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Isolation and Optimization of Biosurfactant-Producing *Pseudomonas spp.* from Orange Peel Waste and Petroleum-Contaminated Soil with Antimicrobial Potential

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Abstract

The demand for sustainable biomolecules has accelerated research on microbial biosurfactants, valued for their biodegradability, low toxicity, and structural diversity. These amphiphilic compounds find applications across pharmaceutical, agricultural, food, cosmetic, and environmental industries, owing to their antimicrobial, antifungal, antioxidant, and anticancer properties. In this study, six bacterial isolates were recovered from orange peel waste and petroleum-contaminated soil in Pune. Primary screening for biosurfactant production was performed using Cetyltrimethylammonium Bromide (CTAB) agar and the Bacterial Adhesion to Hydrocarbons (BATH) assay, followed by secondary confirmation through drop collapse, oil spreading, and emulsification index (E24) tests. Production parameters were optimized using a one-variable-at-a-time approach to assess the effects of carbon concentration, pH, temperature, and incubation period. The most promising isolate was identified as *Pseudomonas spp.* based on Bergey's Manual of Systematic Bacteriology. Thin Layer Chromatography and FTIR spectroscopy confirmed biosurfactant synthesis, with the compound exhibiting notable antimicrobial and antifungal activity. These findings highlight the biosurfactant's potential for diverse biotechnological and pharmaceutical applications.

Keywords

Antimicrobial activity, Biosurfactants, Orange peel waste, Petroleum-contaminated soil, *Pseudomonas spp*.,

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1. INTRODUCTION

Surfactants play an important role in many industries, including detergents, medicines, food processing, cosmetics, and oil recovery.[1,2] However, most commonly used surfactants are synthetic and derived from petroleum.[3] Their long-term use has raised environmental concerns because they can persist in nature, show toxic effects, and depend on non-renewable resources. Therefore, there is a growing need to find safer and sustainable alternatives.

Biosurfactants are natural surface-active compounds produced by microorganisms. These molecules contain both hydrophilic and hydrophobic parts, which allow them to reduce surface and interfacial tension.[4,5] Microorganisms produce biosurfactants to help them utilize insoluble substrates, attach to surfaces, and survive in harsh environments. Compared to synthetic surfactants, biosurfactants are biodegradable, less toxic, and effective under a wide range of environmental conditions.[6,7]

Biosurfactants show great structural diversity. They include glycolipids, lipopeptides, lipoproteins, phospholipids, and polymeric surfactants.[8,9] Common glycolipids such as rhamnolipids, sophorolipids, trehalolipids, and mannosylerythritol lipids are known for their strong emulsifying ability. [10,11]Trehalolipids are mainly produced by species of *Mycobacterium*, *Corynebacterium*, and *Nocardia*,[12,13] while mannosylerythritol lipids are commonly produced by *Pseudozyma* and members of the *Ustilaginaceae*.[14]

Microorganisms have long been recognized as prolific producers of diverse bioactive compounds, among which biosurfactants stand out for their remarkable versatility. These amphiphilic molecules, capable of reducing surface and interfacial tension, extend their utility far beyond simple emulsification. Over the past decades, biosurfactants have emerged as multifunctional agents, demonstrating antimicrobial, antifungal, antiviral, antioxidant, and even anticancer activities. Such properties have positioned them at the crossroads of several disciplines, ranging from environmental biotechnology to agriculture, food industries, cosmetics, pharmaceuticals, and petroleum recovery.[15–17]

In environmental contexts, biosurfactants are particularly valued for their role in bioremediation. By enhancing the solubility and bioavailability of hydrophobic pollutants, they facilitate the removal of hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), and heavy metals through biostimulation and bioaugmentation strategies. Their contribution extends to soil fertility improvement, plant protection under stress conditions, and functioning as natural biocides, thereby offering eco-friendly solutions to pressing ecological challenges.[18]

Compared to synthetic surfactants, biosurfactants offer distinct advantages: they are less toxic, highly biodegradable, and stable across a wide spectrum of temperature, pH, and salinity conditions. These attributes not only make them safer for ecosystems but also enhance their applicability in diverse industrial processes. Recent research has further highlighted their role in the circular bioeconomy, where biosurfactants simultaneously address pollution and valorize agro-industrial waste into value-added products. Advances in microbial biotechnology—such as genetic engineering, metabolic optimization, and improved fermentation strategies—are steadily overcoming production bottlenecks, expanding the scope of biosurfactant applications.[19,20]

Nevertheless, despite their promise, large-scale production remains a challenge. High costs, low yields, and process limitations hinder their widespread adoption. This underscores the importance of identifying efficient biosurfactant-producing microorganisms and optimizing production conditions to achieve economic feasibility.

Against this backdrop, the present study aims to isolate, screen, optimize, and characterize microbial biosurfactants from diverse environmental sources, while evaluating their potential applications. By bridging fundamental microbiology with applied biotechnology, this work contributes to the growing body of knowledge that seeks to harness biosurfactants as sustainable alternatives for environmental and industrial innovation.

2. MATERIALS AND METHODS

2.1 Sample Collection

Environmental samples were collected from Pune, India. Orange peel waste was procured from a local fruit market, while contaminated soil was obtained from a petrol pump site. Samples were collected in sterile polythene bags and transported immediately to the laboratory for further processing. The orange peels were washed thoroughly with distilled water to remove adhering dirt, debris, and possible pesticide residues, and were air-dried prior to use.

2.2 Enrichment and Isolation of Biosurfactant-Producing Bacteria

Biosurfactant-producing bacteria were isolated using a selective enrichment technique. Middlebrook medium was used as the basal medium and supplemented with 2% (v/v) of either orange peel powder or vegetable oil as the sole carbon source to

selectively promote the growth of potential biosurfactant producers.

For the orange peel sample, 1 g of processed orange peel was inoculated into Middlebrook medium containing 2% orange peel powder and incubated at 30 °C in a shaking incubator at 110 rpm for 5 days. Following incubation, 1 mL of the enriched culture was transferred into freshly prepared medium under identical conditions. This enrichment step was repeated three consecutive times to suppress non-target microorganisms and selectively enhance the growth of biosurfactant-producing strains.[21]

For the contaminated soil sample, 1 g of soil was inoculated into Middlebrook medium supplemented with 2% vegetable oil and incubated at 37 °C at 110 rpm for 7 days. After incubation, 1 mL of the culture broth was transferred to fresh medium and incubated under the same conditions. The enrichment process was repeated three times to reduce unwanted microbial populations and Favor oil-degrading, biosurfactant-producing bacteria.[22]

2.2.1 Isolation and Purification of Bacterial Isolates

Following enrichment, aliquots of the culture broth were serially diluted and plated using the spread plate technique. Distinct colonies were selected based on morphological characteristics and purified by repeated streaking on Middlebrook base agar plates. Purified isolates were subsequently subculture on King's B agar to confirm stability and purity.

Pure cultures were maintained at 4 °C for short-term storage. Prior to further screening and characterization studies, isolates were revived under standard growth conditions.

2.3 Characterization of Biosurfactant-Producing Isolates

2.3.1 Morphological Analysis: Gram Staining

Gram staining was performed to determine the Gram reaction and cellular morphology of the isolates. A thin, uniform smear of a pure bacterial culture was prepared on a clean, grease-free glass slide, air-dried, and heat-fixed. The smear was stained with crystal violet (primary stain) for 1 min and gently rinsed with running tap water. Gram's iodine (mordant) was then applied for 1 min, followed by washing with water.

The smear was decolorized with 95% ethanol for approximately 30 s and immediately rinsed to prevent over-decolorization. Subsequently, the slide was counterstained with safranin for 1 min, washed, air-dried, and observed under a light microscope using an oil immersion objective (100×). Gram-positive cells appeared purple, whereas Gram-negative cells appeared pink. Cell shape and arrangement were also recorded.[23]

2.3.2 Biochemical Characterization

Biochemical tests were conducted to differentiate and preliminarily identify the bacterial isolates up to the genus level. Standard assays, including carbohydrate metabolism tests, enzyme detection tests, IMViC tests, and oxidative–fermentative tests, were performed following established microbiological procedures.[24]

2.3.2.1 Indole Test

The indole test was carried out to determine the ability of the organism to degrade tryptophan to indole. A loopful of bacterial culture was inoculated into 1% tryptone broth and incubated at 37 °C for 24 h. After incubation, a few drops of Kovac's reagent were added to the culture tube. Formation of a cherry red ring at the surface indicated a positive result, whereas the absence of red coloration (yellow layer) indicated a negative result.

2.3.2.2 Methyl Red Test

The methyl red (MR) test was performed to detect mixed acid fermentation of glucose. A loopful of the test culture was inoculated into sterile MR broth and incubated at 37 °C for 24 h. After incubation, a few drops of methyl red indicator were added. Development of a red or pink color indicated a positive result, while a yellow color indicated a negative result.

2.3.2.3 Voges–Proskauer Test

The Voges–Proskauer (VP) test was used to determine the production of acetoin (acetyl methyl carbinol) from glucose fermentation. A loopful of culture was inoculated into sterile VP broth and incubated at 37 °C for 24 h. Following incubation, Barritt's reagents (α-naphthol and potassium hydroxide) were added. Development of a cherry red color indicated a positive result, whereas a yellow-brown color indicated a negative result.

2.3.2.4 Citrate Utilization Test

The citrate utilization test was performed using Simmon's citrate agar slants to evaluate the ability of isolates to utilize citrate as the sole carbon source. The test organism was streaked onto the surface of the agar slant and incubated at 37 °C for 24–48 h. Growth accompanied by a color change of the medium from green to blue indicated a positive result due to alkalinization of the medium (pH > 7.6). No growth and no color change indicated a negative result.

2.3.3 Enzyme detection test

Enzyme detection assays were performed to further characterize the biosurfactant-producing isolates. Microbial enzymes function as biochemical catalysts and may be classified as exoenzymes (extracellular) or endoenzymes (intracellular). In the present study,

catalase and oxidase activities were evaluated using standard microbiological procedures.[25]

2.3.3.1 Catalase Test

The catalase test was conducted to determine the ability of isolates to decompose hydrogen peroxide (H_2O_2) into water and oxygen. A small portion of a 24 h-old bacterial culture was exposed to 3% (v/v) hydrogen peroxide on a clean glass slide. Immediate and vigorous formation of oxygen bubbles indicated a positive catalase reaction, whereas the absence of bubbling or only weak bubble formation indicated a negative result.

2.3.3.2 Oxidase Test

The oxidase test was performed to detect the presence of cytochrome c oxidase, an enzyme involved in the bacterial electron transport chain. A well-isolated colony was smeared onto filter paper impregnated with oxidase reagent (N, N, N',N'-tetramethyl-p-phenylenediamine dihydrochloride). Development of a dark blue to purple coloration within 10–30 s was recorded as a positive result due to the formation of indophenol blue. No color change within the specified time indicated a negative reaction.

2.4 Biosurfactant Extraction

Biosurfactant extraction was performed from enriched cultures derived from both orange peel and petroleum-contaminated soil samples. Bacterial isolates obtained from the orange peel sample were inoculated into Middlebrook medium broth supplemented with 2% (v/v) orange peel powder, whereas isolates from contaminated soil were inoculated into Middlebrook broth containing 2% (v/v) vegetable oil as the sole carbon source. Cultures were incubated under optimized conditions: 30 °C for 5 days (orange peel isolates) and 37 °C for 7 days (soil isolates) with continuous shaking.

After incubation, cultures were centrifuged at 5000 rpm for 20 min at 4 °C to separate the bacterial biomass. The cell-free supernatant was carefully collected and subjected to solvent extraction using a chloroform: methanol mixture (1:1, v/v). The solvent mixture was added to the supernatant and transferred to a separating funnel for phase separation. After allowing sufficient time for separation, the biosurfactant-containing fraction was recovered as a white precipitate. The extracted biosurfactant was collected, air-dried, and stored at 4 °C for subsequent characterization and application studies.[26]

2.5 Screening of Biosurfactant-Producing Microorganisms

Screening of biosurfactant production was performed using pure cultures, cell-free supernatants, and extracted biosurfactant fractions. Both primary and secondary screening assays were employed to confirm biosurfactant activity.[27]

2.5.1 Primary Screening

2.5.1.1 CTAB Agar Plate Assay

The CTAB agar plate assay was used as a semi-quantitative method to detect the production of glycolipid and other anionic biosurfactants. Isolates were inoculated onto mineral salt agar plates supplemented with cetyltrimethylammonium bromide (CTAB) and methylene blue. Plates were incubated under appropriate conditions. The formation of dark blue halos around colonies indicated a positive result due to the formation of an insoluble ion pair complex between CTAB, methylene blue, and the secreted anionic biosurfactant.

2.5.1.2 BATH Assay (Bacterial Adhesion to Hydrocarbons)

The BATH assay was performed to evaluate the cell surface hydrophobicity of the isolates. Cultures were grown in Middlebrook medium supplemented with hydrocarbons (vegetable oil or selected alkanes) and incubated under optimized conditions. After incubation, cultures were examined visually. Biosurfactant-producing strains showed hydrocarbon droplet dispersion or formation of a distinct emulsion layer, indicating enhanced interaction between the aqueous phase and hydrocarbons.

2.5.2 Secondary Screening

2.5.2.1 Drop Collapse Assay

The drop collapse assay was carried out to assess surface activity. A drop of vegetable oil was placed on a clean, grease-free glass slide. An aliquot of cell-free supernatant or extracted biosurfactant was gently added onto the oil surface. Observation was made after 1 min. Collapse or spreading of the drop indicated positive biosurfactant activity, whereas a stable, intact drop indicated a negative result. Distilled water was used as a negative control.

2.5.2.2 Oil Spreading Technique

Petri dishes containing 20 mL of distilled water were overlaid with 1 mL of vegetable oil or crude oil to form a thin oil layer. A drop of extracted biosurfactant was carefully placed at the center of the oil surface. Displacement of oil and formation of a clear zone indicated biosurfactant activity. The diameter of the clear zone was measured to estimate activity. Distilled water served as a negative control.

2.5.2.3 Emulsification Index (E24) Assay

The emulsification index (E24) was determined by mixing equal volumes of vegetable oil and extracted biosurfactant in a test tube, followed by vertexing for 2 min. The mixture was allowed to stand undisturbed

at room temperature for 24 h. The emulsification index was calculated using the following formula:

$$E24(\%) = \frac{Height\ of\ emulsion\ layer}{Total\ height\ of\ liquid\ column} x\ 100$$

Higher E24 values indicated greater emulsifying activity of the biosurfactant.

2.6 Optimization of Biosurfactant Production

Optimization studies were performed to evaluate the effects of carbon source concentration, temperature, pH, and incubation period on biosurfactant production by isolates obtained from orange peel and petroleum-contaminated soil samples. All experiments were conducted in triplicate under aseptic conditions using Middlebrook medium as the basal medium. Biosurfactant production was assessed using standard screening assays described previously.[28]

2.6.1 Effect of Carbon Source Concentration

- **Sample 1 – Orange Peel Isolates**
  Dried orange peel powder was used as the sole carbon source at concentrations of 1%, 2%, and 3% (w/v). Fifty milliliters of Middlebrook medium (pH 7.0) were dispensed into 250 mL Erlenmeyer flasks, sterilized, and inoculated with the respective isolates. Cultures were incubated at 30 °C for 5 days in a shaking incubator at 110 rpm.
- **Sample 2 – Petroleum Soil Isolates**
  Vegetable oil was used as the sole carbon source at concentrations of 1%, 2%, and 3% (v/v). Fifty milliliters of Middlebrook medium (pH 7.0) were prepared in 250 mL Erlenmeyer flasks and inoculated with soil isolates. Cultures were incubated at 37 °C for 7 days at 110 rpm.

2.6.2 Effect of Temperature

- **Sample 1 – Orange Peel Isolates**
  Cultures were incubated at three different temperatures (room temperature, 30 °C, and 37 °C) in Middlebrook medium supplemented with 2% (w/v) orange peel powder (pH 7.0). Incubation was carried out for 5 days at 110 rpm.
- **Sample 2 – Petroleum Soil Isolates**
  Cultures were incubated at room temperature, 30 °C, and 37 °C in Middlebrook medium containing 2% (v/v) vegetable oil (pH 7.0) for 7 days at 110 rpm.

2.6.3 Effect of pH

- **Sample 1 – Orange Peel Isolates**
  The pH of Middlebrook medium containing 2% (w/v) orange peel powder was adjusted to 3, 5, 7, and 8 using 0.1 N HCl or 0.1 N NaOH prior to sterilization. The medium was inoculated and incubated at 30 °C for 5 days at 110 rpm.
- **Sample 2 – Petroleum Soil Isolates**
  Middlebrook medium supplemented with 2% (v/v) vegetable oil was adjusted to pH 3, 5, 7, and 8 using 0.1 N HCl or 0.1 N NaOH. After inoculation, cultures were incubated at 37 °C for 7 days at 110 rpm.

2.6.4 Effect of Incubation Period

- Sample 1 – Orange Peel Isolates
  Cultures were incubated for 1, 3, and 5 days in Middlebrook medium containing 2% (w/v) orange peel powder (pH 7.0) at 30 °C and 110 rpm.
- Sample 2 – Petroleum Soil Isolates
  Cultures were incubated for 1, 3, 5, and 7 days in Middlebrook medium containing 2% (v/v) vegetable oil (pH 7.0) at 37 °C and 110 rpm.

  The optimal conditions for biosurfactant production were determined based on maximum emulsification index and other screening parameters.

2.7 Quantification of Biosurfactant Dry Weight

The biosurfactant produced under optimized conditions was quantified by determining its dry weight. The culture broth was centrifuged at 5000 rpm for 20 min at 4 °C to remove bacterial cells. The resulting cell-free supernatant was collected and mixed with chloroform: methanol (1:1, v/v) for solvent extraction. The mixture was transferred to a separating funnel and allowed to stand until clear phase separation occurred.

The biosurfactant fraction, appearing as a white precipitate/sediment, was carefully collected for further processing. A pre-weighed Whatman filter paper was used for gravimetric estimation. The precipitate was poured onto the filter paper, and residual solvent was removed by filtration. The retained sediment was air-dried at room temperature until constant weight was achieved. After complete drying, the filter paper containing the biosurfactant was weighed again. The dry weight of crude biosurfactant was calculated using the following formula:

Biosurfactant dry weight
= Weight of filter paper after drying
− Weight of empty filter paper

The obtained value was expressed in grams per liter (g/L) of culture broth.

2.8 Characterization of Crude Biosurfactant

2.8.1Thin-Layer Chromatography (TLC) Analysis

Crude biosurfactant samples were analyzed by thin-layer chromatography (TLC) to determine their chemical nature. Approximately 0.1 g of crude extract was dissolved in methanol (100 µg/mL), and aliquots were spotted onto pre-coated silica gel TLC plates using a glass capillary, maintaining a distance of 1 cm from the lower edge.

The plates were developed in a solvent system consisting of chloroform:methanol:water (65:15:2, v/v/v) in a saturated chamber until the solvent front reached the upper limit. Plates were air-dried, and the retardation factor (Rf) values were calculated using the formula:

$$Rf = \frac{Distance\ traveled\ by\ the\ sample}{Distance\ traveled\ by\ the\ solvent\ font}$$

Detection of functional groups was performed by exposing the plates to iodine vapor (for lipid detection), spraying with Molisch's reagent (for carbohydrates), and spraying with 1% ninhydrin solution (for free amino groups). The appearance of characteristic color reactions indicated the presence of respective biomolecular components.

2.8.2 Foaming Assay

Foaming activity of the crude biosurfactant was evaluated by vigorously shaking 5 mL of culture supernatant in a graduated test tube for 2 min. After allowing the foam to stabilize, foam height was measured. The foaming percentage was calculated as:

$$\boldsymbol{Foaming}\ (\%) = \frac{\boldsymbol{Height\ of\ foam}}{\boldsymbol{Total\ height\ of\ liquid\ column}} \boldsymbol{x}\ \mathbf{100}$$

Distilled water was used as a negative control.

2.9 Antimicrobial Activity

The antimicrobial activity of crude biosurfactant was evaluated by the agar well diffusion method against Escherichia coli. The bacterial suspension was adjusted to 0.5 McFarland turbidity standard and inoculated into molten Luria–Bertani (LB) medium, which was subsequently poured onto Mueller-Hinton agar plates.

Wells of 8 mm diameter were aseptically punched using a sterile cork borer, and 200 µL of crude biosurfactant extract was dispensed into each well. Streptomycin was used as a positive control. Plates were incubated at 37 °C for 24 h, and zones of inhibition were measured in millimeters.

2.10 Antifungal Activity

Antifungal activity was assessed using the agar well diffusion method against Candida albicans NCIM-3471. The fungal inoculum was adjusted to 0.5 McFarland turbidity and incorporated into molten LB medium before being poured onto Mueller–Hinton agar plates. Wells (8 mm diameter) were prepared and filled with 200 µL of crude biosurfactant extract. Plates were incubated at 37 °C for 24 h, and the diameter of inhibition zones was recorded.

3. RESULTS

3.1 Isolation of Bacteria

Bacterial isolates were successfully obtained from both orange peel and petroleum-contaminated soil samples using Middlebrook 7H9 medium as shown in Table 1. Colonies from orange peel (O1–O3) were larger (≈2 mm), round to irregular in shape, and pigmented creamy to orange, with mucoid or smooth consistency. In contrast, petroleum soil isolates (S1–S3) were smaller (≈0.1 mm), irregular to lobate in shape, and pigmented creamish-brown to orange, with butyrous to dry consistency. All isolates were opaque, exhibited entire margins, and were identified as Gram-negative bacteria (Table 1).

These morphological differences suggest that isolates from petroleum soil may be adapted to hydrocarbon-rich environments, reflected in their smaller colony size and dry texture compared to the nutrient-rich orange peel isolates.

Table 1: Morphological Characteristics of Isolates

Morphological characteristics of Isolates	Sample (orange peel)			Sample (petroleum soil)		
	O1	O2	O3	S1	S2	S3
Size	2mm	2mm	2mm	0.1mm	0.1mm	0.1mm
Shape	Round	Round	Irregular	Irregular	lobate	Round
Color	Creamy	Yellow	Orange	Creamish brown	Yellow	Orange
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Consistency	Mucoid	Butyrous	Smooth	Butyrous	Dry	Butyrous
Elevation	Convex	Concave	Flat	Flat	Flat	Flat
Margin	Entire	Entire	Entire	Entire	Entire	Entire

Gram Nature	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative

3.2 Characterization of Biosurfactant-Producing Isolates

The isolates showed different biochemical patterns, with most being positive for indole and catalase tests. None of the samples showed oxidase activity, while citrate use was common (Table 2).

Table 2: Biochemical Characterization of Isolates (+) positive test & (-) negative test

Biochemical test	Indole	Methyl red	Voges- Proskauer	Citrate	Catalase	Oxidase
Sample O1	+	-	-	+	+	-
Sample O2	+	+	-	+	+	-
Sample O3	+	+	-	+	+	-
Sample S1	+	-	-	+	+	-
Sample S2	+	-	-	+	+	-
Sample S3	+	+	-	-	-	-

3.3 Optimization of Biosurfactant Production

Growth of biosurfactant-producing isolates varied with changes in carbon source concentration, temperature, pH, and incubation period. Sample 1 (orange peel) showed better turbidity at 2% carbon source, 30°C, pH 3–7, and after 5 days of incubation, while Sample 2 (petroleum soil) grew well at 2–3% carbon source, 37°C, pH 5–8, but showed limited growth until later incubation.

Parameters/factors Affecting	Sample 1 (orange peel)	Observation	Sample 2 (petroleum soil)	Observation
% Carbon source concentration	1%	Less turbid	1%	Less turbid
	2%	Growth/turbidity	2%	Growth/turbidity
	3%	Less turbid	3%	Growth/turbidity
Temperature	30°C	Growth/turbidity	30°C	Less turbid
	34°C	Less turbid	34°C	Less turbid
	37°C	Less turbid	37°C	Growth/turbidity
pH	3	Growth/turbidity	3	Less turbid
	5	Growth/turbidity	5	Growth/turbidity
	7	Growth/turbidity	7	Growth/turbidity
	8	Less turbid	8	Growth turbidity
Incubation period	Day 1	No turbidity/growth	Day 1	No turbidity/growth
	Day 3	Less turbid	Day 3	Less turbid
	Day 5	Growth/turbidity	Day 5	Less turbid

3.4 Screening For Biosurfactant Production

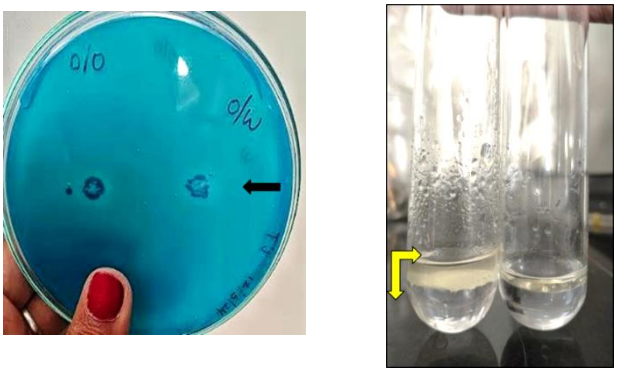
3.4.1 Primary Screening

Initial screening of bacterial isolates was performed using the CTAB agar assay and the BATH assay. (Figure 1 a & b)

a) **CTAB Assay:** Several isolates produced dark hollow or clear zones around colonies, indicating secretion of anionic surfactants.

b) **BATH Assay:** Stable emulsions were observed in cultures supplemented with hydrocarbons, confirming the ability of selected isolates to adhere to and emulsify hydrophobic substrates.

These results suggest that multiple isolates from both orange peel and petroleum soil samples possess biosurfactant-producing potential.



(a) **(b)**

Figure 1: Primary screening of biosurfactant production
(a) CTAB: Clear zone around the blue colony (b) BATH Test - Stable emulsion

3.4.2 Secondary Screening of Isolates

The secondary screening of the selected isolates confirmed their ability to produce extracellular biosurfactants through three distinct surface-activity assays.

Surface Activity and Oil Displacement

a) **The Drop Collapse Assay:** This assay served as a rapid preliminary indicator of surface tension reduction. The isolates showed instantaneous droplet collapse, suggesting high concentrations of surface-active agents. (Figure 2a)

b) **Oil Spreading Technique:** where the culture supernatants successfully displaced the oil phase. The largest zone of clearance was observed for isolate indicating superior surface activity compared to the other strains. (Figure 2b)

c) **Emulsification Stability:** The ability to stabilize hydrocarbon-water mixtures was quantified using the Emulsification Index (E_{24}). Most isolates exhibited the capacity to emulsify [Type of Hydrocarbon, e.g., kerosene], but stability varied significantly. The stable emulsion layers remained intact after 24 hours, suggesting that the produced biosurfactants possess strong emulsifying properties suitable for bioremediation applications. (Figure 2c)

The Emulsification Index after 24 hours (E24%) is calculated using the following formula:
E24(%) = 3/5 x 100 = 60%

(a) **(b)** **(c)**

Figure 2: (a) drop collapse assay (b) oil spreading assay (c) emulsification assay

3.5 Quantification of Biosurfactant Dry Weight

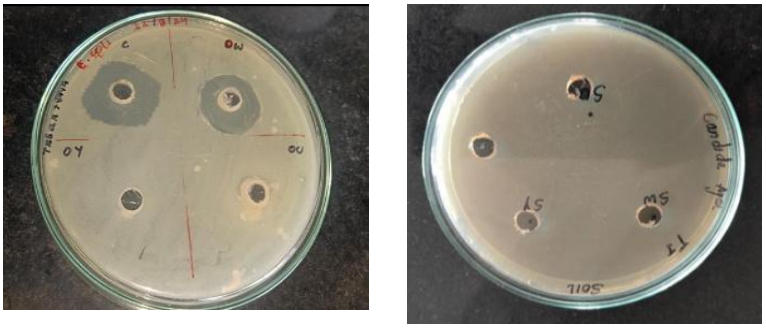
The dry weight of biosurfactant varied with carbon source concentration and isolate type. In Sample 1 (orange peel), maximum yield was observed at 2% concentration, especially in the mixed culture (1.006 g). In Sample 2 (petrol pump), biosurfactant production was also highest at 2% in the mixed culture (0.029 g), with lower yields at 1% and 3% (Table 4).

Table 4: Gravimetric analysis of biosurfactant

% Carbon source Concentration	Sample 1 (Orange peel)	Surfactant produced in grams		Sample 2 (petrol pump)	Surfactants produced in grams
Isolates 1%	O1	0.003	Grams	S1	0.006 Grams
	O2	0.000	Grams	S2	0.001 Grams
	O3	0.002	Grams	S3	0.003 Grams
	Mixed (O1, O2, O3)	0.006	Grams	Mixed (S1, S2, S3)	0.009 Grams
Isolates 2%	O1	0.452	Grams	S1	0.013 Grams
	O2	0.021	Grams	S2	0.007 Grams
	O3	0.033	Grams	S3	0.009 Grams
	Mixed (O1, O2, O3)	1.006	Grams	Mixed (S1, S2, S3)	0.029 Grams
Isolates 3%	O1	0.007	Grams	S1	0.020 Grams
	O2	0.002	Grams	S2	0.003 Grams
	O3	0.005	Grams	S3	0.010 Grams
	Mixed (O1, O2, O3)	0.015	Grams	Mixed (S1, S2, S3)	0.058 Grams

3.6 Antimicrobial Activity

The biosurfactant extracts showed clear zones of inhibition against both bacterial and fungal test organisms. The antibacterial activity (Figure 3a) displayed distinct inhibition zones, while antifungal activity (Figure 3b) also demonstrated measurable suppression of fungal growth.



(a) **(b)**

Figure 3: Zone of inhibition (a) antibacterial activity (b) antifungal activity

3.7 Characterization of Crude Biosurfactant

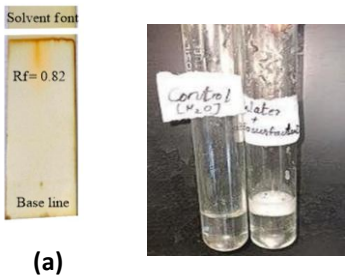
a) Thin Layer Chromatography (TLC) Analysis

TLC was performed for preliminary identification of the crude biosurfactant. The extract was spotted on Silica Gel 60 F_{254} plates and developed in chloroform: methanol: water (65:15:2, v/v/v). Spots were visualized using iodine vapors and anthrone reagent (for carbohydrates). The appearance of a pale yellow/brown spot with an Rf value of 0.82 was observed, indicating the presence of indicated glycolipid nature. (Figure 4a)

b) Foaming Method (Foam Stability Test)

Foaming activity was assessed by vigorously shaking 10 mL of cell-free supernatant in a graduated cylinder for 2 minutes. Foam height was measured immediately, and stability was monitored up to 24 h. Persistent foam indicated effective surface-active properties. (Figure 4b)

Foaming height % = 1.5/5 x 100 = 30%



(a) **(b)**

Figure 4: Characterization of Crude Biosurfactant (a)TLC (b) Foam Stability Test

DISCUSSION

The present study demonstrated that both orange peel waste and petroleum-contaminated soil serve as rich reservoirs of biosurfactant-producing bacteria, though the isolates from each source displayed distinct morphological and physiological traits. Colonies derived from orange peel were larger, pigmented, and mucoid, reflecting the nutrient-rich environment of fruit waste, whereas soil isolates were smaller and drier, consistent with adaptation to hydrocarbon stress (Table 1). All isolates were Gram-negative, aligning with previous reports of genera such as *Pseudomonas* and *Acinetobacter* being prolific biosurfactant producers[29].

The frequent indole and catalase positivity (Table 2) suggests these isolates share basic survival traits useful for biosurfactant production. The lack of oxidase activity and variation in methyl red and citrate tests point to metabolic differences that may affect their growth and biosurfactant efficiency in different environments.[30]

Various parameter affects biosurfactant production depends strongly on environmental conditions, with each isolate showing distinct preferences (Table 3). Sample 1 favored moderate carbon concentration and lower temperature, suggesting adaptation to organic waste environments. In contrast, Sample 2 thrived at higher carbon levels and elevated temperature, reflecting its origin from petroleum-contaminated soil. These differences highlight the ecological influence on metabolic activity and the need for tailored optimization strategies to maximize biosurfactant yield.

Screening assays confirmed biosurfactant activity through CTAB halo formation, BATH tests (Figure 1), drop collapse and oil spreading (Figure 2) each reinforcing the ability of the isolates to reduce surface tension and interact with hydrophobic substrates. The emulsification index of 60% highlighted strong emulsifying potential, a property crucial for applications in bioremediation and oil recovery. Biochemical profiling revealed metabolic diversity, with most isolates testing positive for indole, citrate utilization, and catalase activity, while oxidase negativity suggested variations in electron transport pathways.[31]

Gravimetric analysis (Table 4) showed that mixed cultures of orange peel isolates yielded the highest biosurfactant (1.006 g), reflecting synergistic metabolism, while soil isolates still displayed activity in hydrocarbon-rich environments. The crude biosurfactant demonstrated antimicrobial and antifungal properties (figure 3), suggesting biomedical potential through membrane disruption. Such antimicrobial and antibiofilm activities, particularly from GRAS bacteria, highlight promising applications in infection control, food preservation, and environmental remediation.[32]

Characterization of the crude biosurfactant by TLC (figure 4) indicated glycolipid nature, supported by carbohydrate-reactive staining and foaming assays.[33] Glycolipids are widely recognized for their dual hydrophilic–hydrophobic structure, which explains the observed emulsifying and foaming properties.[34]

Optimization experiments revealed that biosurfactant yield was strongly influenced by culture parameters, with 2% carbon source concentration, temperatures of 30–37°C depending on isolate origin, and pH 5–7 being optimal. Production peaked around day 5 for orange peel isolates and day 7 for soil isolates, suggesting biosurfactant synthesis during late exponential or stationary phases.

Overall, the findings highlight that agro-waste substrates such as orange peel not only provide cost-effective raw material for biosurfactant production but also contribute to waste valorisation and sustainability. The study underscores the dual benefit of harnessing microbial diversity from contrasting environments—nutrient-rich fruit waste and hydrocarbon-polluted soils—to obtain biosurfactants with strong emulsifying and antimicrobial properties.

CONCLUSION

The present study demonstrates that environments such as orange-peel waste and petroleum-contaminated soil harbour diverse microorganisms capable of producing biosurfactants with significant surface-active and antimicrobial properties. The isolates obtained showed effective emulsification, surface tension reduction, and moderate antimicrobial activity, indicating their potential for applications in environmental and industrial biotechnology. Optimization of culture conditions revealed that biosurfactant production is influenced by carbon source concentration, temperature, pH, and incubation period. Notably, agro-waste substrates such as orange peel proved to be an economical and sustainable resource for biosurfactant production. These findings highlight the potential of utilizing microbial resources from diverse environments for eco-friendly biosurfactant production. Further studies focusing on molecular identification, structural characterization, and process scale-up will help to explore their applicability in bioremediation, pharmaceutical, and industrial sectors.

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CONFLICT OF INTERESTS
There are no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT
The manuscript incorporates all datasets produced or examined throughout this research study.

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REFERENCES
(1) Aisha; Batool, I.; Iftekhar, S.; Taj, M. B.; Carabineiro, S. A. C.; Ahmad, F.; Khan, M. I.; Shanableh, A.; Alshater, H. Wetting the Surface: A Deep Dive into Chemistry and Applications of Surfactants. *Cleaner Chemical Engineering* 2025, *11*, 100197. https://doi.org/10.1016/j.clce.2025.100197.
(2) Chowdhury, S.; Shrivastava, S.; Kakati, A.; Sangwai, J. S. Comprehensive Review on the Role of Surfactants in the Chemical Enhanced Oil Recovery Process. *Ind. Eng. Chem. Res.* 2022, *61* (1), 21–64. https://doi.org/10.1021/acs.iecr.1c03301.
(3) Tian, W.; Yao, J.; Liu, R.; Zhu, M.; Wang, F.; Wu, X.; Liu, H. Effect of Natural and Synthetic Surfactants on Crude Oil Biodegradation by Indigenous Strains. *Ecotoxicol. Environ. Saf.* 2016, *129*, 171–179. https://doi.org/10.1016/j.ecoenv.2016.03.027.
(4) Gayathiri, E.; Prakash, P.; Karmegam, N.; Varjani, S.; Awasthi, M. K.; Ravindran, B. Biosurfactants: Potential and Eco-Friendly Material for Sustainable Agriculture and Environmental Safety—A Review. *Agronomy* 2022, *12* (3), 662. https://doi.org/10.3390/agronomy12030662.
(5) Hsu, C.-Y.; Mahmoud, Z. H.; Hussein, U. A.-R.; Abduvalieva, D.; Alsultany, F. H.; Kianfar, E. Biosurfactants: Properties, Applications and Emerging Trends. *S. Afr. J. Chem. Eng.* 2025, *53*, 21–39. https://doi.org/10.1016/j.sajce.2025.04.002.
(6) Sundaram, T.; Govindarajan, R. K.; Vinayagam, S.; Krishnan, V.; Nagarajan, S.; Gnanasekaran, G. R.; Baek, K.-H.; Rajamani Sekar, S. K. Advancements in Biosurfactant Production Using Agro-Industrial Waste for Industrial and Environmental Applications. *Front. Microbiol.* 2024, *15*. https://doi.org/10.3389/fmicb.2024.1357302.
(7) Markam, S. S.; Raj, A.; Kumar, A.; Khan, M. L. Microbial Biosurfactants: Green Alternatives and Sustainable Solution for Augmenting Pesticide Remediation and Management of Organic Waste. *Curr. Res. Microb. Sci.* 2024, *7*, 100266. https://doi.org/10.1016/j.crmicr.2024.100266.
(8) Bjerk, T. R.; Severino, P.; Jain, S.; Marques, C.; Silva, A. M.; Pashirova, T.; Souto, E. B. Biosurfactants: Properties and Applications in Drug Delivery, Biotechnology and Ecotoxicology. *Bioengineering* 2021, *8* (8), 115. https://doi.org/10.3390/bioengineering8080115.
(9) Antonioli Júnior, R.; Poloni, J. de F.; Pinto, É. S. M.; Dorn, M. Interdisciplinary Overview of Lipopeptide and Protein-Containing Biosurfactants. *Genes (Basel).* 2022, *14* (1), 76. https://doi.org/10.3390/genes14010076.
(10) Thakur, P.; Saini, N. K.; Thakur, V. K.; Gupta, V. K.; Saini, R. V.; Saini, A. K. Rhamnolipid the Glycolipid Biosurfactant: Emerging Trends and Promising Strategies in the Field of Biotechnology and Biomedicine. *Microb. Cell Fact.* 2021, *20* (1), 1. https://doi.org/10.1186/s12934-020-01497-9.
(11) Cortés-Sánchez, A. de J.; Hernández-Sánchez, H.; Jaramillo-Flores, M. E. Biological Activity of Glycolipids Produced by Microorganisms: New Trends and Possible Therapeutic Alternatives. *Microbiol. Res.* 2013, *168* (1), 22–32. https://doi.org/10.1016/j.micres.2012.07.002.
(12) Bages-Estopa, S.; White, D. A.; Winterburn, J. B.; Webb, C.; Martin, P. J. Production and Separation of a Trehalolipid Biosurfactant. *Biochem. Eng. J.* 2018, *139*, 85–94. https://doi.org/10.1016/j.bej.2018.07.006.
(13) Kuyukina, M. S.; Ivshina, I. B.; Baeva, T. A.; Kochina, O. A.; Gein, S. V.; Chereshnev, V. A. Trehalolipid Biosurfactants from Nonpathogenic Rhodococcus Actinobacteria with Diverse Immunomodulatory Activities. *N. Biotechnol.* 2015, *32* (6), 559–568. https://doi.org/10.1016/j.nbt.2015.03.006.
(14) Sajna, K. V.; Sukumaran, R. K.; Jayamurthy, H.; Reddy, K. K.; Kanjilal, S.; Prasad, R. B. N.; Pandey, A. Studies on Biosurfactants from Pseudozyma Sp. NII 08165 and Their Potential Application as Laundry Detergent Additives. *Biochem. Eng. J.* 2013, *78*, 85–92. https://doi.org/10.1016/j.bej.2012.12.014.
(15) Vigneshwaran, C.; Vasantharaj, M. J. K.; Sivasubramanian, V. A Review on Biosurfactants and Its Environmental Applications. *IOSR J. Environ. Sci. Toxicol. Food Technol.* 2016, *10* (08), 152–160. https://doi.org/10.9790/2402-100802152160.
(16) Pacwa-Płociniczak, M.; Płaza, G. A.; Piotrowska-Seget, Z.; Cameotra, S. S. Environmental Applications of Biosurfactants: Recent Advances. *Int. J. Mol. Sci.* 2011, *12* (1), 633–654. https://doi.org/10.3390/ijms12010633.
(17) Garg, R.; Garg, R.; Anjum, A.; Eddy, N. O.; Watandost, H. Comprehensive Review of the Synthesis and Sustainable Applications of Biosurfactants. *Discover Applied Sciences* 2025, *7* (10), 1168. https://doi.org/10.1007/s42452-025-07603-z.
(18) Elayaperumal, S.; Sivamani, Y.; Bhattacharya, D.; Lahiri, D.; Nag, M. Eco-Friendly Biosurfactant Solutions for Petroleum Hydrocarbon Cleanup in Aquatic Ecosystems. *Sustainable Chemistry for the Environment* 2025, *9*, 100207. https://doi.org/10.1016/j.scenv.2025.100207.
(19) Markam, S. S.; Raj, A.; Kumar, A.; Khan, M. L. Microbial Biosurfactants: Green Alternatives and Sustainable Solution for Augmenting Pesticide Remediation and Management of Organic Waste.

Curr. Res. Microb. Sci. 2024, *7*, 100266. https://doi.org/10.1016/j.crmicr.2024.100266.

(20) Fernandes, N. A. T.; Simões, L. A.; Dias, D. R. Comparison of Biodegradability, and Toxicity Effect of Biosurfactants with Synthetic Surfactants. In *Advancements in Biosurfactants Research*; Springer International Publishing: Cham, 2023; pp 117–136. https://doi.org/10.1007/978-3-031-21682-4_6.

(21) Rani, M.; Weadge, J. T.; Jabaji, S. Isolation and Characterization of Biosurfactant-Producing Bacteria from Oil Well Batteries with Antimicrobial Activities Against Food-Borne and Plant Pathogens. *Front. Microbiol.* 2020, *11*. https://doi.org/10.3389/fmicb.2020.00064.

(22) Al-Marri, S.; Eldos, H. I.; Ashfaq, M. Y.; Saeed, S.; Skariah, S.; Varghese, L.; Mohamoud, Y. A.; Sultan, A. A.; Raja, M. M. Isolation, Identification, and Screening of Biosurfactant-Producing and Hydrocarbon-Degrading Bacteria from Oil and Gas Industrial Waste. *Biotechnology Reports* 2023, *39*, e00804. https://doi.org/10.1016/j.btre.2023.e00804.

(23) Maina, J.; Maina Mathara, J.; M. Kikuvi, G.; Wafula, E. Isolation and Identification of Bacteriocin-Producing <I & gt;Bacillus & lt;/I>Spp from <I>Rastrineobola</I > <I>Argentea </I>(Omena) with Activity against Bovine Mastitis Bacterial Pathogens. *Journal of Food Security* 2021, *9* (2), 62–75. https://doi.org/10.12691/jfs-9-2-4.

(24) Powers, E. M.; Latt, T. G. Simplified 48-Hour IMVic Test: An Agar Plate Method. *Appl. Environ. Microbiol.* 1977, *34* (3), 274–279. https://doi.org/10.1128/aem.34.3.274-279.1977.

(25) Valizadeh, S.; Enayatizamir, N.; Ghomsheh, H. N.; Motamedi, H.; Moghadam, B. K. Characterization of the Biosurfactant Production and Enzymatic Potential of Bacteria Isolated from an Oil-Contaminated Saline Soil. *International Microbiology* 2023, *26* (3), 529–542. https://doi.org/10.1007/s10123-022-00318-w.

(26) Banat, I. M.; Franzetti, A.; Gandolfi, I.; Bestetti, G.; Martinotti, M. G.; Fracchia, L.; Smyth, T. J.; Marchant, R. Microbial Biosurfactants Production, Applications and Future Potential. *Appl. Microbiol. Biotechnol.* 2010, *87* (2), 427–444. https://doi.org/10.1007/s00253-010-2589-0.

(27) Sidkey, N.; Mohamed, H.; Elkhouly, H. Evaluation of Different Screening Methods for Biosurfactant Producers Isolated from Contaminated Egyptian Samples Grown on Industrial Olive Oil Processing Waste. *Br. Microbiol. Res. J.* 2016, *17* (4), 1–19. https://doi.org/10.9734/BMRJ/2016/28437.

(28) Thomas, S.; Joshy, G.; Subbiah, K. Optimization of Biosurfactant Production Using Chrysene Degrading Bacteria Isolated from Marine Water. *J. Pure Appl. Microbiol.* 2022, *16* (4), 2580–2591. https://doi.org/10.22207/JPAM.16.4.25.

(29) Ben Belgacem, Z.; Bijttebier, S.; Verreth, C.; Voorspoels, S.; Van de Voorde, I.; Aerts, G.; Willems, K. A.; Jacquemyn, H.; Ruyters, S.; Lievens, B. Biosurfactant Production by *Pseudomonas* Strains Isolated from Floral Nectar. *J. Appl. Microbiol.* 2015, *118* (6), 1370–1384. https://doi.org/10.1111/jam.12799.

(30) Elhamrouni, I.; Alhammadi, E.; Ishak, M. Y. Study on Biodegradation of Used Engine Oil in a Stirred Batch Bioreactor by Ochrobactrum Intermedium and Bacillus Paramycoides Isolates. *Sci. Rep.* 2025, *15* (1), 36707. https://doi.org/10.1038/s41598-025-20748-6.

(31) Valizadeh, S.; Enayatizamir, N.; Ghomsheh, H. N.; Motamedi, H.; Moghadam, B. K. Characterization of the Biosurfactant Production and Enzymatic Potential of Bacteria Isolated from an Oil-Contaminated Saline Soil. *International Microbiology* 2023, *26* (3), 529–542. https://doi.org/10.1007/s10123-022-00318-w.

(32) De Giani, A.; Zampolli, J.; Di Gennaro, P. Recent Trends on Biosurfactants with Antimicrobial Activity Produced by Bacteria Associated with Human Health: Different Perspectives on Their Properties, Challenges, and Potential Applications. *Front. Microbiol.* 2021, *12*. https://doi.org/10.3389/fmicb.2021.655150.

(33) Dias, M. A. M.; Rossini, E. L.; de Britto, D.; Nitschke, M. Production and Characterization of a Novel Glycolipid Biosurfactant from Bradyrhizobium Sp. *Fermentation* 2025, *11* (8), 471. https://doi.org/10.3390/fermentation11080471.

(34) Khalfallah, A. Structure and Applications of Surfactants. In *Surfactants - Fundamental Concepts and Emerging Perspectives*; Intech Open, 2024. https://doi.org/10.5772/intechopen.111401.