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Exploring Biological Laboratory Approach on Production, Characterization and Environmental Applications of Biosurfactant Produced by *Bacillus spizizenii* (S1)

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A B S T R A C T

A crude oil tolerant bacterial strain, *Bacillus spizizenii* S1, was isolated from petroleum hydrocarbon contaminated soil and identified through 16S rRNA sequencing showing 98.3% similarity to *Bacillus spizizenii* and deposited in GenBank under the accession number PV533893. The bacterial strain produced a highly effective biosurfactant when grown on natural substrates, with cotton seed providing the highest yield of 54% at a 1.5% concentration. The biosurfactant showed multiple strong surface-active properties, including excellent oil displacement, foaming and emulsifying abilities. It also exhibited efficient hydrocarbon degradation in DCPIP assays. In soil column studies, treatment with 1% biosurfactant achieved 76 % crude oil removal within 10 days, performing significantly better than synthetic surfactants such as SDS and Triton X-100. GC-MS analysis revealed enhanced solubilization and breakdown of hydrophobic compounds in the treated soil. Phytotoxicity tests using black gram and maize showed better root and shoot growth, confirming its low toxicity and environmental safety. Overall, *Bacillus spizizenii* S1 and its biosurfactant demonstrate great potential as an eco-friendly and effective solution for cleaning up petroleum contaminated environments.

Introduction

Crude oil, is primarily found in porous rock formations within the Earth's crust and is processed into a wide range of chemical products. It is a complex mixture of hydrocarbons along with nitrogen, sulfur, and oxygen-containing compounds and serves as a major source of global energy. However, petroleum hydrocarbons are among the most persistent pollutants, causing significant environmental damage, threatening human health, and impacting aquatic ecosystems (Christova *et al.,* 2019). Petroleum based products plays a major role in the modern society. According to US Environmental Protection Agency (EPA) direct exposure to polycyclic

aromatic hydrocarbons can cause death (Adipah *et al.,* 2019). Crude oil extraction employs various techniques, including steam flooding, cyclic steam injection, and miscible or non-miscible methods (Zhang *et al.,* 2019). Over the past decade, oil spills have released over one billion gallons of crude into the environment, with an estimated six million tons entering oceans annually, severely affecting ecosystems, public health, and marine food chains (Zhang *et al.,* 2019; Rather *et al.,* 2025). Workers at petrochemical sites have also been reported to face higher risks of lung cancer and other chronic illnesses (Devatha *et al.,* 2019).

Traditional physical and chemical remediation methods, though effective, are often expensive, energy-intensive, and environmentally damaging (De Rocchi *et al.,* 2021). In contrast, microbial bioremediation offers an eco-friendly and cost-effective alternative, capable of complete hydrocarbon degradation in contaminated soils and waters (Chunyan *et al.,* 2023). Biosurfactants, surface-active compounds produced by bacteria, fungi, and algae, play a critical role in enhancing hydrocarbon degradation due to their interfacial activity and diverse molecular structures. The global biosurfactant market has also shown substantial growth, increasing from USD 3.99 billion in 2016 to USD 5.52 billion in 2022, with a projected compound annual growth rate (CAGR) of 5.5% through 2026 (Almansoory *et al.,* 2019; Soltanighias *et al.,* 2019; Bellebcir *et al.,* 2023).

Despite advances in microbial remediation using species like *Pseudomonas aeruginosa*, *Bacillus cereus*, and *Marinobacter* sp., many strains face limitations such as low biosurfactant yield, substrate specificity, or poor environmental adaptability (Al-Marri *et al.,* 2023; Deivakumari *et al.,* 2020). The crude oil and its components are hydrophobic and mostly impossible to microbial enzymes but it can be solubilize using surfactant which makes the molecule for the degradation (Varjani *et al.,* 2021). According to (Das *et al.,* 2023) asphaltene is a polycyclic aromatic hydrocarbon with high molecular weight possesses a unique structure present in crude oil. Apart from bacteria the micro-organisms include fungi, actinomycetes plays a crucial role in the crude oil degradation and notable producers of biosurfactant with secondary metabolites (Bellebcir *et al.,* 2023). Biosurfactant produced as a secondary metabolite through *Bacillus sp* (Parthasarathy *et al.,* 2025). In the present study, bacterial isolates from crude oil-contaminated soil were evaluated for hydrocarbon tolerance, degradation ability, and biosurfactant production using natural substrates. The most efficient isolate was identified at the molecular level and further assessed for biosurfactant properties, surface activity, and bioremediation potential through column experiments and phytotoxicity analysis to determine environmental safety and practical applicability.

Materials and Methods

Screening of biosurfactant producing bacteria

The soil sample were collected from the crude oil contaminated area near Mamangam, Salem, Tamil Nadu, India. the soil samples were collected aseptically using sterile polythene bags and transferred into Bioremediation laboratory, Department of Microbiology, Periyar University, Salem, Tamil Nadu, India for further processing. The soil samples were suspended into the sterile distilled water and serially diluted and spread plate technique was performed on nutrient agar and incubated at 37ºC for 24 hours. After incubation, pick the colonies and subculture on the nutrient agar and incubated at 37C for 24 hr. The obtained isolates were stored in nutrient agar slants at 4ºC for further studies. The bacterial isolates of various genera were identified based on their morphological and biochemical characters based on Bergy's Manual of Determinative Bacteriology (Parthasarathy *et al.,* 2025). To screen the crude oil tolerance bacterial isolates by spot plate assay. The MSM (minimal salt agar medium) was supplemented with crude oil at various concentrations from 0.5 to 1% was prepared and sterilized at 15lbs, 121ºC for 20 minutes. The medium with respective concentration of crude oil was transferred into the Petri dish and allowed to solidify. The bacterial isolates were inoculated and incubated at 37ºC for 24 to 48 hours and observed for bacterial growth (Gyasi *et al.,* 2024).

Phylogenetic characterization of potential bacterial strain

The potent bacterial strain S1 was subjected to molecular characterization through 16S rRNA gene sequencing. The genomic DNA of the isolate was extracted by using minor modification of a procedure outlined (Wang *et al.,* 2009). The extraction was with phenol-chloroform extraction protocol and agarose gel electrophoresis to verify DNA quantify and quality. 16s ribosomal RNA was amplified by using the PCR method with Taq DNA polymerase and primers 27F (5′-

AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The conditions for thermal cycling were as follows; denaturation of the target DNA at 95ºC for 4minutes, followed by 30 cycles at 94ºC for 1 minute, primer annealing at 50ºC for 1minute and primer extension at 62ºC for 1 minute. At the of the cycling, the reaction mixture was held at 72ºC for 10minutes and then cooled to 4ºC. The amplified sequence was analyzed using the NCBI BLAST tool, and the gene sequence was submitted to GenBank. Phylogenetic tree was constructed by neighbor joining method using Mega X software.

Biosurfactant production in MSM with various natural substrates

The five different natural substrates such as cotton seed, corn powder, groundnut cake, coconut cake and rice husk were used to produce biosurfactant using potent bacterial strain S1. The mineral salt medium was prepared and sterilized at 121ºC for 15 lbs. The potential bacterial strain S1 and 1% crude oil was inoculated in the medium and kept in rotary shaker at 37ºC and 120rpm for 7 days. After the incubation period, the MSM broth was centrifuged at 5000 rpm for 20 mins.

The supernatant was collected for the extraction and transferred into the into the separating funnel 500 ml. The biosurfactant was extracted through organic solvents such Chloroform and Methanol in the ratio of 2:1 (v/v) (Thirumurugan *et al.,* 2023). The biosurfactant was extracted and estimated by gravimeter analysis. The solvents remained in the extraction was evaporated through fume hood and then collected the biosurfactant sample and stored for further analysis. The weight of the biosurfactant was calculated according to this Eq. (1):

Biosurfactant (%) = [(final-initial)/initial] X 100

Optimization of cotton seed extract as carbon substrate on biosurfactant production

The cotton seeds were optimized for the biosurfactant production at various concentration (0.5%, 1%, 1.5%, 2% and 2.5%) using potent bacterial strain S1. The mineral salt medium was prepared and sterilized at 121ºC for 15 lbs. The potential bacterial strain S1 and 1% crude oil was inoculated in the medium and kept on a rotary shaker at 37ºC and 120rpm for 7 days. After the incubation period, the MSM broth was centrifuged at 5000 rpm for 20 mins. The supernatant was collected for the extraction and transferred into the into the separating funnel 500 ml. The biosurfactant was extracted through organic solvents such Chloroform and Methanol in the ratio of 2:1 (v/v) (Thirumurugan *et al.,* 2023). The biosurfactant was extracted and estimated by gravimeter analysis. The solvents remained in the extraction was evaporated using a fume hood and then collected the biosurfactant sample and stored for further analysis.

Hydrolysis of carbon sources by the selected bacterium

The hydrolysis of carbon sources was evaluated using starch hydrolysis and carbohydrate fermentation tests. For starch hydrolysis, starch agar medium was prepared, sterilized at 121°C for 15 minutes, inoculated with bacterial strains by streaking, and incubated at 37°C for 24 hours. After incubation, the plates were flooded with iodine solution, and the appearance of clear zones around the colonies indicated starch hydrolysis (Vanitha *et al.,* 2019). Carbohydrate fermentation was tested to determine the ability of microorganisms to ferment specific sugars. Phenol red was used as a pH indicator, and Durham tubes were inserted into the fermentation broth to detect gas production. The broth was prepared, sterilized, inoculated with bacterial cultures, and incubated at 37°C for 24 hours. A color change from red to yellow indicated acid production due to fermentation, while gas accumulation in the Durham's tube confirmed gas production.

Bacterial biosurfactant on oil spread assay

Oil spreading assay technique was conducted based on the experimental method of (Das *et al.,* 2023). 20 ml of crude oil was poured into the petri dish containing 50 ml of double distilled water. Then 10 µl of bacterial suspension was added on center of the crude oil. The halo formation was the indication of biosurfactant production.

Bacterial biosurfactant on Foaming index

Foaming index of the biosurfactant in the potent culture medium was determined by shaking the supernatant (10 ml) for 2 min, (Borah *et al.,* 2017) and then foaming was calculated according to the following formula:

Foaming % = (Height of the foam / Total height) × 100

Bacterial biosurfactant on Emulsification index (E24)

The emulsification index was investigated by adding crude oil to biosurfactant 2:4 ratio (v/v). The solution was vortexed with high speed for 2 min and incubated for 24 h.

After the specified time, emulsification percentage was calculated through the height of emulsion layer (Ansari *et al.,* 2021). In addition, the emulsification index was determined for petrol, engine oil, palm oil, diesel, and petrol similar to the steps on which performed for crude oil. The emulsification index was calculated by formula:

E24 = (Height of the emulsified layer/ Total height of solution) × 100

Determination of ionic charge on bacterial biosurfactant

The ionic charge of the biosurfactant produced by the potent bacterial strains was assessed using the agar double diffusion method, as described by (Kumari *et al.,* 2021). A low-hardness agar medium (1% agar without nutrients) was prepared, and two wells were made at a distance of 1.5 cm from each other.

One well was filled with 30 µL of the biosurfactant solution, while the other was filled with a reference compound of known ionic charge.

Sodium dodecyl sulfate (SDS) was used as the anionic control, and barium chloride served as the cationic control. The plates were incubated at room temperature for 48 hours. The formation of a visible precipitation line between the wells indicated ionic interactions, thus revealing the ionic nature of the biosurfactant.

2,6 dichlorophenol indophenol (DCPIP) assay

For a rapid and simple selection of hydrocarbon oxidizing isolates, an assay based on the redox indicator 2, 6-dichlorophenol indophenol (DCPIP, Sigma) was used (Ljesevic *et al.,* 2020). Briefly, a bacterial suspension (1 ml) was added to 9 ml of sterile MSM supplemented with crude oil (10 ml) in a sterile glass test tube. The final concentration of DCPIP was 0.16 mg mL-1. Cultures were incubated at 28°C with agitation (180 rpm) for 24 h. The color of the medium was subsequently observed, and the sample was evaluated as positive for microbial hydrocarbon-degrading ability if colorless (degraded) and negative for microbial hydrocarbon-degrading ability if blue (not degraded).

FTIR characterization of the biosurfactant

FTIR analysis was performed to determine the functional groups of extracted biosurfactant with a Kbr pellet (Shimadzu, IR spirit) range between 400 and 4000cm^{-1} (Zargar *et al.,* 2022; Parthasarathy *et al.,* 2025).

Glass column study on crude oil degradation using bacterial biosurfactant

The removal of crude oil from contaminated soil was carried out using different lab scale column packed with soil and the setup was made as shown in the figure. This is modified set up of bioleaching of heaving metal process reported by (Ayyasamy *et al.,* 2009). The column of 45 cm height and 4 cm width in diameter. Prior to use, each column was washed and sterilized 4-5 times with absolute alcohol (99%). About 3 kg of crude oil contaminated soil was packed in each column under aseptic conditions and closed tightly with holed caps. Each hole was connected with silicon tubes. The tube from the top of the column was inserted into the collection vessel and the tube from the bottom was to the reservoir containing 500 ml of synthetic water. Biosurfactants were obtained from MSM medium with substrates as the carbon sources respectively. Approximately 500 ml of biosurfactant at a concentration of 1% was prepared in a 1L Erlenmeyer flask. The following protocol (Column 1 to 4) was used for the different type of biosurfactant used in the column study:

(A) Column 1: Control (Tap water)
(B) Column 2: 1% Biosurfactant
(C) Column 3: 1% SDS
(D) Column 4: 1% Triton X 100

The samples were passed continuously through the soil column (down to up-flowing) for up to 10 days using a peristaltic pump at a flow rate of 20ml/h. Every 24 hrs, the effluents were collected from each column and total crude oil contents were analyzed gravimetrically. And also, the extracts were collected from the column outlets and used for plant growth in the pot culture experiment.

After completion of the column test, the soil samples were taken out from the columns, dried overnight at room temperature and analyzed the presence of crude oil content by the diethyl ether extraction method and the potential degradation rate was confirmed by GC-MS analysis.

GC-MS characterization of the biosurfactant

GC-MS analysis was performed to the treated and untreated column sample to identify the compounds present in the samples. The mass spectrum was recorded using an instrument Shimadzu (2010 Plus) at Periyar University, Salem, equipped with a silica capillary column (SH-Rxi-5Sil MS column, 30 × 0.25 mm I.D., film thickness 0.25 µm) (Zargar *et al.,* 2022). A 1.0 µl sample of the was injected. Helium was used as the carrier gas. The temperature program was set from Initial temp 50°C for 4 min, ramp 10°C/min to 240 °C hold 2 min and increase 8°C/min to 280 °C hold for 8 min at a rate of 1 mL/min. The scan range was 50–550 Da. The identification of compounds was carried out by comparing their mass spectra with those in the Wiley and NIST 2017 libraries.

Phytotoxicity assay on treated and untreated crude oil contaminated soil

The crude oil contaminated soil was treated in lab-scale column setup with different treatments. After the treatment the soil was dried to remove excess water in the soil. The column 1 and column 2 treated samples were prepared in the different ratio and mixed with fertile soil (20, 40, 60, 80 and 100%) and fertile soil was maintained as control. Further, the soil was filled in small gardening seedling tray with dimension of 54 cm × 26 cm × 4.5 cm. The seedling tray has 50 cavities and of thickness 0.6 mm is used for this study. Each seedling tray containing of 100g of soil and irrigated with the tap water. Seeds of *Vigna mungo* (black gram) and *Zea mays* (Maize) was taken and surface sterilized with $CuSo_4$ solution for 2-3 min and washed with sterile distilled water thoroughly (Deivakumari *et al.,* 2020). Four seeds of each were taken and planted in each cup. Slightly pressed and allowed to germinate. Periodically moisture content was maintained. The growth parameters were assessed at every 24 hours for 10 days. After 10^{th} day, the plants were uprooted and washed thoroughly with distilled water and length of the roots and shoot were measured. All the experiments were conducted in triplicate.

Statistical analysis

All experiments were performed in triplicate, and the results were expressed as mean ± standard deviation (SD). Graphs were generated using Origin Pro 8.5 software. One-way ANOVA with Tukey's post-hoc test was applied, and a p value < 0.05 was considered statistically significant.

Results and Discussion

Selection of potential biosurfactant producing bacterial strain

Totally 10 bacterial isolates were isolated in this soil sample and the isolates were screened with crude oil tolerance by spot plate assay. Among 10 bacterial isolates, strain S1 were shows growth on the MSM agar plates and these isolates were further screened with different experiments. The molecular level identification of the strain S1 were performed based on the 16S rRNA sequence obtained from PCR amplification of the encodes the ribosomal RNA were aligned to those available in the NCBI database for comparison with other sequence of the gene database. The bacterial strain S1 was closely related with 98.3% similarity of *Bacillus spizizenii*. Therefore, the stain was submitted in the NCBI GenBank as *Bacillus spizizenii* strain S1 with accession number was PV533893 and phylogenetic tree were constructed using MEGA X software (Fig 1).

Optimum conditions of natural substrates on biosurfactant production

The production of biosurfactants varied significantly depending on the natural substrate utilized. Among the tested substrates, cotton seed maximum biosurfactant yield (45%), indicating its suitability as an efficient carbon and nutrient source for microbial metabolism. Corn powder (27%) and coconut oil cake (21%) resulted in moderate biosurfactant production, and rice husk (4%) and groundnut oil cake (3%) exhibited minimal production. The effect of cotton seed concentration in mineral salt medium on biosurfactant production was evaluated (Fig 2). Biosurfactant yield increased progressively with substrate concentration up to 1.5%, where the maximum production (54%) was recorded. At lower concentrations, such as 0.5% (15%) and 1.0% (27%), the biosurfactant yield was comparatively low, indicating insufficient carbon availability for optimal

microbial activity. Further increases in substrate concentration resulted in a decline in production, with 2.0% and 2.5% yielding only 22% and 11%, respectively (Fig 3). The reduction at higher concentrations could be due to substrate inhibition or the accumulation of metabolic by-products that negatively influenced microbial growth and biosurfactant synthesis.

Carbon source utilization by *Bacillus spizizenii* (S1)

In the starch hydrolysis assay, a clear zone of 12 mm was observed around the bacterial colonies (Fig 4a). In the carbohydrate fermentation test, the inoculated tubes changed color from red to yellow with gas production, while the control tubes remained red (Fig 4b).

Determination of surfactant properties

Oil spread assay

The oil spread assay was conducted to evaluate the surface activity of the biosurfactant from the different natural substrate. The visualized clear zone was observed under visible light. The biosurfactant derived from cotton seed exhibited the largest clear zone diameter (3.4 ± 0.2 cm), indicating superior surface-active potential compared to other tested substrates.

Groundnut oil cake (2.0 ± 0.2 cm) and corn powder (2.1 ± 0.3 cm) demonstrated moderate displacement activity, whereas coconut oil cake (1.7 ± 0.1 cm) and rice husk (1.6 ± 0.3 cm) showed comparatively lower activity (Fig 5 & Table 1). These results suggest that the cotton seed is the effective substrate among the tested for the biosurfactant production in terms of oil displacement efficiency.

Foaming index

The foaming ability of biosurfactants derived from different natural substrates was evaluated by measuring foam height. Among the tested substrates, cotton seed-derived biosurfactant exhibited the highest foaming activity (3.5 ± 0.1 cm), followed by coconut oil cake (2.0 ± 0.2 cm). In contrast, biosurfactants obtained from rice husk (1.5 ± 0.3 cm), groundnut oil cake (1.5 ± 0.2 cm), and corn powder (0.5 ± 0.1 cm) showed relatively lower foam stability (Table 2). The superior foaming property of cotton seed aligns with its high biosurfactant yield and oil displacement activity, suggesting that cotton seed provides favorable components for producing surface-active and foam-forming molecules.

Emulsification index (E24)

The emulsification index of the biosurfactant was tested against different hydrophobic substrates. The highest emulsification index was observed with crude oil (35%), followed by engine oil (28%) and petrol (22%), indicating strong emulsifying potential of the biosurfactant with hydrocarbon substrates. In contrast, lower emulsification values were recorded with diesel (14%) and palm oil (8%), suggesting limited emulsification stability (Fig 6a). The ionic charge of the biosurfactant was determined using the agar diffusion method. A distinct precipitation line was observed when the biosurfactant was placed opposite to sodium dodecyl sulfate (SDS), an anionic surfactant, whereas no precipitation was detected against barium chloride, a cationic compound (Fig 6b). This interaction pattern confirms that the biosurfactant exhibits a cationic nature, as it formed ionic complexes with the negatively charged SDS but did not interact with the positively charged barium ions. The hydrocarbon degradation potential of the bacterial strain S1 was assessed using the redox indicator 2,6-dichlorophenol indophenol (DCPIP) (Fig 6c). A distinct color change from blue to colorless was observed in the test system containing strain S1, whereas the control sample without bacterial inoculation retained its original blue color.

FTIR Characterization of biosurfactant produced by *Bacillus spizizenii* (S1)

The FTIR analysis was performed for the biosurfactant to identify the functional groups and bonds present in the sample (Fig 9). The biosurfactant produced by Bacillus spizizenii S1 showed peaks at 3281.80, 1370.99cm^{-1} (O-H stretching), 2923.93, 2856.63 and 1462.16 cm^{-1} (C-H stretching), 1657.45 cm^{-1} (C-H bending), 1079.61 cm^{-1} (C-O stretching) and 720.93 cm^{-1} (C=C).

Refinement of crude oil contaminated soil by lab-scale column approach

Crude oil contaminated soil was treated using lab scale column with different treatments and the experiment was carried out for 10 days. Effluent was collected every 24 hours and analyzed gravimetrically to check the removal

of crude oil. Among the four treatments, the column treated with 1% biosurfactant (column 2) showed the highest removal efficiency when compared to other treatments. The removal started from day 1 and gradually increased every day, and by the end of 10th day, around 76% of crude oil was removed from the soil (Fig 7). The SDS-treated column (column 3) showed moderate removal with 48%, and Triton X-100 (column 4) showed around 24% removal and it is toxic to environment. The control column with only distilled water (column 1) showed very low removal, as it had no surfactant activity. The continuous flow of the treatments using peristaltic pump at 20 ml/hr helped in releasing oil from the soil matrix. The effluents collected from biosurfactant-treated columns became less oily after 6–7 days and showed visible reduction in oil content. After the column test, the soil was removed, dried and visually observed. Biosurfactant treated soil appeared less greasy and had less oil smell compared to control.

GC-MS analysis for lab-scale column approach

The GC-MS analysis was performed for the control and treated column sample to identify the chemical compound present in the sample (Fig 8a, 8b & Table 3-4). In the control column, 20 compounds were identified, including esters, ketones, aromatic derivatives, halogenated phenyl esters, and steroid-like structures, with major peaks observed for a triazole derivative (10.83%), cyclohexanone (10.79%), and a dioxa-thiaoctane derivative (10.43%) eluting between 27–34 min. In the biosurfactant-treated column (1% biosurfactant), 20 compounds were also detected but with a distinct profile dominated by hydrophobic and lipid-associated metabolites, such as iodo-alkanes, long-chain hydrocarbons, fatty acid esters, oxygenated terpenoids, and heterocyclic molecules. The biosurfactant column exhibited more evenly distributed peaks, with the highest contributions from a nicotinonitrile derivative (7.99%), cis-5,8,11-eicosatrienoic acid (5.65%), and a triazolopyrimidine carboxylate (7.60%), eluting between 17–37 min. Comparison of the two treatments control column primarily yielded esters, ketones, and aromatic derivatives, the biosurfactant column was enriched with hydrophobic, lipid-like, and heterocyclic bioactive compounds, particularly iodo-alkanes and fatty acid derivatives. The broader spectrum and altered retention times suggest that the biosurfactant enhanced solubilization and recovery of membrane-associated and nonpolar metabolites that were not efficiently extracted with water. This shift highlights the potential of biosurfactants in improving metabolite profiling by enabling detection of compounds.

Phytotoxicity study

In the phytotoxicity study, *Vigna mungo* showed a reduction in shoot length from 13 cm in the control to 5 cm at 100% soil in column 1, whereas biosurfactant-treated column 2 maintained higher shoot lengths, with 12 cm at 20–40% soil and 9 cm at 100% soil.

Root length in column 1 decreased from 3 cm in the control to 1 cm at 100% soil, while column 2 sustained values between 2–3 cm (Table 5). In *Zea mays*, shoot length declined from 14 cm in the control to 6 cm at 100% soil in column 1, while column 2 maintained higher shoot lengths, with 12 cm at 20–40% soil and 10 cm at 100% soil. Root length in column 1 decreased from 4 cm in the control to 2 cm at 100% soil, whereas column 2 sustained values between 3–4 cm. Overall, the biosurfactant treated column exhibited lower phytotoxicity and supported better shoot and root development compared to column 1 (Table 6).

Table.1 Oil spread assay of biosurfactant produced from varying substrates

Substrates Sources	Clear zone in diameter (cm)
Cotton seed	3.4 ± 0.2
Rice husk	1.6 ± 0.3
Coconut cake	1.7 ± 0.1
Groundnut cake	2.0 ± 0.2
Corn powder	2.1 ± 0.3

Table.2 Foaming Index of biosurfactant produced from varying substrates

Natural substrates	Foam height (cm)
Cotton seed	3.5±0.1
Rice husk	1.5±0.3
Coconut cake	2.0±0.2
Groundnut cake	1.5±0.2
Corn powder	0.5±0.1

Table.3 GC-MS table of column provided with 1% distilled water

S. No	Retention Time	Area %	Height %	Compound Name
1	27.695	3.63	4.33	2,6-Dibromo-3-trifluoromethyl-4-nitrophenyl-beta-phenylpropionate
2	28.105	10.83	4.83	1H-1,2,3-Triazol-1-amine, N-[(4-methoxyphenyl) methylene]-4,5-dimethyl
3	29.745	4.27	5.86	Succinic acid, 3-methylbut-2-yl cis-hex-2-en-1-yl ester
4	28.849	10.79	7.78	2-ethyl-4-methoxycyclohexan-1-one
5	29.950	10.43	6.01	1,8-Dioxa-5-thiaoctane, 8-(9-borabicyclo [3.3.1] non-9-yl)-3-(9-borabicyclo [3.3.1] non-9-yloxy)-1-phenyl
6	30.030	4.93	5.03	(2R,3R,4aR,5S,8aS)-2-Hydroxy-4a,5-dimethyl-3-(prop-1-en-2-yl) octahydronaphthalen-1(2H)-one
7	30.140	4.72	4.38	(1S,4R,5R)-1,3,3-Trimethyl-2-oxabicyclo [2.2.2] octan-5-ol
8	30.310	7.94	4.43	3beta-Trimethylsiloxy-5alpha,6alpha-epoxycholestane
9	30.375	3.86	4.46	1-Hydroxy-4-hydroxymethyl-2,2,5,5-tetramethyl-3-imidazoline-3-oxide
10	30.525	5.51	5.00	Pyrimido (5,4-e)-1,2,4-triazine-5,7(1H,6H)-dione, 1,3,6-trimethyl-, 4-oxide
11	30.955	4.33	4.46	Silane, chlorodiethylheptyloxy
12	31.675	2.32	5.11	trans-2-Decen-1-ol, trifluoroacetate
13	32.930	1.17	4.66	3-Quinolinecarboxylic acid, 7-chloro-4-hydroxy
14	33.269	2.48	5.20	2-Methylvaleric acid, cyclohexylmethyl ester
15	33.301	1.43	5.02	Methyl 4-(6-methyl-4-oxoheptan-2-yl) cyclohexane-1-carboxylate
16	33.365	3.44	4.32	2,7-Dimethyl-2,7-octanediol
17	32.505	5.71	5.01	Dimethylmalonic acid, dodecyl pentachlorophenyl ester
18	33.537	4.33	5.95	8-(2-octylcyclopropyl) octanal
19	34.080	2.12	4.06	Terephthalic acid, dec-4-enyl heptyl ester
20	34.131	5.67	4.57	2-Trimethylsiloxy-6-hexadecenoic acid, methyl ester

Fig.1 Phylogenetic tree for potential bacterial strain *Bacillus spizizenii* S1

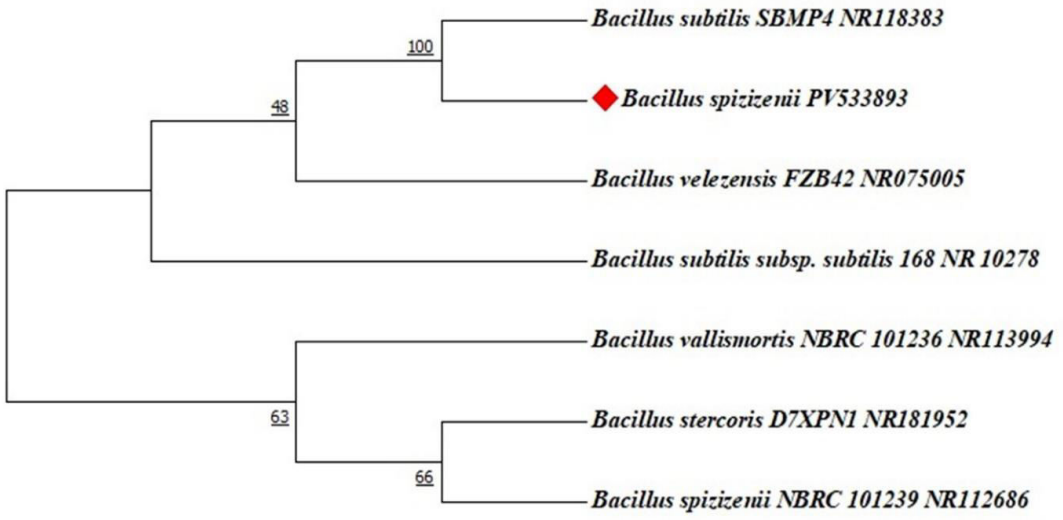


Table.4 GC-MS table of column provided with 1% bio-surfactant

S. No	Retention Time	Area %	Height %	Compound Name
1	17.179	2.80	4.71	3-(4-fluorophenyl) sulfonyl-1-morpholin-4-ylpropan-1-one
2	18.910	3.64	5.82	alpha-D-Xylopyranoside, methyl, 2,4-dimethanesulfonate
3	18.943	4.33	7.30	1-iododododecane
4	20.014	4.74	5.43	1-iodotetradecane
5	20.085	2.80	4.22	5-butylnonane
6	21.055	2.78	5.46	1-iodo-2-methylundecane
7	22.051	3.63	6.10	1-iodo-2-methylundecane
8	22.739	7.09	4.92	2,6,10,15-tetramethylheptadecane
9	23.009	5.13	6.48	1-iodotetradecane
10	26.451	2.79	4.07	2-bromododecane
11	29.675	6.11	4.34	4-hexyl-2-methyl-5-octyl-1,3-dioxolane
12	30.106	5.23	4.27	1-(3-Ethylcyclobutyl) ethenone
13	34.708	5.24	4.44	(2E)-1-Ethoxy-3,7-dimethyl-2,6-octadiene
14	35.190	4.80	4.27	(1S,2E,7E,10R,11E)-10-Methoxycembra-2,4,7,11-tetraene
15	35.264	5.65	4.96	cis-5,8,11-Eicosatrienoic acid, trimethylsilyl ester
16	35.505	7.99	4.71	4-Methoxymethyl-6-methyl-2-(2-oxo-2-piperidin-1-yl-ethylsulfanyl)-nicotinonitrile
17	36.812	6.17	5.35	Cyclohexyl methyl prop-2-enyl carbonate
18	36.883	5.92	4.66	Octahydrochromen-2-one
19	37.150	7.60	4.35	Ethyl 7-amino- [1,2,4] triazolo[1,5-a] pyrimidine-6-carboxylate
20	37.364	5.54	4.13	2-(4-Morpholinyl)-2-oxoethyl 2-pyridinyl sulfide

Table.5 Phytotoxicity assay of black-gram with different column treated soil

Treatment column	Soil ratio					
	Control (cm)	20%	40%	60%	80%	100%
Shoot length						
Column 1	13±0.3	07±0.4	07±0.2	06±0.5	06±0.4	05±0.2
Column 2	13±0.4	12±0.3	12±0.3	10±0.6	10±0.3	09±0.3
Root length						
Column 1	03±0.3	02±0.2	02±0.2	02±0.3	01±0.2	01±0.2
Column 2	03±0.2	03±0.4	03±0.3	02±0.3	02±0.2	02±0.3

Table.6 Phytotoxicity assay of maize plant with different column treated soil

Treatment column	Soil ratio					
	Control (cm)	20%	40%	60%	80%	100%
Shoot length						
Column 1	14±0.2	10±0.4	09±0.3	07±0.4	07±0.4	06±0.3
Column 2	14±0.3	12±0.2	12±0.3	11±0.4	10±0.2	10±0.4
Root length						
Column 1	04±0.2	02±0.2	02±0.3	02±0.2	02±0.3	02±0.1
Column 2	04±0.3	04±0.1	03±0.2	03±0.4	03±0.2	03±0.2

Fig.2 Biosurfactant production with natural substrates

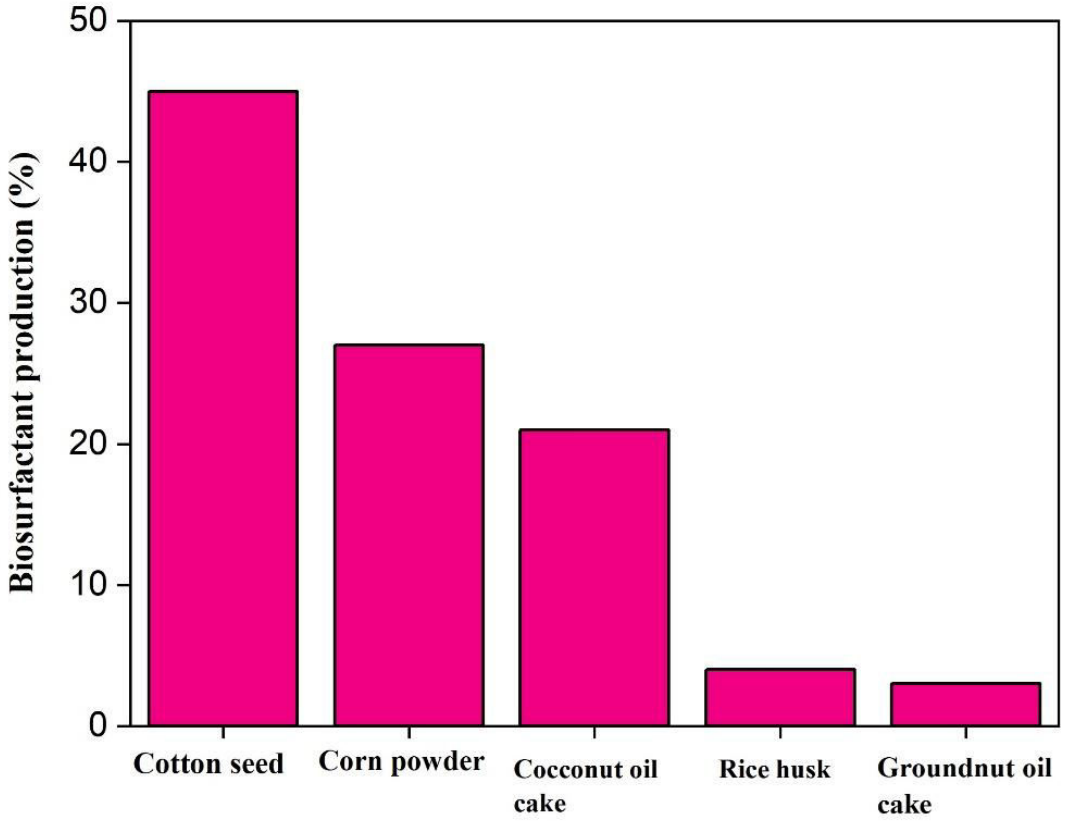


Fig.3 Optimization of cotton seed for biosurfactant production with various concentration

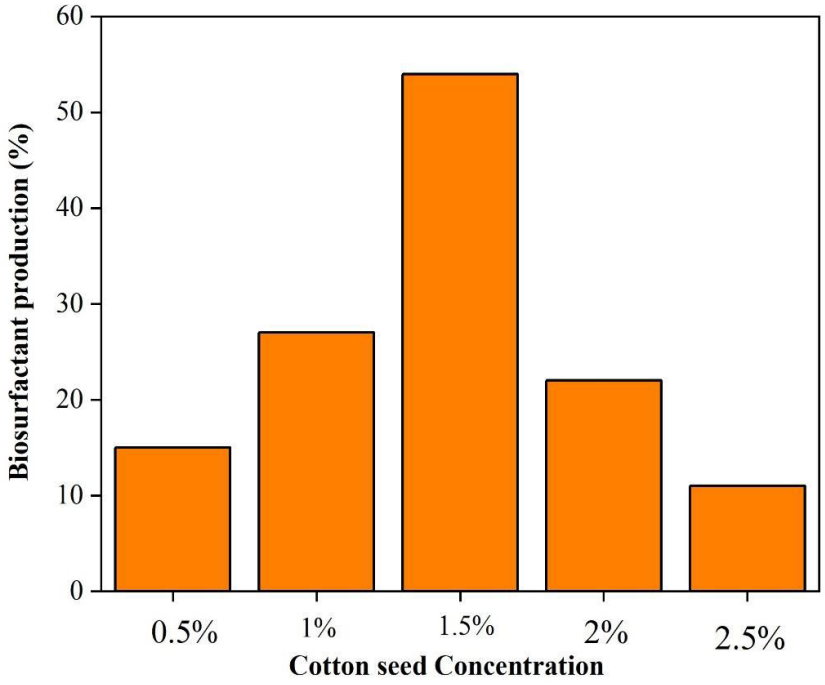


Fig.4 Hydrolysis of carbon source using potent strain S1 a) Starch hydrolysis; b) Carbohydrate fermentation test

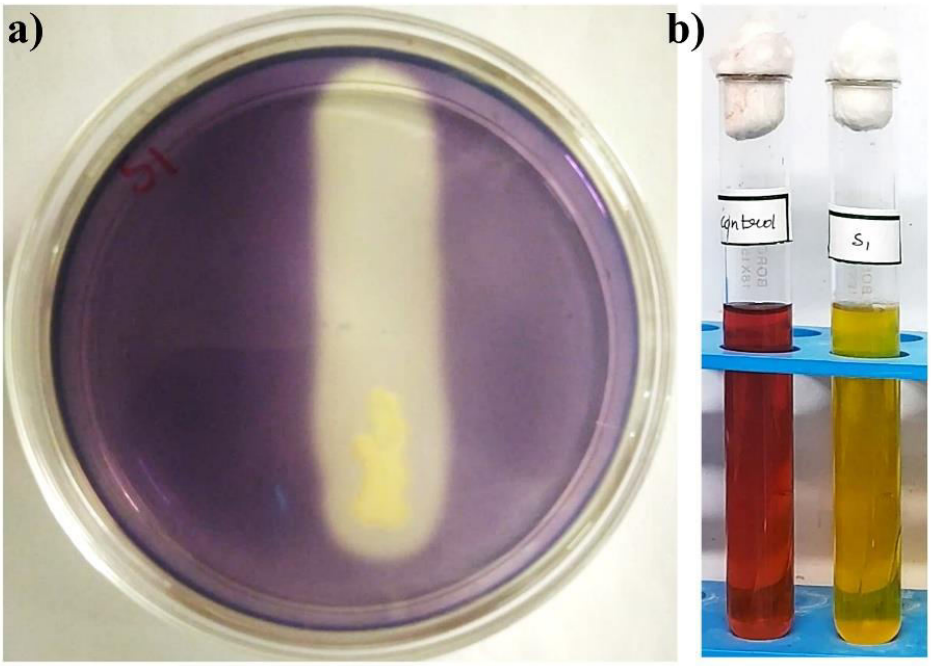


Fig.5 Oil displacement assay a) Cotton seed; b) Rice husk; c) Coconut cake; d) Groundnut cake; e) Corn powder

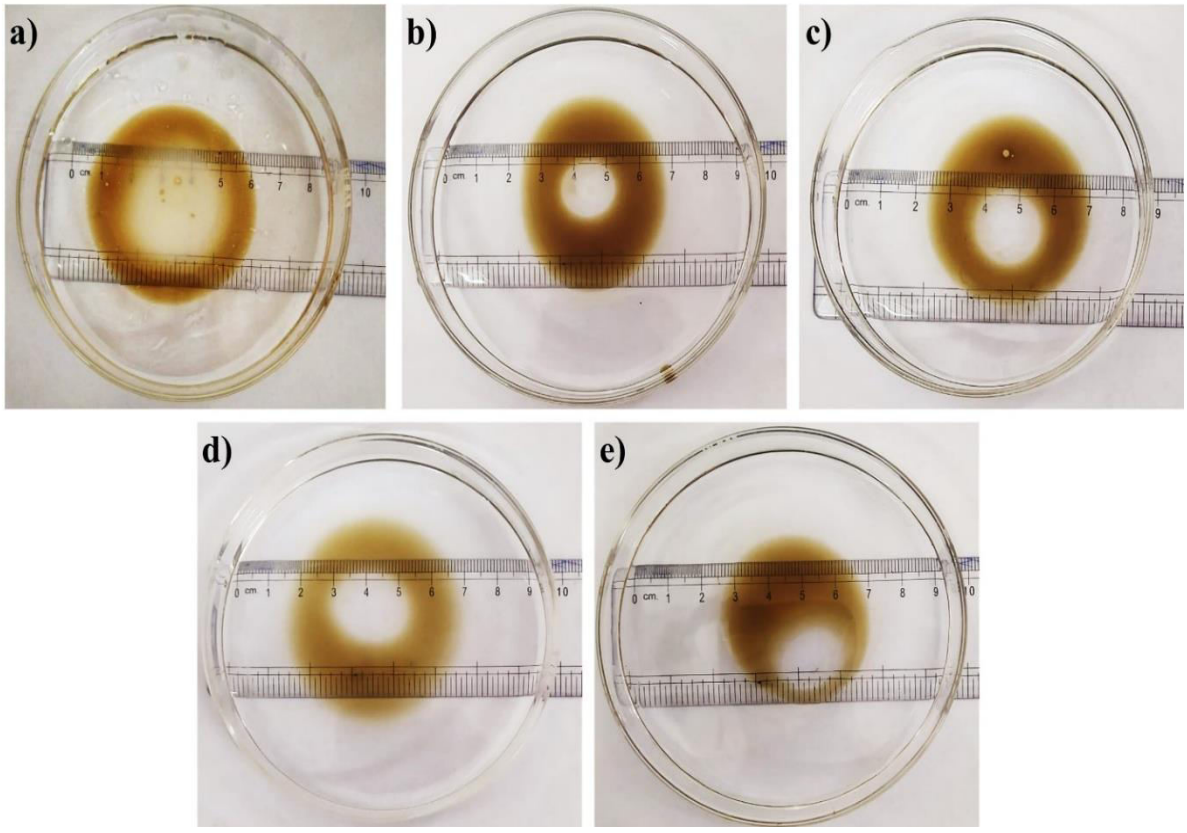


Fig.6 a) Emulsification Index (E24); b) Ionic Charge; c) DCPIP assay

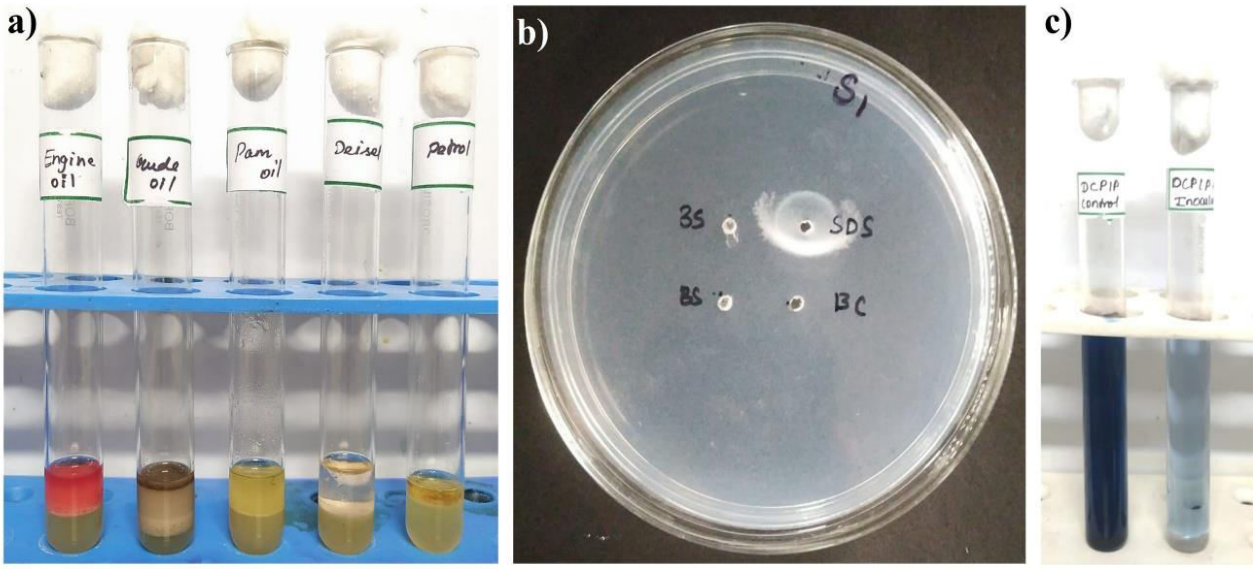


Fig.7 Removal of crude oil in contaminated soil using biosurfactant, Triton-X 100, SDS through various column treatments

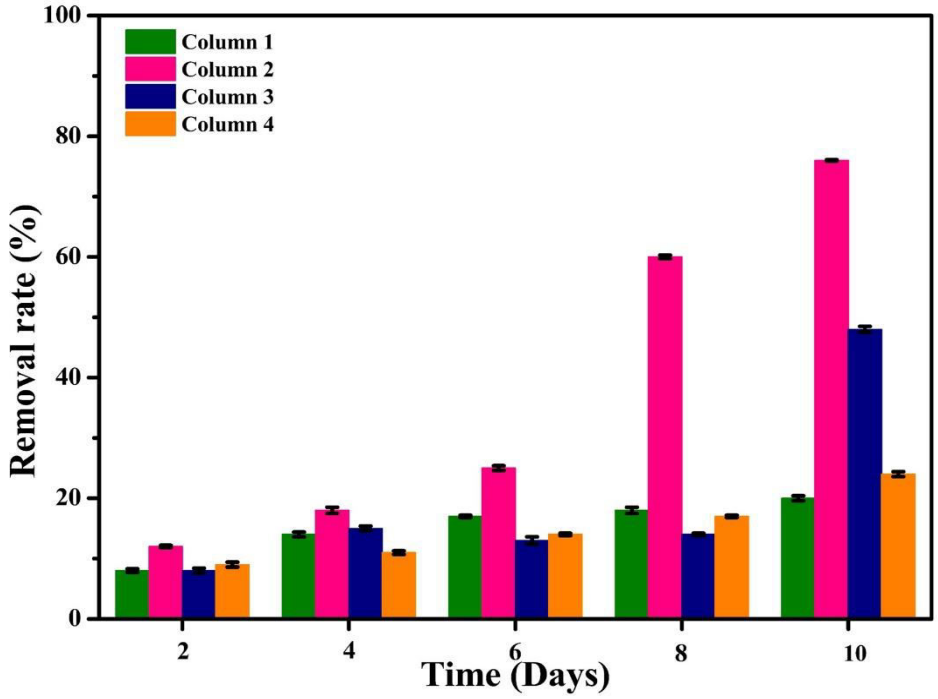


Fig.9 FTIR analysis of the biosurfactant

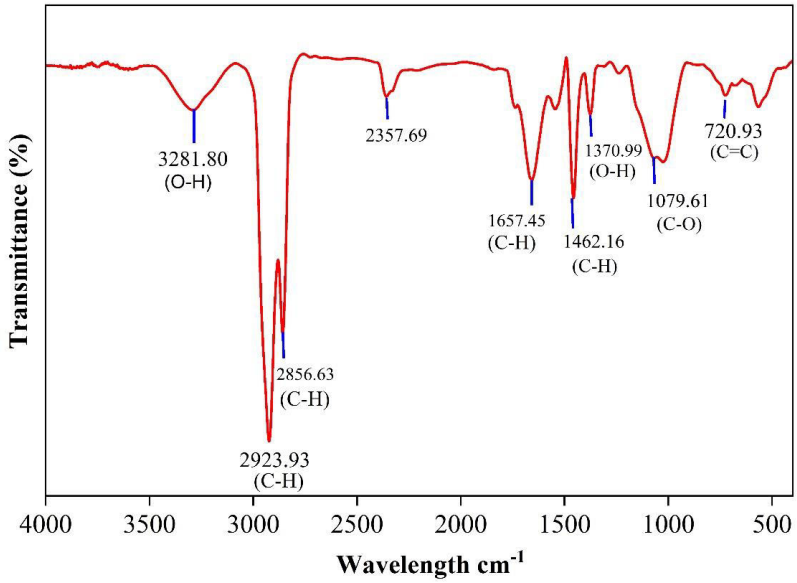
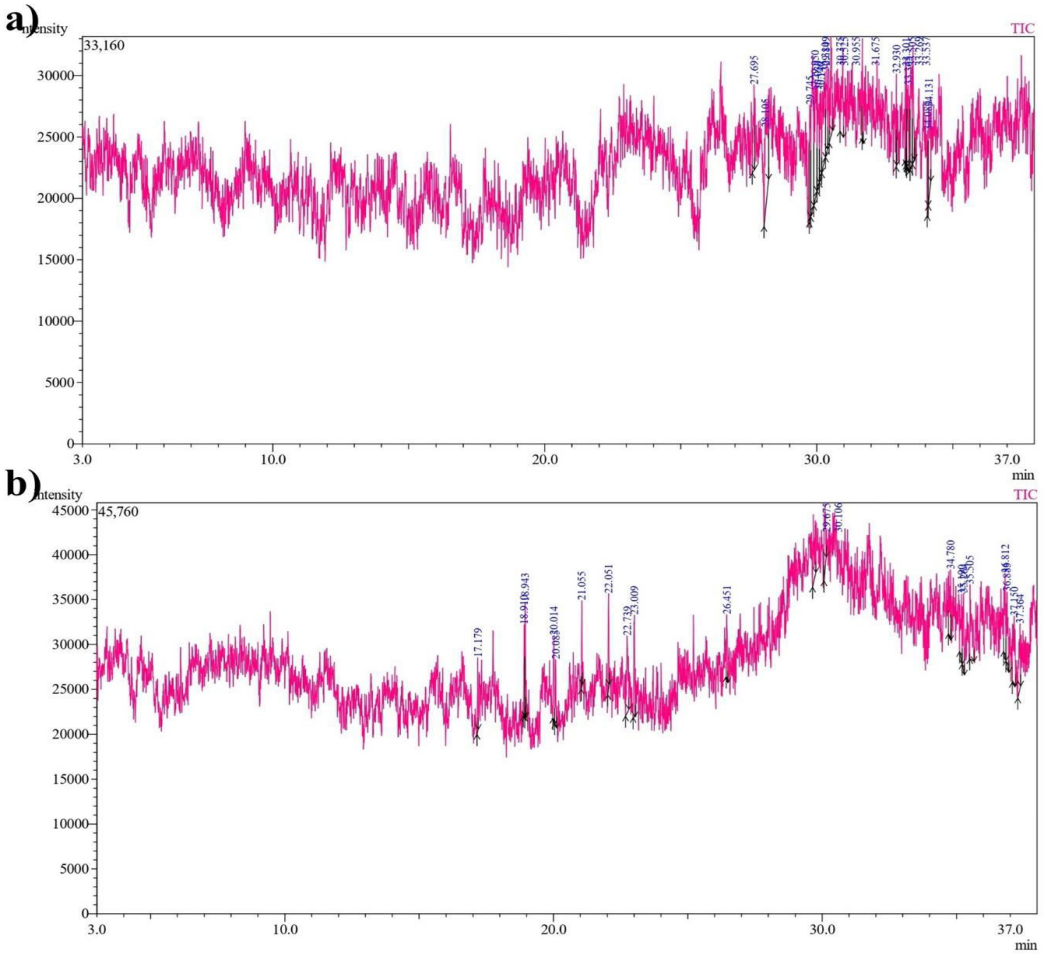


Fig.8 a) GC-MS chromatogram of control column (Treatment 1); b) GC-MS chromatogram of biosurfactant column (Treatment 2)



In the present study, crude oil-contaminated soil was collected from Mamangam, Salem, Tamil Nadu, India. Among the ten bacterial isolates obtained, strain S1 showed high tolerance to crude oil, efficiently produced biosurfactant, and degraded hydrocarbons effectively. The strain S1 was biochemically characterized as a Gram-positive *Bacillus* sp. and molecularly identified through 16S rRNA sequencing as *Bacillus spizizenii* (S1), with the NCBI accession number PV533893.

Similar studies have reported that *Bacillus thuringiensis* (SSL1) and *Bacillus cereus* (SSL3) showed high tolerance to crude oil and efficiently degraded crude oil hydrocarbons while producing biosurfactants (Das *et al.,* 2023). Previous research has also revealed that thermophilic bacteria such as *Bacillus* and *Geobacillus* isolated from oil-contaminated sites effectively degrade hydrocarbons and form biofilms that contribute to corrosion reactions (Elumalai *et al.,* 2019). Five natural carbon sources were evaluated for biosurfactant production, among which cottonseed showed the highest yield. Optimization experiments indicated maximum biosurfactant production at 1.5% cottonseed concentration. Likewise, sucrose has been shown to enhance microbial growth and biosurfactant yield (Nayarisseri *et al.,* 2018; Parthipan *et al.,* 2017). Sugarcane molasses, a low-cost substrate, has also been reported to support maximum biosurfactant production by *Lactobacillus* strains (Mouafo *et al.,* 2018).

In this study, *Bacillus spizizenii* S1 exhibited strong oil displacement, foaming, and emulsification activity with cottonseed as the substrate. The biosurfactant produced was cationic in nature and formed an ionic complex with SDS. DCPIP assay results confirmed that strain S1 could degrade hydrocarbons present in crude oil and utilize them as a carbon source. A previous study reported that strain OCNW9 showed the highest foaming activity and oil spreading diameter of 45 mm on paraffin oil, with emulsification rates of 85% for xylene and 86% for hexane (Ewida *et al.,* 2019). Similarly, four strains (PET1, PET3, GAR1, TP2) isolated from oil-contaminated sites also tested positive for biosurfactant production (Rizvi *et al.,* 2024). Other reports have shown that *Candida lipolytica* and other *Candida* species produce anionic biosurfactants (Rufino *et al.,* 2014; Luna *et al.,* 2013). The isolate HDB5 exhibited higher petroleum hydrocarbon degradation efficiency (27.5%) compared to other isolates (Veerapagu *et al.,* 2019), while *Pseudomonas borborid* achieved complete degradation of petroleum hydrocarbons within 120 hours (Balogun *et al.,* 2013). According to (Ljesevic *et al.,* 2020) the biodegradability of petroleum hydrocarbons was higher in both strains of Rhodococcus sp RNP05 and Planomicrobium sp RNP01 which reduces the blue to colourless of the DCPIP solution. Lab-scale column studies demonstrated higher crude oil removal efficiency in biosurfactant-treated columns compared to untreated controls. Another study indicated that oil content in disturbed and undisturbed soil columns was richer in aromatic compounds at deeper depths, while resin content was lower (Zhang *et al.,* 2022). The biosurfactant-assisted system (BAS) achieved 88.7% hydrocarbon reduction after 60 days, whereas other treatments (NA, BS) showed comparatively lower reductions (Napp *et al.,* 2022).

GC-MS analysis of the control column revealed major peaks between 27–35 minutes, corresponding to triazole derivatives and cyclohexanone, commonly found in crude oil. In contrast, the biosurfactant-treated soil column exhibited hydrophobic, lipid-associated metabolites and heterocyclic compounds between 17–37 minutes, consistent with previous findings (Rather *et al.,* 2025). (Ljesevic *et al.,* 2024) reported that GC-FID analysis (Lower molecular weight compounds degraded faster, while heavier fractions persisted longer (Popoola *et al.,* 2021). (Zargar *et al.,* 2022) reported that obtained peaks during FTIR analysis were perfectly matches with LC-MS spectrum. Gordonia sp. IITR 100 produced biosurfactant are rich in lipids and carbohydrates confirms by the TLC plate assay. The biosurfactant produced by CIG -6A^{T} was characterized using FTIR reports that functional groups and bands presence with lactonic SL (Kumari *et al.,* 2021). Phytotoxicity assays using *Vigna mungo* and *Zea mays* demonstrated healthy plant growth in treated soil, with germination success ranging from 20% to 100%. Enhanced growth performance in treated soil indicated reduced toxicity and improved soil quality (Deivakumari *et al.,* 2020). In the previous study (Parthasarathy *et al.,* 2025) plant growth exhibits 80% on seed germination, 97% on leaf and roots elongation which indicates the treated samples are free from toxicity.

In conclusion, the crude oil–tolerant strain *Bacillus spizizenii* S1 was isolated and identified with 98.3% similarity through 16S rRNA sequencing. Cotton seed was found to be the most effective substrate, yielding 54% biosurfactant with strong oil displacement, foaming, and emulsifying activities. The cationic biosurfactant efficiently degraded hydrocarbons and

achieved 76% crude oil removal in soil within 10 days, surpassing synthetic surfactants. GC–MS analysis confirmed enhanced breakdown of hydrophobic compounds, while phytotoxicity tests showed improved plant growth in treated soil. In conclusion, *B. spizizenii* S1–derived biosurfactant shows great potential for eco-friendly bioremediation of petroleum-contaminated environments.

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Authors Contribution

Sona Shanmugasundaram, Dharanisha Vaithilingam and Pudukadu Munusamy Ayyasamy have equally contributed to this work (Conceptulization, Methodology, Investigation and Original draft Writing) Revising the draft and manuscript editing: Sona Shanmugasundaram Pudukadu Munusamy Ayyasamy, Gopikirshnan Venugopal: Review and editing. Funding Acquistion: Sona Shanmugasundaram

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Data availability

All data generated or analyzed in this study are included in this article.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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