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BIOCHEMICAL ATTRIBUTES AND SCREENING OF POTENTIAL BIOSURFACTANT-PRODUCING SOIL ACTINOBACTERIA

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Abstract
The current research aims to examine the biosurfactant-producing ability of microbial isolates by emulsification activity and hydrocarbon adhesion. Microbial isolates were cultivated in acceptable culture medium, and cell-free supernatants were utilised to measure emulsification index (E24) using three hydrocarbons: hexane, petroleum oil, and crude oil. The bacterial adhesion to hydrocarbons (BATH) experiment was conducted to measure cell surface hydrophobicity and the capacity of cells to attach to various hydrocarbons. Emulsification tests indicated that only 10% of the isolates exhibited negative emulsification activity, while approximately 50% displayed significant emulsification indices with all three hydrocarbons. Petroleum oil generated the highest average E24 values, suggesting higher substrate compatibility for biosurfactant detection. Stable emulsification was also reported with hexane and crude oil for numerous isolates. The BATH test demonstrated that most isolates displayed highest adhesion toward hexane (92.23 ± 0.51 to 52.15 ± 1.42%) demonstrating a high level of hydrophobicity and implying robust biosurfactant-mediated cell–hydrocarbon interaction. Together, our data demonstrate that a majority of the isolates exhibit high emulsifying activity and hydrocarbon-binding potential (73.00 ± 0.1 to 4.00 ± 0.1%). The combined examination of emulsification index and hydrocarbon adhesion reveals that most isolates contain high biosurfactant-producing potential (95.25 ± 0.02 to 18.00 ± 0.02%). These results emphasise their potential for use in bioremediation, hydrocarbon degradation, and other industrial processes requiring effective natural surfactants.

KEYWORDS: Actinobacteria; Biosurfactant; Screening; Secondary metabolites: Molecular identification.

INTRODUCTION

Biosurfactants are also microbes through produced surfactant agents these are heterogenous group of secondary bioactive compounds. Biosurfactants are organic compounds including both hydrophilic and hydrophobic (Chakraborty et al., 2015). They belong to 6 different types such as lipopeptide, glycolipids, neutral lipids, fatty acids, and polysaccharides biosurfactants (Farooq et al., 2024). Biosurfactants are non- toxic, biodegradable, biocompatible, and essential at lower concentrations, these are distinguishing characteristics. They are more selective and have important foaming characteristics. These are function and under conditions of high salinity, pH, temperature (Kugaji et al., 2025). Biosurfactants are produced by different screening methods like oil spreading or oil displacement method, hemolytic activity, lipase activity, cetyltrimethylammonium bromide (CTAB), Bacterial Adhesion to hydrocarbons (BATH) assay, emulsification assay, emulsification index, drop collapsing method, and penetration method. Biosurfactants are produced extracellularly and intra cellularly, have potential range of applications in several fields are there in various industries such as agriculture field, pharmaceutical, cosmetics, medical fields, textile manufacture, detergents, food processing, and oil recovery etc.
Biosurfactants produced by the different types of microorganisms such as *Sphingobacterium, Rhodococcus sp, Bacillus lichen forms, Ochrubacterium sp* strain and *actinobacteria caloaceticus*, but until at present Actinomycetes *nocardiosis* strain. Biosurfactants can be used in bioremediation of surfactant and various viral lipid membrane, actinomycetes or actinobacteria are aerobic gram-positive bacteria and widely distributed in environmental and habitat terrestrial, oceans etc. Actinobacteria that containing a high G+C content about 57-75% in their DNA. It was at least biosurfactant production from hill region microorganisms are metabolically and physiologically different from terrestrial organisms required to unfavorable conditions. Hill and rock environments like alkalinity, salinity, temperature etc, the result was chance for the presence of new bioactive molecules in this microorganism. Actinobacteria are very important sources for bioremediation, pharmaceuticals, agriculture clinical important enzymes, antibiotics, and bioactive compounds. This study reveals the potential biosurfactant producing actinobacteria isolated from hill, and their efficacy towards the oil recovery (Shubhrasekhar et al., 2013).

MATERIAL AND METHODS

Collection of soil samples and isolation of actinomycetes

Soil samples were collected from various locations in Molkalamuru nearby Nunkappa hill, Anjaneya hill and Molkalamuru forest. Soil samples were collected in clean polythene bags at the depth of 15 to 20 cm. 500 g of soil sample from each place was collected using sterile polyethene bag, the samples were air dried and stored in room temperature, air dried soil samples were subjected for serial dilution and inoculation into the medium, the pH of medium was adjusted to 7.0. The plates were incubated for 7 to 14 days at 37 °C. The pure cultures were maintained on starch casein agar and subsequently on 15% glycerol stock for long term preservation (Thampayak et al., 2008; Meenakshi et al., 2024).

Morphological features of actinomycetes

The most popular detection technique in bacteriology is colony morphology, which involves identifying bacteria based on their phenotypic traits on solid media, such as color, form, surfaces, elevation, edges, opacity, and consistency (Nalini et al., 2021).

Biochemical characterization of actinomycetes

Actinomycete isolates were characterized by a range of biochemical assays. These tests include the Gram's staining, catalase test, indole test, methyl red test, Vogues Proskauer (VP) test, citrate utilization test and urea hydrolysis test. The following standard protocols were performed (Sony et al., 2025).

Gram's staining

The culture suspension was smeared onto a clean glass slide, air-dried, and heat-fixed, followed by Gram staining and microscopic observation under oil immersion (100×) (Pooja et al., 2015).

Indole test

The test culture was inoculated into the sterile tryptone broth and incubated for 30 °C for 24 h. After incubation 0.5 mL of Kovács' reagent was added to tryptone broth. Formation of pink colour in the alcohol layer indicated a positive reaction (Gharbi et al., 2025).

Methyl red test

The test culture was inoculated with sterile glucose phosphate (GPB) broth and incubated at 30 °C for 24 h. After incubation, 5 to 6 drops of methyl red indicator was added to the GPB broth, mixed well and observed for a bright red colour as an indication of a positive test. The appearance of yellow colour indicates a negative test (Manjunatha et al., 2025).

Voges-Proskauer test

Sterile glucose phosphate broth was inoculated with bacterial suspension and incubated at 30 °C for 24 h. After incubation 0.5 mL of (40 g of potassium hydroxide and 0.3 g of creatinine in 100 mL distilled water) was added to each tube. The tubes were then placed in a water bath maintained at 30 °C, for 4 h. A positive reaction was denoted by the development of eosin pink colour in 2-5 min (Perez-Corral et al., 2022).

Citrate utilization test

The culture was inoculated onto sterile Simon's citrate agar slant test tubes were incubated for 24 h at 30 °C. Change in the colour of the media from green to blue indicates a positive result (Oyedoh et al., 2025).

Urea hydrolysis test

Sterile Christensen's urea broth was inoculated with 0.1 mL of bacterial culture suspension. Formation of purple-pink colour after incubation for 24 h at 30 °C indicates a positive test (Marianah et al., 2025).

Catalase test

On a clean glass cavity slide, pure culture was scraped off from sterile starch casein agar and 0.5 mL solution of 3% hydrogen peroxide then observe for effervescence (Shivaveerakumar and Hiremath, 2019; Babu et al., 2025).

Triple sugar iron agar test

The Culture was inoculated in to sterile triple sugar iron agar slant and butt and positive results were confirmed by observing change in the colour of the TSI slant (Chaudhary et al., 2013).

Gelatinase activity

The gelatine liquefaction test was performed by centrally inoculating the culture suspension into sterile gelatine agar tubes, followed by incubation at 30 °C for 24 hours. After incubation 1.5% acidified mercuric chloride solution was added to the tubes as an indicator. The appearance of a clear zone surrounding the bacterial growth, resulting from gelatin liquefaction, was considered a positive reaction for gelatinase activity (Mehta and Jadeja, 2022).

Screening of biosurfactants

The production of biosurfactant can be screened by different methods like qualitative and quantitative namely, drops collapse method, oil spreading method, hemolytic activity method, lipase activity method, CTAB Agar method, (Cetyltrimethylammonium bromide), BATH Assay, (Bacterial addition of hydrocarbons), emulsification assay and emulsification index.

Hemolytic activity was determined using blood agar plates prepared with sheep blood. The bacterial isolates were streaked onto the agar surface and incubated under appropriate conditions. The formation of a clear zone surrounding the colonies indicated positive hemolytic activity. Lipase activity were evaluated using tributyrin agar medium supplemented with rhodamine dye. After incubation, the development of pink-coloured colonies under appropriate visualization conditions confirmed positive lipase activity (Rizvi et al., 2024).

Drop collapse method

The wells of a polystyrene 96-well microplate lid were coated with 2 mL of crude oil and allowed to dry at 22 °C for 24 h. Subsequently, 5 mL of filtered cell-free supernatant was carefully added to the center of the oil-coated well. Observations were recorded after 1–2 min. Biosurfactant production was considered positive when the oil drop flattened, whereas rounded oil droplets indicated a negative result, reflecting the absence of biosurfactant activity (Rani et al., 2020).

Oil spreading method

The oil spreading method was performed using polystyrene Petri dishes (100 mm × 15 mm). Each Petri dish was filled with 50 mL of distilled water, over which 20 mL of crude oil was carefully layered. Subsequently, 10 µL of filtered cell-free supernatant was gently pipetted onto the center of the oil layer. After 1 min, the diameter of the clear zone formed on the oil surface was measured and compared with that of the negative control. The formation of a clear zone indicated positive biosurfactant activity (Shah et al., 2016).

Hemolytic activity method

Actinomycetes was growing on blood agar plate (BAP), then 1.5 mL sterilized sheep blood was pipetted into the conical flask carry 500 mL of blood agar medium, plates were incubated for 5 to 7 days at 37 °C. After incubation, the plates were observed for the presence of clear zones surrounding the colonies with the appearance, considered as positive result, the plates without zone formation on the plate negative (Hamed et al., 2021).

Lipase activity assay

The lipase activity assay was performed with tributyrin agar plates incorporated with Rhodamine dye as an indicator. The pure actinomycetes cultures were inoculated on plates and incubated at 37 °C for 7 days. After incubation clear zone was observed around the colonies, produced by pink color colonies indicating lipase positive result. No formation of clear zone around the colony indicates negative test for lipase (Arifiyanto et al., 2020).

CTAB agar assay

The starch casein agar media was prepared using a Methylene blue (MB 0.2 mg/mL) and carbon sources (2%) in addition to Cetyltrimethylammonium bromide (CTAB, 0.5 mg/mL), The appearance of a dark blue zone around the colonies indicated a positive result for biosurfactant production (Satpute et al., 2008).

Emulsification index (EI)

The emulsification index (EI) was determined by mixing the cell-free supernatant obtained from a 7-day-old actinomycete culture with hydrophobic substrates (kerosene, engine oil, olive oil, and palm oil) in a 1:1 ratio. The mixtures were vortexed vigorously for 2 min and then allowed to stand for 24 h. The EI was subsequently calculated using the standard formula (Ravinder et al., 2022).

$$EI\ (\%) = (\text{height of the emulsion layer} / \text{total height of the liquid column}) \times 100 \quad (1)$$

Emulsification assay

The cell-free supernatant and hydrophobic substrate were mixed in a 1:3 ratio and vortexed for 2 min to ensure thorough mixing. After 24 h of incubation, the aqueous phase was carefully collected, and its absorbance was measured at 400 nm (Qi et al., 2025).

Bacterial adhesion to hydrocarbons (BATH) test

Cell surface hydrophobicity was determined using the bacterial adhesion to hydrocarbons (BATH) method, as described by Rosenberg et al. (1980). Bacterial cell pellets were harvested and washed twice with buffer salt solution The washed cells were resuspended in the same buffer and adjusted to an optical density (OD) of approximately 0.5 at 610 nm. Subsequently, 2 mL of the cell suspension was transferred into sterile test tubes and 100 µL of crude oil was added. The mixtures were vortexed vigorously for 3 minutes to ensure proper interaction between bacterial cells and the hydrocarbon phase. After shaking the tubes were allowed to stand undisturbed for 1 hour to permit complete phase separation. The OD of the aqueous phase was then measured at 610 nm using a spectrophotometer. The percentage of bacterial cells adhering to crude oil was calculated using the standard formula based on the decrease in absorbance (Zoueki et al., 2010).

$$\text{Cell adherence}\ (\%) = [1 - (OD_{\text{shaken with oil}} / OD_{\text{original}})] \times 100 \quad (2)$$

where OD is the optical density at 610 nm of the aqueous phase after ($OD_{\text{shaken with oil}}$) and before ($OD_{\text{original}}$) mixing with the hydrocarbon.

Molecular Characterization through 16S rRNA Analysis

The molecular identification and validation of actinobacteria strain were carried out via 16S rRNA sequencing. PCR amplification of intergenic spacer region and sequencing of 16S rRNA gene of the isolated actinobacteria strain was done at Barcode Biosciences Pvt. Ltd. Bangalore. The resulting gene sequences were used in a homology search in Gene Bank through BLAST tools (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the homology of sequences were studied, further multiple sequence alignments was performed using the Clustal W tool available on the EBI server, based on sequence similarity scores, phylogenetic tree was constructed to understand the evolutionary relationship with other identical sequences (Krishnasamy et al., 2026).

RESULTS AND DISCUSSION

Collection of soil samples and isolation of actinomycetes

A total of 20 soil samples were collected from different locations, including Nunkemale siddeshwara Hill, Molkalamuru Forest, Davangere university Campus, Molkalamuru Hill, and Molkalamuru-Anjaneya Hill. The sampling sites were located between latitudes 14.66796061°N and 15.66796066°N and longitudes 76.646462177°E and 77.646462184°E. From these soil samples a total of 175 bacterial isolates were obtained. For isolation, one gram of soil sample was placed in nine millilitres of sterile double-distilled water and dilution factor 10^{-1} to 10^{-7} dilutions were enabling the selective isolation of distinct colonies. Consistent with earlier reports on soil actinomycete diversity (Ayoib et al., 2024). These isolates are preserved for starch casein agar medium glycerol stock. These isolates were initially screened for biosurfactant production and exhibited strong biosurfactant producing capabilities (Elkhawaga, 2018), standard graph values obtained from the bacterial isolates are presented in Figure 1.

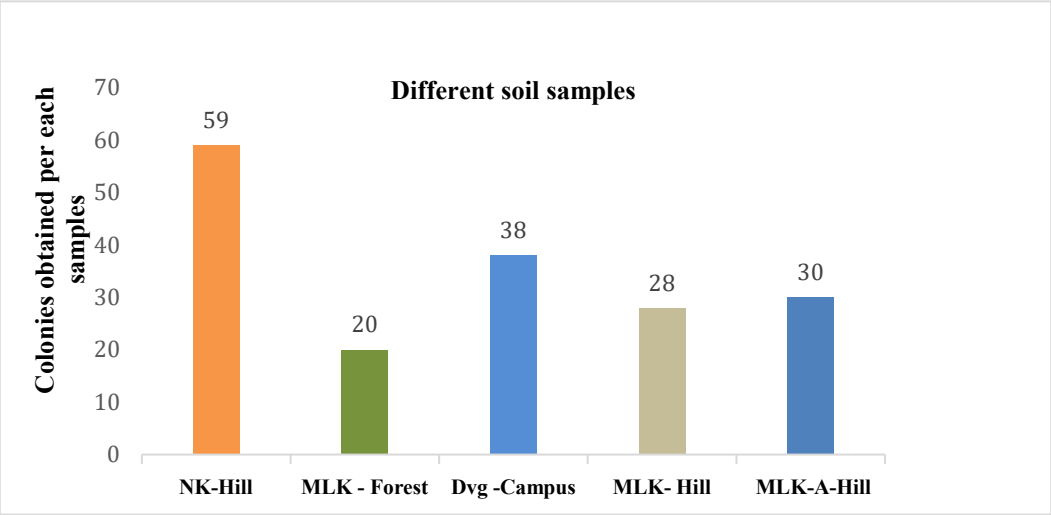


Figure 1. The collection of soil samples and isolation of actinomycetes.

Morphological features of actinomycetes

The morphological characters of the selected potential isolates SVS 1, SVS 2, SVS 3, SVS 4, SVS 5, SVS 6 SVS 7, SVS 8, SVS 9, SVS 10 SVS 11, and SVS 12, showed purple colour for Gram's staining like Gram-positive bacteria and observed open spirals and hairy like long hyphal structures with spore chain formation and branched filamentous like fungi under trinocular microscope at 100× magnification, these morphological features closely resemble earlier reports describing the characteristic structures of soil actinomycetes (Panatula et al., 2022; Meenakshi et al., 2025), as shown in the Table 1.

Table 1. Morphological features of the actinomycete isolates.

Sl No	Size	Shape	Elevation	Margin	Color	Texture	Opacity	Other	Pigmentation
SVS1	Large	Round	Raised	Entire	White	Smooth	Opaque	Brittle	White
SVS2	Small	Round	Dome shaped	Entire	White	Smooth	Opaque	Brittle	Creamish white
SVS3	Small	Regular	Umbonate	Lobate	Creamish white	Dull	Opaque	Brittle	White pigment
SVS4	Small	Round	Raised	Entire	White	Smooth	Opaque	Brittle	White pigment
SVS5	Small	Round	Raised	Entire	White	Smooth	Transferent	Brittle	Transferent pigment
SVS6	Large	Round	Raised	Entire	White & block	Rough	Opaque	Brittle	White &block pigment
SVS7	Small	Round	Raised	Entire	Cream white	Rough	Opaque	Brittle	No pigment
SVS8	Large	Irregular	Convex	Entire	Block color	Rough	Opaque	Brittle	Black pigment
SVS9	Large	Irregular	Raised	Undulate	Red color	Rough	Transferent	Brittle	Red pigment

SVS10	Small	Round	Raised	Entire	White &block	Rough	Opaque	Brittle	No pigment
SVS11	Large	Round	Raised	Entire	White	Smooth	Opaque	Brittle	No pigment
SVS12	Large	Irregular	Raised	Undulate	Red color	Rough	Transferent	Brittle	Red pigment

Biochemical test for actinomycete isolates

All 12 actinomycete isolates were found to be Gram-positive, showed violet coloration under light microscopy, which confirmed the presence of a thick peptidoglycan cell wall typical of actinomycetes, below in Table 2. This observation is consistent with earlier studies reporting the predominance of Gram-positive *Streptomyces* like organisms in soil samples (Sapkota et al., 2020).

Table 2. Biochemical characterization of the actinomycete isolates.

Sl No	Samples Code	Gram's Staining	Catalase Test	Indole Test	Methyl Red Test	VP Test	Gelatine Test	CUT Test	Urease Test	TSI Test
1	SVS 1	+	+	+	+	+	+	+	+	+
2	SVS 2	+	+	+	+	+	+	+	+	+
3	SVS 3	+	+	+	+	+	+	+	+	+
4	SVS 4	+	+	+	+	+	+	+	+	+
5	SVS 5	+	+	+	+	+	+	+	+	+
6	SVS 6	+	+	+	-	+	+	+	+	+
7	SVS 7	+	+	+	+	+	+	+	+	+
8	SVS 8	+	+	+	+	+	+	+	+	+
9	SVS 9	+	+	+	-	+	+	+	+	+
10	SVS 10	+	+	+	+	+	+	+	+	+
11	SVS 11	+	+	+	+	+	+	+	+	+
12	SVS 12	+	+	+	+	+	+	+	+	+

+, positive; -, negative; VP, Voges-Proskauer; CUT, citrate utilization test; TSI, triple sugar iron agar test.

Gelatin hydrolysis

All isolates showed clear zones on gelatin medium, indicating positive gelatinase activity, below in Table 2. Similar extracellular protease production has been widely reported in soil derived *Streptomyces* and related actinomycetes, supporting their saprophytic and proteolytic nature (Sapkota et al., 2020).

Indole production

Indole-positive isolates developed a pink ring after the addition of Kovács reagent, confirming tryptophan degradation below in Table 2. This result agrees with previous reports showed variable indole and indole acetic acid (IAA) production among soil *Streptomyces* isolates (Shatri, 2024).

Methyl red and Voges–Proskauer tests

Most isolates were MR-positive, while all isolates were VP-positive, indicating the ability to utilize both mixed-acid and acetoin butanediol fermentation pathways, below in Table 2. Similar mixed MR–VP patterns among soil actinomycetes have been reported earlier and reflect metabolic diversity (Khirennas et al., 2023).

Citrate utilization, urease and catalase tests

All isolates showed positive citrate utilization, urease activity, and catalase activity, below in Table 2, demonstrating their ability to use diverse carbon and nitrogen sources and to tolerate oxidative stress. These biochemical traits are commonly reported in soil actinomycetes (Madushanka et al., 2025).

Triple sugar iron (TSI) agar

The TSI test showed variable colour reactions, indicating differences in carbohydrate fermentation patterns among the isolates below in Table 2. Similar variability in TSI profiles among actinomycetes has been reported previously, highlighting their metabolic heterogeneity (Nayarisseri et al., 2018).

Screening and production of biosurfactant

A total number of 12 potential isolates of actinomycetes of different morphology were subjected to preliminary screening for biosurfactant. Primary and secondary screening of biosurfactant production was done by various screening methods, hemolytic activity, oil spreading method, CTAB blue agar plate method, and lipase activity method, quantitative methods include by emulsification indexed method, emulsification assay and BATH assay. Out of the 12 isolates, 6 isolates exhibited good responses in one or more preliminary experiments, suggesting their ability to generate surface-active chemicals. These qualitative approaches showed to be very efficient and practical for processing large numbers of isolates, as previously reported by biosurfactant screening (Uyar and Avcı, 2023).

Drop collapse method

The drop-collapse method is a rapid, sensitive, and cost-effective technique used for the preliminary screening of biosurfactant production. The method requires a minimal sample volume, is easy to perform. A positive result

for biosurfactant production was indicated by flattening of the oil drop, whereas rounded oil droplets were scored as negative, indicating the absence of biosurfactant production for SVS2 isolate, as shown in Figure 2. These findings are consistent with the observations of Eldin et al. (2019), who also reported the effectiveness of the drop-collapse method as a rapid and reliable screening of biosurfactant.

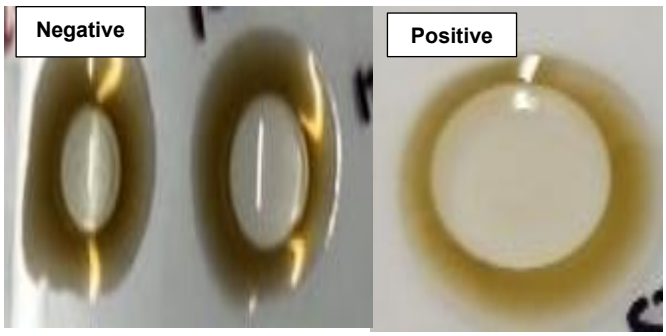


Figure 2. Drop collapse method.

Oil spreading method

The oil spreading method is a rapid and easy-to-perform assay that requires only a small volume of sample. In this study, the SVS2 isolate was initiate to be positive in the oil-spreading assay, showing a transparent zone with a diameter of 8 cm. This result demonstrates that a significant proportion of the actinomycete isolates possess surface-active (biosurfactant-producing) properties, as shown in Table 3 and Figure 3. Similar observations were reported by Schalchli et al. (2023).

Table 3. Diameter (mm) of clear zones formed by microbial isolates in the oil spreading method.

Sl No	Sample code	Zone of inhibition (mm) for standard deviation
1	SVS 1	6 ± 0.02
2	SVS 2	4 ± 0.01
3	SVS 3	6 ± 0.03
4	SVS 4	3 ± 0.04
5	SVS 5	1 ± 0.02
6	SVS 6	2 ± 0.05
7	SVS 7	8 ± 0.04
8	SVS 8	4 ± 0.03
9	SVS 9	3 ± 0.02
10	SVS 10	2 ± 0.04
11	SVS 11	1 ± 0.02
12	SVS 12	2 ± 0.03

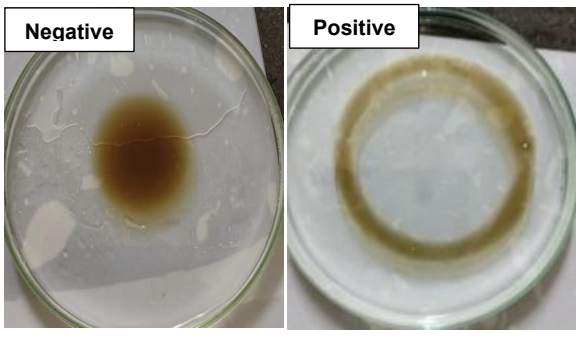


Figure 3. Oil spreading method.

Hemolytic activity method

Hemolytic activity serves as an effective primary screening method for the detection of biosurfactant-producing microorganisms. In this study of the SVS2 isolate indicates positive hemolytic activity as shown in Table 4 and Figure 4. The consistent with previous findings where haemolysis has been reported as a reliable first-line indicator for detecting microbial biosurfactant (Shoeb et al., 2015). Similar studies have also emphasized that hemolytic assays despite being qualitative provide rapid and valuable preliminary evidence of biosurfactant production, making them suitable for screening large numbers of isolates (Carrillo et al., 1996; Banat et al., 2010). Hemolytic activity was confirmed by the presence of a clear zone of catalysis surrounding the colonies. The isolate showed β-hemolytic activity, indicated by a clear zone of catalysis on blood agar.

Table 4. Diameter (mm) of clear zones formed by microbial isolates showing hemolytic activity.

Sl No	Sample code	Zone of inhibition (mm) for standard deviation
1	SVS 1	6 ± 0.03
2	SVS 2	4 ± 0.04
3	SVS 3	1 ± 0.03
4	SVS 4	4 ± 0.02
5	SVS 5	3 ± 0.04
6	SVS 6	2 ± 0.02
7	SVS 7	9 ± 0.01
8	SVS 8	4 ± 0.02
9	SVS 9	6 ± 0.012
10	SVS 10	5 ± 0.02
11	SVS 11	4 ± 0.01
12	SVS 12	5 ± 0.02

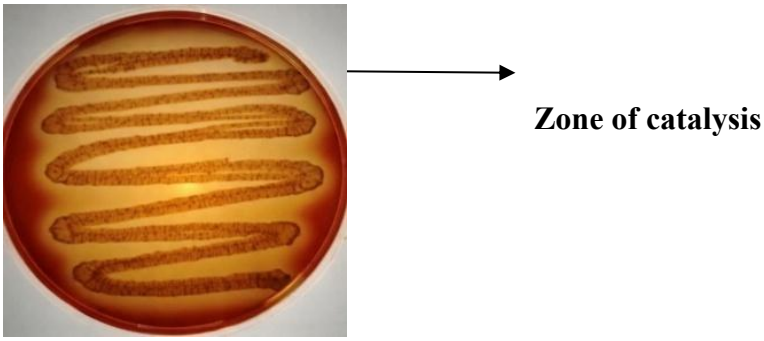


Figure 4. Hemolytic activity.

Lipase activity method

Tributyrin agar medium is widely used for the detection of extracellular lipase activity, as the enzyme hydrolyses tributyrin oil present in the medium. Lipase catalyses the breakdown of complex lipids into simpler components. Tributyrin oil is a triglyceride composed of glycerol and fatty acids. The presence of tributyrin oil renders the medium opaque, and lipase activity is indicated by the formation of a clear zone around the colonies due to hydrolysis of the lipid substrate. Rhodamine dye indicator was used in lipase activity test. Lipase enzymes produced by microorganisms hydrolyse tributyrin; therefore, the SVS2 isolate formed a clear halo zone around the colony, indicating positive lipase activity. Actinobacteria will split the attach of glycerol and saturated fatty acids so that they may be transformed into finished by-products that will be used to production energy and further biological process, as shown in Table 5 and Figure 5. These observations are consistent with earlier findings reported by Arifiyanto et al. (2020).

Table 5. Diameter (mm) of clear zones formed by microbial isolates showing lipase activity.

Sl No	Sample code	Zone of inhibition (mm) for standard deviation
1	SVS 1	7 ± 0.01
2	SVS 2	6 ± 0.02
3	SVS 3	5 ± 0.04
4	SVS 4	7 ± 0.03
5	SVS 5	3 ± 0.02
6	SVS 6	2 ± 0.02
7	SVS 7	8 ± 0.05
8	SVS 8	6 ± 0.02
9	SVS 9	5 ± 0.01
10	SVS 10	3 ± 0.02
11	SVS 11	2 ± 0.03
12	SVS 12	4 ± 0.04

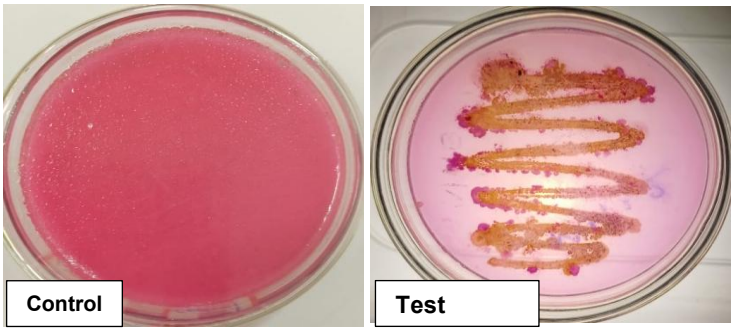


Figure 5. Lipase activity.

Blue agar plate method

The CTAB agar plate technique is a semiquantitative assay for the precipitation of extracellular glycolipids or other anionic surfactants. Blue agar plate method shown by SVS2 isolate positive result produced blue colour colonies. The assay was developed, and the current study results showed that approximately half of the isolates were positive. The results represented in Figure 6. These results are similar to the findings of Javadi et al. (2021), who established the CTAB methods dependability for first biosurfactant screening. Earlier studies have also supported this observation by Siegmund and Wagner (1991), developed the methylene blue-CTAB assay and demonstrated its effectiveness in identifying glycolipid producing microorganisms, while Walter et al. (2010) reported similar detection rates ranging from 30-60% among soil isolates.

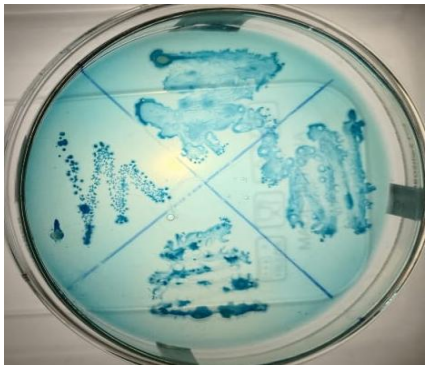


Figure 6. Blue agar plate method.

Bacterial adhesion to hydrocarbons (BATH) test

The ability of actinomycetes SVS2 to adhere to hydrocarbons is a characteristic feature of biosurfactant-producing microorganisms. expand the bacterial bond to hydrocarbons technique, an easy photometric assay to calculate the hydrophobicity of microorganism. The results revealed a wide variation in actinomycetes adhesion among the potential isolates, with the highest cell adherence recorded at 92.23 ± 0.51%, while the lowest adherence was observed at 52.15 ± 1.42%. These findings indicate strong surface hydrophobicity in certain actinomycete isolates, suggesting their high potential for biosurfactant production and hydrocarbon interaction. The method evaluates the adhesion ability of actinomycetes to various liquid hydrocarbons using three hydrocarbons in the assay, with maximum adhesion observed toward hexane. Furthermore, this approach enables the simultaneous finding of biosurfactant production and hydrocarbon degradation on hydrocarbon-overlaid agar plates. Rosenberg et al. (1980) reported similar findings, confirming the BATH test as a credible indication of hydrocarbon-degrading and biosurfactant-producing actinomycetes. Previous investigations (Pacwa-Płociniczak et al., 2011; Das and Chandran, 2011) found that isolates with significant adherence to hydrophobic substrates, such as hexane and hexadecane, had higher hydrocarbon degradation capacity. Manikkasundaram et al. (2023) reported the capacity to detect both biosurfactant synthesis and hydrocarbon breakdown by topping agar plates with hydrocarbons.

Emulsification index (E24)

Emulsification Index is one of the standards to influence the capability of biosurfactants. Emulsifying index (E24 affect the efficiency of biosurfactant and are specified as a percentage (%) of the height of the emulsified layer divided by the total height of the liquid column. Among the tested isolates, SVS2 showed the highest activity with a percentage of 95%, while the lowest recorded percentage among the isolates was 18%. The result was recorded as 95.25± 0.2%, showing complete uniformity among the measured values. The lowest percentage value was 18.00 ± 0.2%, indicating no variation among the observations. In the current study, a number of isolates demonstrated positive emulsification ability and exhibited good emulsification indices in the oil emulsification method, as a shown Figure 7. These results emphasise the isolates potential for producing surface-active compounds with wide hydrocarbon affinity. Nalini et al. (2021) made similar discoveries, reporting that

biosurfactant-producing strains commonly exhibit high E24 values, especially when exposed to long chain hydrocarbons. The considerable emulsification capacity shown in our isolates is also consistent with the findings of Satpute et al. (2008), who noted that effective emulsifiers often form stable emulsions for more than 24 hours.

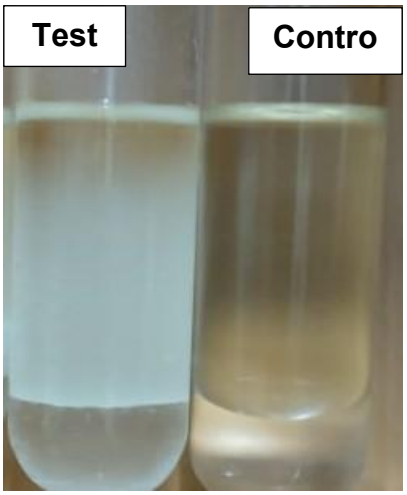


Figure 7. Emulsification index.

Emulsification assay

The emulsification test was used as an indirect screening method, with the presence of biosurfactants in the cell-free broth resulting in hydrocarbon emulsification. The tested isolates, SVS2 showed the highest percentage (73%), whereas the lowest percentage recorded was 4%. The percentage value was 73.00 ± 0.1%, indicating no variation among the observations. The percentage value was 4.00 ± 0.1%, indicating no variation among the observations. In our study, petroleum oil had the strongest emulsification activity, as shown in Figure 8, suggesting a higher interaction between biosurfactant molecules and mid-chain hydrocarbons. Shubhrasekhar et al. (2013) reported similar findings, noting that petroleum substrates produced higher E24 values. Gudina et al. (2013) found that petroleum oil provides better emulsification stability than lighter hydrocarbons. Bezza and Chirwa (2017) found comparable results, which suggest strong emulsification with petroleum and crude oil. Overall, our findings demonstrate that isolates with higher E24 values are effective biosurfactant makers with intriguing industrial applications.

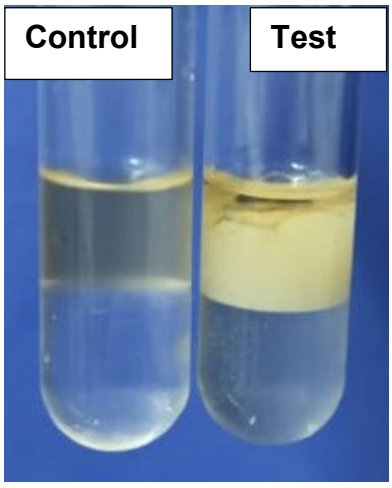


Figure 8. Emulsification assay showing formation of the emulsion layer.

Molecular Characterization through 16S rRNA Analysis

The selected actinomycete isolate SVS2 was identified by 16S rRNA gene sequencing and BLASTN analysis against the NCBI database. The isolate exhibited 99% sequence similarity to *Nocardia wallacei*, suggesting its possible identity as this species (Figure 9). Previous studies have demonstrated that 16S rRNA gene sequencing is an important molecular tool for identifying Nocardia species (Conville et al., 2018). However, closely related species may exhibit high sequence similarity, which can limit definitive species-level identification. Multilocus sequence analysis using genes such as gyrB, secA1, hsp65, and rpoB can improve identification accuracy (Conville et al., 2018; Xiao et al., 2016).

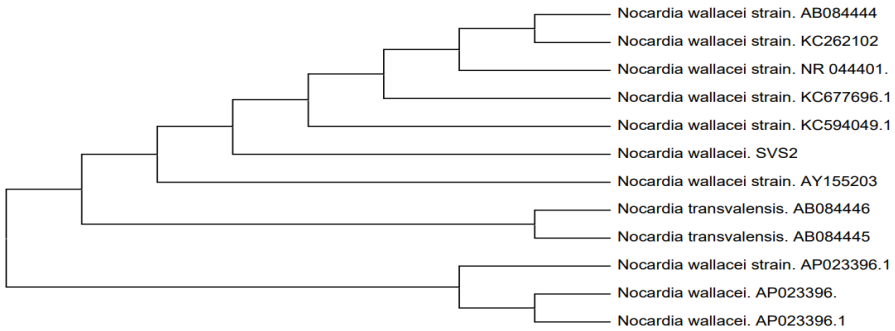


Figure 9. Phylogenetic analysis of the selected actinobacterial isolate SVS2 based on 16S rRNA gene sequences, showing its evolutionary relationship with closely related species, including *Nocardia wallacei*.

CONCLUSION

Bacteria that produce biosurfactants are an environmentally kindly reserve for synthetic surfactants in a variety of area such as cosmetics, agriculture and petroleum recovery due to their effectiveness in bioremediation, low toxicity, stability under extreme environmental conditions. When it comes to interpret industrial and environmental problems, their special qualities and adaptability make them indispensable. Achieving cost-effective large-scale production and realising their full potential require on-going research and technological innovation.

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DECLARATIONS

Consent for Publication We confirm that the manuscript has been read and approved by all named authors. Conflict of Interest The authors declare no conflict of interest

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