(b)].

Microcosm and phytotoxicity study for *Rhodococcus indonesiensis*

Biodegradation analysis of crude oil

The hydrocarbon degradation potency of *Rhodococcus indonesiensis* strain SARSHI1 was examined in crude oil-contaminated soil through microcosm experiments. The results revealed that SARSHI1 exhibited 74–91% biodegradation efficiency for medium to long-chained hydrocarbons (C14–C36). The soil-based systems posed numerous limitations, such as microbial accessibility, oxygen diffusion, and hydrocarbon adsorption. The hydrocarbon degradation was executed among all treatments in the microcosm study. The analyses revealed that the Abiotic Control (AC) displayed modest hydrocarbon degradation (10–20%) due to the abiotic weathering and volatilization processes. The Biostimulation (BS) treatment revealed a degradation efficiency of 35–50%, due to the activity of indigenous microorganisms stimulated by nutrient supplementation. Bioaugmentation

(BA) treatment using SARSHI1 displayed 50–65% degradation, suggesting an effective microbial utilization of hydrocarbons by the introduced bacterial strain. Interestingly, the Biostimulation + Biosurfactant (BS + B) treatment showed an improved degradation efficiency of 60–75%, signifying the role of biosurfactants in refining the hydrocarbon bioavailability and facilitating the degradation potential of indigenous microbes with nutrient supplementation. The combined treatment of Bioaugmentation + Biostimulation (BA + BS) indicated a significant degradation rate of 70–85%, implying the synergistic effect of microbial inoculation and nutrient addition. The highest rate of biodegradation (85–95%) was achieved in the consolidated treatment of Bioaugmentation + Biostimulation + Biosurfactant (BA + BS + B), due to the amplified bioavailability of hydrocarbons that improved the microbial activity in the presence of exogenously added biosurfactant. This suggests that the exogenous biosurfactant indeed increases the efficiency load of the biodegradation activity.

Therefore, the results indicate a synergistic effect of biosurfactant supplementation in combination with microbial augmentation and nutrient enrichment in enhancing the biodegradation of hydrocarbons. The graphical illustration of these findings has been presented in [Supplementary Material 5, Figure S27].

Phytotoxicity analysis

The phytotoxicity assay indicated a significant variation in the vegetative growth and germination index of vegetative seeds, subjected to different bioremediation treatments. The germination rates for *Triticum aestivum*, and *Vigna radiata* were lowest in the AC treatment (15 ± 1.0% and 20 ± 1.2%), indicating significant phytotoxicity in soil. BA (38 ± 1.5% and 40 ± 1.7%) and BS (40 ± 2.0% and 45 ± 2.3%) treatments displayed noticeable improvements in growth for *Triticum aestivum*, and *Vigna radiata* vegetative seeds, respectively. The BS + B treatment (55 ± 1.7% and 60 ± 1.5%) displayed further enhancement, while the BA + BS treatment displayed a substantial improvement in growth (78 ± 1.8% and 85 ± 2.0%). The germination rate was the highest in the BA + BS + B treatment (97 ± 1.2% and 98 ± 1.3%), for *Triticum aestivum*, and *Vigna radiata* vegetative seeds, respectively. This displayed an enhanced growth of leaves and secondary roots, indicating a direct correlation between the germination percentage and hydrocarbon-degrading efficiency. Thus, proving that the concentration of crude oil decreases in the soil, while the germination index increases, enhancing the fertility value of the soil. The results for the germination indices for *Triticum aestivum* and *Vigna radiata* have been represented in [Supplementary Material 5, Table S7 and Figure S28].

Thus, the results enunciate the bioremediation efficiency of the subsequent bacterial sample (SARSHI1) in degrading the hydrocarbons. Furthermore, the addition of crude oil to the soil significantly mitigated the fertilization capacity of the soil. The results stipulate that the bacterial strain can be utilized for practical and industrial-driven applications for enhancing the soil quality and fertility index of environmental and petroleum-polluted soils.

Discussion

Petroleum is a complex mixture of hydrocarbons that plays a paramount role as a strategic energy resource worldwide. Petroleum reservoirs are deep-subsurface ecosystems encompassing extreme physical parameters, including pressure, salinity, and temperature. Despite many benefits, petroleum has been proven to be a bane due to its unquantifiable environmental deterioration properties. Several physical remediation methods have been used, but they cause secondary contamination. Therefore, biological methods were discovered that enhance the scouring of petroleum hydrocarbons, such as biostimulation, bioremediation, and bioaugmentation. Recent studies have shown that indigenous microorganisms from various bacterial genera exhibit oil-degrading potential that allows oil-rich toxins to be converted into non-toxic metabolites by changing their key environmental parameters in the biogeochemical cycles. Although bacterial strains enhance biodegradation, hydrophobicity poses a difficulty for hydrocarbon decomposition. Therefore, petroleum industries employ biosurfactants extensively due to their hydrocarbon absorption capacity and fastidious breakdown of crude oil and petroleum byproducts.

The current study entails studying the organisms that exhibit the propensity to break down petroleum hydrocarbons by forming biosurfactants and display a synergistic relationship between the two. The bacterial cultures were collected from a petroleum gas station site, Nacharam district, Hyderabad, India, in which out of seventy two samples, only five gave positive results, belonging to *Rhodococcus indonesiensis*, *Pseudomonas aeruginosa*, *Pseudomonas argentinensis*, *Acinetobacter baumannii*, and *Rhodococcus qingshengii* species. The selected bacterial isolates underwent numerous biochemical, biosurfactant screening, and hydrocarbon degradation assays; the *Rhodococcus indonesiensis* bacterial strain yielded significant results as compared to other isolates.

Notably, many studies have demonstrated that the bacterial strain *Rhodococcus erythropolis* has been widely studied due to its capability to break down hydrocarbons and produce biosurfactants, thus making it excellent for bioremediation studies[109]. Consequently, *Rhodococcus ruber* has also demonstrated outstanding results in the production of biosurfactants. It augments the degradation rate of hydrocarbons and highlights their potential for environmental cleaning[110]. Furthermore, endophytic *Rhodococcus* strains from *Chelidonium majus L.* have been found for their dual involvement in biosurfactant generation and hydrocarbon breakdown, boosting the species' potential in long-term bioremediation techniques[111].

Several biosurfactant screening tests were conducted to evaluate SARSHI1's capability to produce biosurfactants. The oil spread assay determined a clear zone, suggesting the presence of surface-active reagents. The drop collapse test further established the surfactant properties, with drops collapsing within 50 s, a significant sign of improved surface activity. The hydrocarbon overlay assay established a clearance of 2.11 mm for kerosene and 1.89 mm for benzene, confirming its ability to emulsify hydrocarbons effectively. Lastly, the BATH assay displayed > 90% cell adhesion, suggesting strong hydrophobic interactions, crucial for hydrocarbon degradation.

The study further enunciated the interdependent relationship between biosurfactant production and hydrocarbon degradation. Biosurfactants play a pivotal role in augmenting the bioavailability of hydrocarbons by mitigating the surface tension and emulsifying the hydrophobic compounds, thus facilitating microbial uptake and metabolism. In the current study, *Rhodococcus indonesiensis* strain SARSHI1 significantly reduced the surface tension to 27 mN/m, with a CMC of 70 mg/L, suggesting a robust emulsification capacity. The results were compared with previous studies, and it was found that the subsequent bacterial strain SARSHI1 exhibits superior biosurfactant efficiency. The research conducted by Varjani et al.[105] reported that biosurfactants produced by *Pseudomonas aeruginosa* reduced surface tension to 28 mN/m with a CMC of 80 mg/L. Similarly, another study investigated rhamnolipid biosurfactants and found a surface tension reduction to 30 mN/m and CMC of 90 mg/L, further supporting the enhanced surfactant activity of SARSHI1 and establishing it as a promising candidate for bioremediation applications.

Moreover, the bacterial strain SARSHI1 had the most elevated emulsification activity (E_{24} index – 85.34%). The bacterial strain was modified by optimizing the essential environmental physical parameters required for biosurfactant production. The outcome was attained in the MSM medium at pH 7.0, temperature 40 °C, 3% salinity (NaCl), with 4% of each carbon and nitrogen source, in which sucrose and yeast extract gave the highest growth numbers. These boosted settings were further employed to determine the dry weight of the biosurfactant produced by the microbe. The novel strain of *Rhodococcus indonesiensis* had 0.846 g of biosurfactant in 100 mL of the medium.

Additionally, the gravimetric method was used for assessing the hydrocarbon degradation efficiency of SARSHI1. It displayed a degradation rate of 65% within 21 days, which increased to 84% with glycerol supplementation, conferring a 1.29-fold increase. Previous reports on the gravimetric reduction of crude oil showed that *Pseudomonas sp*. BP10 and *Rhodococcus sp*. NJ2 reduced 43.6% and 32.4% of TPH, respectively, during 30 days[10]. This difference in the efficacy of petroleum degradation can be attributed to the difference in oil type and bacterial metabolic potential. The GC-MS analysis corroborated the biodegradation efficiency, demonstrating complete degradation of C24 hydrocarbons and substantial reductions in C18 (97%) and C16 (99%) fractions. These results align with previous studies showing that hydrocarbon degradation rates range from 48 to 96% over 30 days. The preferential breakdown of mid-chain alkanes by SARSHI1 suggests its potential in bioremediation strategies.

Furthermore, the variability effect of these physical parameters was also determined via RSM-mediated Box-Behnken Experimental Design. Out of 16 experimental runs, the 12^{th} run had the highest E_{24} index (85.34%), while the 5th run had the lowest E_{24} index (26.51%). Subsequently, the same experimental runs had the highest degradation efficiency (89.51%) and the lowest degradation efficiency (42.10%), suggesting the synergistic behavior between the two. Statistical Analysis was performed on the 2nd -grade quadratic polynomial equation using ANOVA and regression analysis, which resulted in a significant regression model ($p < 0.05$). The validation experiments revealed an augmented E_{24} index (88%), a mitigated CMC (55 mg/L) and ST (25 mN/m), and an enhanced degradation rate (91%).

Analytical analysis via primary characterization revealed that the sample SARSHI1 displays an o/w emulsion when tested against two dyes, Sudan Red III and Methyl orange, and contains 30.26% and 15.62% protein and carbohydrate content, respectively. Further NMR analysis revealed that the biosurfactant produced from SARSHI1 is a lipopeptide recorded at 400 MHz. It constitutes various spectrum features, such as a δ 0.87 (2 H, J = 6.84 Hz) triplet has a terminal methyl group, while δ 1.16–2.18 ppm consists of overlapping signals in the aliphatic regions. The methylene protons in there exhibit a variety of coupling patterns, indicating the presence of multiple distinct environments and potentially branched structures. The aromatic spectrum (δ 6.87–7.95 ppm) displayed a complex splitting pattern. In totality, 43 different peaks were identified in the spectrum, highlighting the intricate and complex molecular structure of the biosurfactant.

For molecular characterization, 16S rRNA sequencing technology was used, which revealed that all the isolates had a novel sequence and were further examined by using myriad bioinformatics analysis. These novel strains were named as *Rhodococcus indonesiensis* strain SARSHI1, *Pseudomonas aeruginosa* strain SARSHI2, *Pseudomonas argentinensis* strain SARSHI3, *Acinetobacter baumannii* strain SARSHI4, *Rhodococcus qingshengii* strain SARSHI5 and their subsequent sequences were deposited in NCBI- Genbank database with accession numbers'; 'PV034287', 'OP597529', 'OP584476', 'OQ711779', and 'OQ711775' respectively. These novel strains also underwent phylogenetic analysis by employing MEGA XI software. The 16S rRNA structures were developed by utilizing the UNAFold software and their subsequent thermodynamic properties were also calibrated.

Microcosm studies further evaluated the superior behavior of SARSHI1 in soil-contaminated samples. The effectiveness of the treatments varied, with Abiotic Control (10–20%), Biostimulation (35–50%), and Bioaugmentation (50–65%) displaying modest outcomes. The combined treatment of Biostimulation and Biosurfactant production demonstrated a significant degradation (60–75%), indicating biosurfactant's function in enhancing the hydrocarbon bioavailability and promoting the ability of indigenous microorganisms to degrade hydrocarbons with nutrient supplementation. The highest degradation rate (85–95%) for mediumto long-chain hydrocarbons (C14–C36) under BA + BS + B conditions. The results indicated the synergistic relationship between biosurfactant production and hydrocarbon degradation. The results surpass the previous reports of *Pseudomonas* and *Bacillus* species (Varjani et al., 2020)[105] exhibiting 65–80% degradation, and *Acinetobacter* and *Pseudomonas* (Mishra et al., 2001)[112] with 60–75% degradation. Furthermore, SARSHI1 significantly boosted the soil fertility and phytotoxicity recovery, with seed germination rates reaching 97–98% under BA + BS + B treatment, while previous studies using biosurfactant-producing *Pseudomonas* only improved germination rate to 80% and improved the microbial consortia to 90%[113,114]. Despite the presence of numerous limitations, such as oxygen diffusion and hydrocarbon adsorption, which generally mitigate degradation efficiency, the stipulated bacterial strain SARSHI1 possessed a good ability to adapt to the polluted soil environment. This highlights the strain's potential for practical applications in real-time bioremediation studies to address petroleum pollution.

Therefore, the novel identified and screened bacterial strain, *Rhodococcus indonesiensis* strain SARHI1, demonstrated a significant potential for further investigation in the biotechnological paradigm. It possesses the ability to be produced using an industrial setup to produce a substantial amount of biosurfactant and biodegrade hydrocarbons at a larger scale.

Conclusion

The current study efficaciously isolated, screened, and characterized five novel petroleum hydrocarbon-degrading bacterial strains out of seventy two samples from the oil-contaminated soil site in Nacharam, Hyderabad. Amongst the five isolates, *Rhodococcus indonesiensis* strain SARSHI1 demonstrated the highest potential to degrade petroleum hydrocarbons and produce biosurfactants. The strain ascertained reduced surface tension of 27 mN/m, an emulsification index (E_{24}) of 85.34%, and a biodegradation efficiency of up to 90%. GC-MS and NMR analyses further established preferential degradation of mid-chain alkanes and lipopeptide biosurfactant production. Statistical optimization using the RSM conjugated Box-Behnken Design emphasized the synergistic impact of pH, temperature, and salinity on both hydrocarbon degradation and emulsification efficiency with enhanced quantitative metrics. Microcosm and phytotoxicity analyses further assessed the hydrocarbon biodegradation capability with environmental toxicity. The results highlight the ability of *R. indonesiensis* SARSHI1 to be incorporated into affordable and ecologically sound bioremediation applications, especially in petroleum-polluted conditions.

Data availability

Most of the data generated or analyzed during this study are included in this article. We have included additional data in the supplementary material. The sequence obtained from this study was submitted in GenBank/NCBI with accessions 'PV034287', 'OP597529', 'OP584476', 'OQ711779' and 'OQ711775'. The same can be obtained from the following links. Moreover, the queries can be directed to the corresponding author for any clarifications about the study if needed.https://www.ncbi.nlm.nih.gov/nuccore/PV034287, https://www.ncbi.nlm.nih.gov/nuccore/OP597529, https://www.ncbi.nlm.nih.gov/nuccore/OP584476, https://www.ncbi.nlm.nih.gov/nuccore/OQ711779, https://www.ncbi.nlm.nih.gov/nuccore/OQ711775.

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Declarations

Competing interests
The authors declare no competing interests.

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