Microbiology Research International
Vol. 13(1), pp. 23-29, February 2025
DOI: 10.30918/MRI.131.25.010
ISSN: 2354-2128
Full Length Research Paper

Isolation and identification of biosurfactant-producing bacteria from hydrocarbon-contaminated mechanic workshop soils in Port Harcourt, Nigeria

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Accepted 19 February, 2025

ABSTRACT

Biosurfactants are microbial-derived compounds known for their ability to reduce the surface tension of viscous liquids. They play a key role in solubilizing hydrophobic substrates, such as petroleum. Soils in mechanic workshops are often contaminated with petroleum or crude oil, making them ideal environments for biosurfactant-producing bacteria. This study aimed to isolate and characterize such bacteria from mechanic workshops in Port Harcourt, Nigeria, for potential bioremediation applications. Soil samples were collected from five different mechanic workshop sites in Port Harcourt. Bacterial enumeration revealed counts ranging from 7.85×10^4 to 2.01×10^5 CFU/g, with Sample D having the highest bacterial count and Sample A the lowest. Microbiological tests identified the bacterial isolates as belonging to the genera *Bacillus* (15%), *Sphingomonas* (10%), *Klebsiella* (20%), *Staphylococcus* (20%), *Proteus* (5%), *Escherichia coli* (25%), and *Paenibacillus* (5%). Biosurfactant production was assessed using three screening methods: the oil spreading assay, the drop collapse assay, and foaming capacity. *Bacillus* spp. and *Klebsiella* spp. tested positive for all three assays, indicating their strong biosurfactant-producing potential. *Escherichia coli* was positive for the drop collapse assay but negative for oil spreading and foaming capacity. *Sphingomonas* spp. tested positive for the drop collapse assay and foaming capacity but negative for the oil spreading assay. *Paenibacillus polymyxa* was positive for the oil spreading assay and foaming capacity but negative for the drop collapse assay. *Staphylococcus* spp. was positive only for foaming capacity, while *Proteus* spp. was positive for the oil spreading assay and drop collapse assay but negative for foaming capacity. The study concluded that *Bacillus* spp. and *Klebsiella* spp. exhibited the highest biosurfactant-producing activity. These findings highlight the potential of mechanic workshop soils in Port Harcourt as a valuable source of biosurfactant-producing bacteria, which could be utilized for bioremediation purposes.

Keywords: Biosurfactants, bioremediation, mechanic workshop soils, petroleum contamination, bacterial isolation, screening assays.

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INTRODUCTION

Biosurfactants are surface-active compounds produced by microorganisms, playing a crucial role in microbial metabolism and environmental interactions. These amphiphilic molecules, composed of hydrophilic and hydrophobic domains, reduce surface and interfacial tension, thereby enhancing the bioavailability of hydrophobic compounds (Díaz De Rienzo et al., 2021; Marchant and Banat, 2023). Biosurfactants have gained significant attention due to their applications in bioremediation, pharmaceuticals, agriculture, and the food industry, offering advantages such as biodegradability, low toxicity, and effectiveness under extreme environmental conditions (Muthusamy et al., 2020; Mnif and Ghribi, 2021).

Microorganisms known for biosurfactant production include bacteria, fungi, and actinomycetes. Among bacteria, *Pseudomonas aeruginosa* and *Bacillus subtilis* are well-documented producers, synthesizing rhamnolipids and surfactin, respectively (Silva et al., 2022). Other bacterial genera, such as *Klebsiella, Enterobacter,* and *Acinetobacter,* also contribute to biosurfactant synthesis (Jimoh et al., 2021). Fungal species, including *Candida bombicola* and *Aspergillus niger,* produce sophorolipids and mannosylerythritol lipids, which are valuable in industrial applications (Santos et al., 2023). Actinomycetes, particularly *Streptomyces* spp., have been identified as promising biosurfactant producers, demonstrating potent emulsification and antimicrobial properties (Raja et al., 2024).

Given the growing environmental concerns associated with hydrocarbon pollution, the search for efficient biosurfactant-producing microorganisms has intensified. Hydrocarbon-contaminated environments, such as mechanic workshop soils, serve as reservoirs for microbial populations adapted to hydrocarbon degradation and biosurfactant production (Olawale et al., 2021). The ability of these microorganisms to enhance the solubilization and breakdown of petroleum hydrocarbons makes them valuable candidates for bioremediation strategies (Adetunji and Olaniran, 2022).

This study aims to isolate and identify biosurfactant-producing bacteria from hydrocarbon-contaminated soils collected from mechanic workshops in Port Harcourt, Nigeria. By employing cultural, biochemical, and molecular identification techniques, this research seeks to characterize the potential of these bacterial isolates in biosurfactant production and assess their applicability in environmental remediation.

MATERIALS AND METHODS

Description of study area

Port Harcourt is located in the Niger Delta region of southern Nigeria. The city lies between latitudes 3°37'N and 3°56'N and longitudes 11°10'E and 11°45'E, approximately 50 km from the Atlantic coast. It experiences an annual average precipitation of 3,030 mm and an average temperature of 23°C.

Sample collection

Soil samples were collected from five mechanic workshops in Port Harcourt, Nigeria. The specific locations and their corresponding coordinates were as follows: Emenike (4°47’52.758"N, 6°59’069"E), Agip (4°48’30.96"N, 6°59’9.384"E), Ikoku (4°48’0.57"N, 7°0’32.328"E), Nkpolu-Oroworukwo (4°48’16.584"N, 6°59’9.798"E), and Elekahia (4°49’15.138"N, 7°1’25.494"E). Using a handheld soil auger, 5 g of soil was collected from each site at a depth of 10–15 cm and placed in sterile polythene bags. The collected samples were then transported to the microbiology laboratory in an ice-packed cooler to preserve microbial integrity.

Microbiological Analysis

Serial dilution

Serial dilutions were performed according to the method described by Ogbonna and Inana (2018). One gram (1 g) of each soil sample was aseptically transferred into a sterile test tube containing 9 mL of sterile normal saline and thoroughly mixed using a vortex mixer to obtain a homogeneous suspension. A series of 10-fold serial dilutions (up to 10^{-4}) were then prepared by transferring 1 mL of the initial suspension into a fresh tube containing 9 mL of sterile normal saline. This process was repeated sequentially for subsequent dilutions, ensuring the reduction of microbial load and facilitating bacterial enumeration and isolation.

Inoculation and Incubation

Aliquots (0.1 mL) from the 10^{-2}, 10^{-3}, and 10^{-4} dilutions were aseptically inoculated onto sterile Bushnell Haas agar plates (HiMedia, India) supplemented with crude oil-soaked filter paper, as described by Satpute et al. (2010). The spread plate technique was employed using a sterile bent glass rod to evenly distribute the inoculum across the agar surface. The plates were then incubated in an inverted position at 37°C for 3–5 days under aerobic conditions to allow for the growth of hydrocarbon-degrading bacteria.

Enumeration of bacterial colonies

After incubation, bacterial colonies on the Bushnell Haas agar plates were counted manually. Each plate was inoculated in duplicate with 0.1 mL aliquots from the 10^{-2}, 10^{-3}, and 10^{-4} dilutions, and the average colony count was determined. The results were expressed as colony-forming units per gram (CFU/g) of the soil sample.

Distinct bacterial colonies with unique morphological characteristics (e.g., color, size, margin, and texture) were selected and subcultured onto freshly prepared nutrient agar plates to obtain pure cultures, following the method of Bodour et al. (2003).

Isolation and preservation of bacterial colonies

The pure bacterial isolates were preserved on nutrient agar slants in sterile Bijou bottles and stored at 4°C for further characterization (Fracchia et al., 2012). Long-term

preservation was carried out by storing bacterial isolates in glycerol stocks (15% v/v) at -20°C for subsequent molecular and biochemical analysis (Jha et al., 2016).

Identification and characterization of bacterial isolates

Gram staining

Gram staining was performed to differentiate bacterial isolates into Gram-positive and Gram-negative groups (Rivenson et al., 2023). A thin smear of bacterial culture was prepared on a glass slide, heat-fixed, and sequentially stained with crystal violet for 60 seconds, followed by Lugol's iodine for 60 seconds. The smear was then decolorized with 95% ethanol for 10–15 seconds and counterstained with safranin for 30 seconds. After air drying, the stained slide was observed under an oil immersion microscope at 100× magnification. Gram-positive bacteria appeared purple, while Gram-negative bacteria appeared red (Bergey and Holt, 2000).

Biochemical tests

Motility test: Assessed using the stab culture method in semisolid nutrient agar. Motility was indicated by diffuse growth radiating outward from the stab line (Tille, 2017).

IMViC tests:

Indole Test - A positive result was indicated by a cherry-red ring after the addition of Kovac's reagent (Forbes et al., 2018).

Methyl Red test - A red color indicated stable acid production, while yellow indicated a negative result (MacFaddin, 2000).

Voges-Proskauer test - A red or pink coloration after adding reagents confirmed acetoin production (Holt et al., 1994).

Citrate Utilization test - A blue color change indicated citrate utilization (MacFaddin, 2000).

Catalase test: Oxygen bubbles after adding hydrogen peroxide indicated catalase activity (Cheesbrough, 2006).

Oxidase test: A blue-purple coloration confirmed a positive result (MacFaddin, 2000).

Sugar Fermentation test: A color change from red to yellow indicated acid production, while gas bubbles confirmed gas production (Tille, 2017).

Starch Hydrolysis test: A clear brown zone around bacterial growth indicated starch hydrolysis (Forbes et al., 2018).

Triple Sugar Iron Agar (TSIA) test: Results were interpreted based on color changes, gas production, and hydrogen sulfide (H_2S) precipitation (MacFaddin, 2000).

Screening for biosurfactant production

Biosurfactant-producing bacteria were screened using different qualitative assays, including the oil spread assay, drop collapse assay, and foaming capacity test (Satpute et al., 2010; Yadav et al., 2021).

Oil spread assay: Performed according to Morikawa et al. (2000), a clear zone of oil displacement indicated biosurfactant production.

Drop collapse assay: Following Jain et al. (1991), a collapsed droplet indicated biosurfactant production, while a stable droplet suggested a negative result.

Foaming capacity test: Foaming capacity was determined using the method of Cooper and Goldenberg (1987). Bacterial isolates were grown in Bushnell Haas broth supplemented with 1% crude oil and incubated at 37°C for 48 hours under shaking conditions (150 rpm). After vortexing, the height of foam was recorded, and foaming capacity (%) was calculated using the formula:

$$\text{Foaming capacity (FC) [\%]} = \frac{\text{height of foam}}{\text{total height}} \times 100.$$

RESULTS

Microbiological Count

The bacterial population in soil samples from five different mechanic workshops in a 102-aliquot sample is as follows: Sample A (Emenike) – 7.85×10^4, Sample B (Agip) – 9.9×10^4, Sample C (Ikoku) – 1.68×10^5, Sample D (Nkpolu-Oroworukwo) – 2.01×10^5, and Sample E (Elekahia) – 1.81×10^5 (Table 1).

Table 1. Hydrocarbon Utilizing Bacterial Count of soil samples from mechanic workshops (CFU/g).

Sample Code	x10^2 (CFU/g)
Sample A	7.85×10^4
Sample B	9.9×10^4
Sample C	1.68×10^5
Sample D	2.01×10^5
Sample E	1.81×10^5

KEY: Sample A - Emenike, Sample B - Agip, Sample C - Ikoku, Sample D - Nkpolu-Oroworukwo, Sample E - Elekahia.

Bacterial isolates in different mechanic workshop soil samples

The cultural morphology and biochemical test results of

bacterial isolates obtained from soil samples across different mechanic workshops are presented in Table 2. The findings revealed the presence of three Gram-positive bacterial species belonging to the genera *Staphylococcus spp.*, *Bacillus spp.*, and *Paenibacillus spp.* Additionally, four Gram-negative bacterial species were identified, including *Escherichia coli*, *Proteus spp.*, *Klebsiella spp.*, and *Sphingomonas spp.*

The distribution of bacterial isolates across different mechanic workshop soil samples, as shown in Table 3, varies by location. *Escherichia coli* was present in all five samples (A–E), indicating its widespread occurrence in mechanic workshop soils. *Sphingomonas spp.* was detected in samples A and B but was absent in the other locations, suggesting a more restricted distribution. *Klebsiella spp.* was found in samples A, B, D, and E but was absent in sample C. *Proteus spp.* appeared only in sample B, indicating limited occurrence. *Staphylococcus spp.* was present in samples A, B, C, and E but was absent in sample D. *Bacillus spp.* was detected in samples A, C, and D, while *Paenibacillus spp.* was found only in sample E.

The percentage occurrence of bacterial species in the mechanic workshop soil samples was as follows: *Escherichia coli* (25%), *Sphingomonas spp.* (10%), *Klebsiella spp.* (20%), *Proteus spp.* (5%), *Staphylococcus spp.* (20%), *Bacillus spp.* (15%), and *Paenibacillus spp.* (5%), as represented in Figure 1.

Biosurfactant production screening of bacterial isolates

The biosurfactant production screening of bacterial isolates was conducted using the Oil Spreading Assay (O.S.), Drop Collapse Assay (D.C.), and Foaming Capacity (F.C.), revealing variations in activity among the tested species (Table 4).

Table 2. Biochemical tests result of the bacterial isolate.

S/N	Isolate code	Gram reaction	Gram shape	Surface	Color	CT	OX	MT	SH	CU	IN	MR	VP	GLU	LAC	SUC	Slant	Butt	Gas	H_2S	Probable Organism
1	MEC1	+	Rod	Shiny	Cream	+	+	+	+	+	-	+	+	A	A	A	A	A	-	-	*Bacillus spp*
2	MEC2	-	Rod	Smooth	Cream	+	-	+	-	-	+	+	-	A	A	A	B	A	-	-	*Escherichia coli*
3	MEC3	-	Rod	Shiny	Cream	+	-	-	-	+	-	-	+	A	A	A	A	A	+	-	*Klebsiella spp*
4	MEC4	-	Rod	Smooth	Orange	+	+	+	+	-	-	-	+	A	A	A	A	A	-	-	*Spingomonas spp*
5	MEC5	-	Rod	Shiny	Cream	+	-	-	-	+	-	-	+	A	A	A	B	A	-	-	*Klebsiella spp*
6	MEC6	+	Rod	Smooth	Cream	+	-	+	+	-	-	+	+	A	A	A	A	A	-	-	*Bacillus spp*
7	MEC7	+	Cocci	Smooth	Cream	+	-	-	-	+	-	+	-	N	A	A	B	A	-	-	*Staphylococcus spp*
8	MEC8	-	Rod	Shiny	Cream	+	-	-	-	+	-	-	+	A	A	A	A	A	-	-	*Klebsiella spp*
9	MEC9	-	Rod	Smooth	Cream	+	-	+	-	+	-	+	+	A	N	N	B	B	-	-	*Proteus spp*
10	MEC10	-	Rod	Shiny	Cream	+	-	+	-	+	-	-	+	A/G	A	A	B	A	-	-	*Klebsiella spp*
11	MEC11	+	Rod	Smooth	Cream	+	-	-	+	-	-	+	+	A/G	A	A	B	B	-	-	*Paenibacillus spp*
12	MEC12	+	Cocci	Smooth	Cream	+	-	-	-	-	+	-	+	A	A	N	B	B	-	-	*Staphylococcus spp*

KEY: + = Positive, - = Negative, CT = Catalase, OX = Oxidase, CU = Citrate Utilization, IN = Indole, MT = Motility, SH = Starch Hydrolysis, MR = Methyl Red, VP = Voges-Proskauer, GLU = Glucose, LAC = Lactose, SUC = Sucrose, AG = Acid/Gas, A = Acid, N = Negative, B = Alkaline/Base.

Table 3. Occurrence of bacterial isolates in the different mechanic workshops soil sampled.

Bacterial isolates	Sample A	Sample B	Sample C	Sample D	Sample E
Escherichia coli	+	+	+	+	+
Sphingomonas spp	+	+	-	-	-
Klebsiella spp	+	+	-	+	+
Proteus spp	-	+	-	-	-
Staphylococcus spp	+	+	+	-	+
Bacillus spp	+	-	+	+	-
Paenibacillus spp	-	-	-	-	+

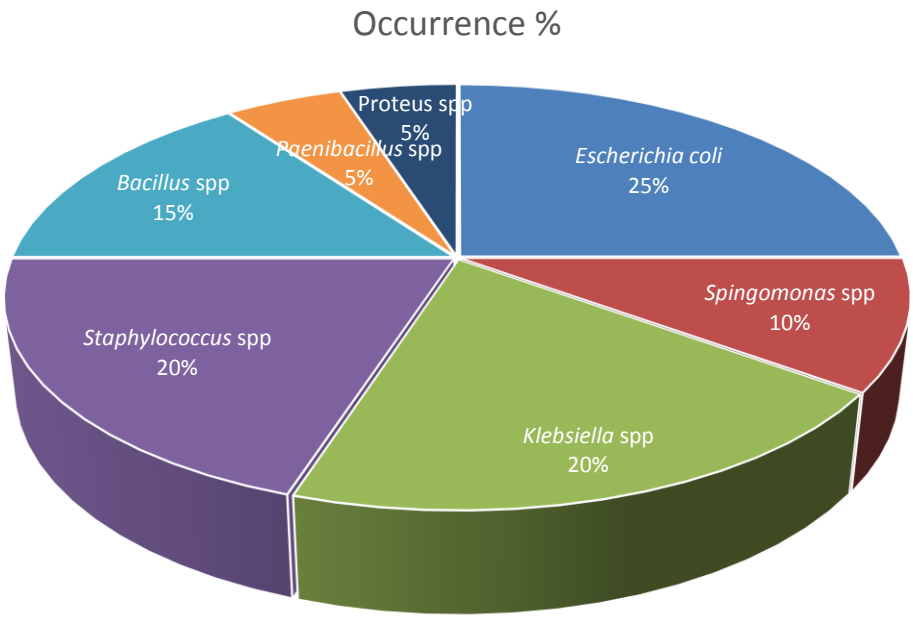

Figure 1. Percentage occurrence of bacterial isolates.

Table 4. Biosurfactant activity of bacterial isolates.

S/N	Isolates	Oil Spreading Assay (O.S)	Drop Collapse Assay (D.C)	Foaming Capacity (F.C) (%)
1	*Bacillus* spp	+	+	14.3
2	*Escherichia coli*	-	+	-
3	*Klebsiella spp*	+	+	3.1
4	*Sphingomonas* spp	-	+	26.3
5	*Paenibacillus polymyxa*	+	-	35.3
6	*Staphylococcus* spp	-	-	16.7
7	*Proteus* spp	+	+	-

KEY: + = Positive, - = Negative.

- *Bacillus spp.* tested positive for both oil spreading and drop collapse assays and exhibited a foaming capacity of 14.3%, indicating moderate biosurfactant production.
- *Escherichia coli* was negative for oil spreading but positive for drop collapse, suggesting surface activity without significant emulsification properties.
- *Klebsiella spp.* tested positive for both oil spreading and drop collapse assays but exhibited a low foaming capacity of 3.1%, indicating limited biosurfactant production.
- *Sphingomonas spp.* demonstrated a strong foaming capacity of 26.3% and was positive for drop collapse but negative for oil spreading, suggesting that its biosurfactant may not efficiently reduce surface tension.
- *Paenibacillus polymyxa* exhibited the highest foaming capacity (35.3%) and was positive for oil spreading but negative for drop collapse, implying strong emulsifying properties.
- *Staphylococcus spp.* tested negative for both oil spreading and drop collapse assays but had a moderate foaming capacity of 16.7%, indicating possible biosurfactant production with limited surface activity.
- *Proteus spp.* was positive for both oil spreading and drop collapse assays but did not exhibit any foaming capacity, suggesting that while it produces biosurfactants, they may not generate stable foams.

DISCUSSION

This study investigated the isolation, identification, and screening of bacteria for biosurfactant production in hydrocarbon-contaminated soils. Hydrocarbon-polluted soils are known to harbor diverse biosurfactant-producing bacteria, which play a crucial role in the biodegradation and bioremediation of oil-contaminated environments. The activity and distribution of these bacteria are influenced by factors such as pollutant type (e.g., crude

oil), essential nutrients, and organic matter content (Ejike et al., 2021; Iram et al., 2023).

The total hydrocarbon-utilizing bacterial counts from soil samples collected at five different sites in Port Harcourt—Sample A (Emenike), Sample B (Agip), Sample C (Ikoku), Sample D (Nkpolu-Oroworukwo), and Sample E (Elekahia)—revealed that Sample D exhibited the highest bacterial count, while Sample A had the lowest. The elevated bacterial population in Sample D is likely attributed to the high levels of hydrocarbon contamination (e.g., used motor oil, petroleum products) and heavy metals commonly associated with mechanic workshops. These findings align with reports showing a positive correlation between contamination levels and bacterial population density, as hydrocarbon-degrading bacteria thrive in polluted environments by utilizing hydrocarbons as carbon and energy sources (Rahman et al., 2022; Okpala et al., 2023).

To facilitate the uptake of insoluble hydrocarbons, these bacteria produce biosurfactants, which enhance hydrocarbon degradation and emulsification. Bacterial isolates identified from the samples included three Gram-positive genera—*Bacillus, Staphylococcus*, and *Paenibacillus*—and four Gram-negative genera—*Escherichia coli, Klebsiella* spp., *Sphingomonas* spp., and *Proteus* spp. Among these, *Escherichia coli, Sphingomonas* spp., and *Staphylococcus* spp. were the most frequently isolated bacteria across all samples. The predominance of *E. coli* (25% occurrence) suggests potential contamination from wastewater and fecal matter of human and animal origin, as previously reported in studies on environmental pollution and microbial risks (Oluwafemi et al., 2022; Adegboye et al., 2023). The presence of *E. coli* in these soils indicates fecal contamination, posing health risks to individuals working in or residing near these environments.

Sphingomonas spp. is another notable isolate frequently recovered from petroleum-contaminated soils. These bacteria exhibit unique capabilities to degrade polycyclic aromatic hydrocarbons (PAHs), making them essential for in situ bioremediation. Studies have identified *Sphingomonas* as a dominant group in microbial communities associated with PAH-contaminated soils due to their efficient degradation pathways and high adaptability in hydrocarbon-rich environments (Zhou et al., 2019; Abioye et al., 2023).

The biosurfactant-producing potential of bacterial isolates was evaluated using multiple screening tests, including oil spreading, drop collapse, and foaming capacity assays. Results revealed that *Bacillus* spp. and *Klebsiella* spp. were positive for all three tests, indicating their robust biosurfactant production capabilities. Other isolates demonstrated varied activity: *E. coli* was positive for drop collapse but negative for oil spreading and foaming capacity, while *Sphingomonas* spp. was positive for drop collapse and foaming capacity but negative for oil spreading. *Paenibacillus polymyxa* exhibited positive results for oil spreading and foaming capacity but was negative for drop collapse. *Staphylococcus* spp. was only positive for foaming capacity, whereas *Proteus* spp. was positive for oil spreading and drop collapse but negative for foaming capacity.

The superior biosurfactant production of *Bacillus* spp. and *Klebsiella* spp. aligns with findings from previous studies, which highlight these genera as prolific biosurfactant producers. Members of the genus *Bacillus* are well known for producing potent lipopeptide biosurfactants, such as surfactins and lichenysins, which exhibit strong emulsification properties and play critical roles in industrial, pharmaceutical, and environmental applications (Goswami et al., 2023; Silva et al., 2023). These biosurfactants also enhance hydrocarbon bioavailability, promoting efficient biodegradation in contaminated environments. Similarly, *Klebsiella* spp. has been reported to produce glycolipid biosurfactants with strong emulsifying potential, making them valuable in hydrocarbon biodegradation, oil recovery, and environmental remediation (Nwaguma et al., 2022). Moreover, biosurfactants derived from *Klebsiella* spp. have promising biomedical applications due to their antimicrobial and anti-adhesive properties, which could be explored for medical and biotechnological advancements (Zulekha et al., 2023).

CONCLUSION

This study successfully isolated and identified biosurfactant-producing bacteria from hydrocarbon-contaminated mechanic workshop soils in Port Harcourt, Nigeria. The presence of diverse hydrocarbon-degrading bacterial genera, including *Bacillus, Klebsiella, Sphingomonas, Staphylococcus,* and *Escherichia coli,* highlights the potential of these microbial communities in bioremediation efforts. The positive results from biosurfactant screening assays, particularly for *Bacillus* and *Klebsiella* species, confirm their ability to produce biosurfactants that enhance hydrocarbon degradation. These findings reinforce the role of indigenous bacteria in the natural attenuation of petroleum pollutants and suggest their applicability in biotechnological and environmental remediation strategies.

Further studies should focus on optimizing biosurfactant production and assessing its efficiency in large-scale bioremediation to maximize its potential for sustainable environmental management.

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