**Journal of Environmental Biology**
p-ISSN: 0254-8704 • e-ISSN: 2394-0379 • CODEN: JEBIDP

JEB®

Original Research Paper
Volume 45 Issue 3 May 2024 pp. 338-348
*DOI: http://doi.org/10.22438/jeb/45/3/MRN-5202*

*J. Environ. Biol.*, 2024

Journal website : www.jeb.co.in ★ E-mail : editor@jeb.co.in

# Biosurfactant production by *Bacillus amyloliquefaciens*, characterization and its potential applications

**C.M. Brinda, R. Ragunathan* and J. Johney**

*Department of Biotechnology, Centre for Bioscience and Nanoscience Research, Coimbatore–641 021, India*
**Received:** 28 November 2023 **Revised:** 25 January 2024 **Accepted:** 20 March 2024

***Corresponding Author Email :** cbnrcindia@gmail.com ***ORCiD:** https://orcid.org/0000-0003-4416-6232

## Abstract

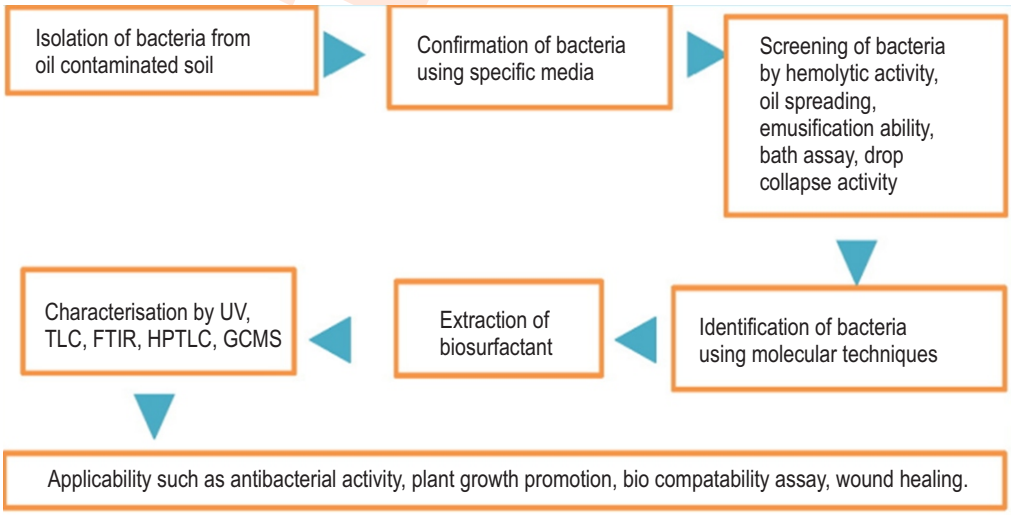
**Aim:** To identify indigenous bacteria responsible for producing biosurfactants in soil contaminated by oil and to investigate their potential uses in the fields of pharmaceuticals and environmental applications.

**Methodology:** The microorganisms of oil-contaminated soil were isolated, screened, and identified using 16S rRNA gene sequencing. *Bacillus amyloliquefaciens* was utilized to produce biosurfactants, and were characterized by UV, TLC, FTIR, HPTLC, and GCMS analysis. Antibacterial activity, plant growth promotion, biocompatibility, and wound healing activity of surfactant were performed.

**Results:** Six bacterial cultures (B1 to B6) were isolated from the soil samples collected from various locations in Kerala and Tamil Nadu. Among the isolates screened for biosurfactant production, *Bacillus amyloliquefaciens* exhibited the highest capacity for biosurfactant production, confirmed through 16S rRNA gene sequencing. The biosurfactant produced was identified as surfactin of lipopeptide nature, characterized by various analytical techniques: UV absorption at 400 nm, TLC and HPTLC (Rf value of 0.58), FTIR (prominent peak at 3819 $cm^{-1}$ corresponding to -OH groups), GCMS with a maximum retention time of 12.03. Surfactin effectively inhibited pathogenic bacteria, enhanced plant growth, was biocompatible, and enhanced the rate of cell migration towards wound scratch.

**Interpretation:** The current study revealed that *Bacillus amyloliquefaciens,* isolated from oil-contaminated soil, is capable of producing biosurfactant surfactin. Additionally, it unveiled a broad spectrum of applications for surfactin, encompassing wound healing, biocompatibility, antimicrobial activity, and promotion of plant growth.

**Key words:** Antibacterial activity, Biocompatibility, *Bacillus amyloliquefaciens*, Biosurfactant, Wound healing.




**How to cite:** Brinda, C.M., R. Ragunathan and J. Johney: Biosurfactant production by *Bacillus amyloliquefaciens,* characterization and its potential applications. *J. Environ. Biol.*, **45,** 338-348 (2024).

## Introduction

Surfactants are organic molecules that reduce the surface tension between liquids, liquid and solid, or gas and liquid. This reduction depends on physical and chemical factors of surfactants such as concentration, temperature, purity, structure and electrolyte concentration. It tends to form a monolayer or bilayer structures at surfaces and interfaces (Faisal *et al.,* 2023). They consists of a hydrophilic head and a hydrophobic tail, enabling them to adsorb and aggregate at interfaces (Bjerk *et al.,* 2021). The hydrophilic part of surfactants interact favorably with polar molecules such as metals, water and other ions. Conversely, the hydrophobic section allows interaction with non-polar substances like hydrocarbons (Zhu and Free, 2020). Biosurfactants are derived from microorganisms like fungi, yeasts and bacteria that function similarly to surfactants. They operate at interfaces altering surface properties like adhesion, wettability and surface tension (Sing *et al.,* 2019). Unlike synthetic surfactants, biosurfactants are biodegradable, environmentally friendly, and often exhibit diverse functionalities, making them attractive for various industrial applications (Araujo *et al.,* 2020). Their ability to function in extreme conditions and their compatibility with biological systems have sparked interest in their use across industries including agriculture, pharmaceuticals, cosmetics and environmental remediation (Nayarisseri *et al.,* 2018).

There are mainly four categories of surfactants: cationic surfactants, anionic surfactants, amphoteric surfactants, and non-ionic surfactants (Falk, 2019). Anionic surfactants have negatively charged ions in the hydrophilic portion. They are mainly used in shampoo, laundry and dishwashing liquids as they possess superior cleansing capacity. Surfactin is an anionic cyclic lipopeptide biosurfactant. Sodium laureth sulfate and Sodium lauryl sulfate are examples of anionic surfactants. Cationic surfactants have positively charged ion head regions and good emulsifying abilities. It is mostly used in hand and bathroom sanitizers. Cetylpyridinium chloride and Adogen are common examples of cationic surfactants (Badmus *et al.,* 2021). Amphoteric surfactants have both anionic and cationic ions in the hydrophilic region. Their application includes household cleaning and personal care products. Betaines and lecithin, are frequently utilized as zwitterionic surfactants. Non-ionic surfactants do not possess either a positive or negative electrical charge. They are commonly used in drug delivery and household cleaners. Polyoxyethylenated alcohol and polyoxyethylenated alkyl phenol are examples of non-ionic surfactants (Nikunj and Tejas, 2017).

Biosurfactants have gained importance as they are non-toxic and environmentally safe. The adoption of biosurfactants has been prompted by the adverse environmental effects caused by synthetic surfactants. (Barakat *et al.,* 2017). Biosurfactants are capable of replacing chemical surfactants in many industrial activities. Commercially, the most important biosurfactants are Lipopeptides and Glycolipids produced by *Bacillus* sp., *Pseudomonas* sp., and *Candida* sp. (Brinda *et al.,* 2023). In pharmaceutical industries recovery of intracellular products and gene delivery can be done through biosurfactants. They can act as emulsifying agents, antimicrobial substances and enzyme inhibitors (Basha *et al.,* 2021). Biosurfactants, with their environmentally friendly nature, aid in the bioremediation process by enhancing the solubility and bioavailability of hydrophobic contaminants. Their unique properties facilitate the emulsification and degradation of pollutants, leading to more efficient and sustainable clean-up of contaminated sites. Biosurfactant-mediated bioremediation offers a promising solution for addressing environmental pollution, while minimizing ecological impact. They have a significant role in oil industry in the recovery of oils and cleaning of oil-contaminated areas. The emulsifying property of biosurfactants makes them ideal for detergents and laundry industries (Mathew *et al.,* 2020). The remarkable properties such as increasing shelf life, impeding the multiplication of pathogenic microorganisms, altering the viscosity, and making them less toxic, make them appropriate in the food industry (Banat *et al.* 2014). Antimicrobial activity, non-lethal properties, foaming, and hydrating properties of microbial surfactants permit them to replace synthetic surfactants in the cosmetic industry. They improve soil quality, eliminate plant pathogens, and enhance the bioavailability of supplements for beneficial microbes (Sarubbo *et al.,* 2022). This study aimed to isolate indigenous bacteria capable of producing biosurfactants from hydrocarbon-contaminated oil in different districts of Kerala and Tamil Nadu regions. Subsequently, the study assessed the potential of these biosurfactants in promoting plant growth, exhibiting antimicrobial activity, facilitating wound healing and determining their biocompatibility.

## Materials and Methods

**Soil sample acquisition:** Soil samples were collected from oil-splattered surfaces at several vehicle garages and gasoline outlets in Kerala and Tamil Nadu, India. Sampling sites, 5 cm deep, were excavated to collect the polluted soil. Three samples were collected from Kalamassery, Aluva, and Palakkad, districts in Kerala, while other samples were collected from Eachanari, Coimbatore and Madukkarai districts in Tamil Nadu. The samples were sealed in sterile containers and transferred to the laboratory for examination.

**Study area:** The study area encompassed various vehicle garages and gasoline outlets located in Kerala and Tamil Nadu, India. Kerala is situated in the south-western region of India. Kalamassery, Aluva and Palakkad in Kerala are characterized by industrial and urban environments, with significant vehicular activity contributing to pollution. Tamil Nadu, located in the south-eastern part of India, is known for its industrial development. Eachanari, Coimbatore, and Madukkarai in Tamil Nadu are notable for their industrial zones, including automobile manufacturing and associated services. The sampling sites were specifically selected from the areas adjacent to vehicle garages and gasoline outlets, where oil splattering is common.

**Isolation of bacteria from contaminated soil:** Five bacterial strains were isolated using serial dilution method, plated on nutrient agar, and incubated for 24 hr at 37 $^0$C as outlined by Sanders (2012). The eminent colonies with various morphological features were re-suspended in the nutrient broth and incubated for 24 hr at 37 $^0$C, and later it was employed for further studies.

**Biochemical and morphological assessment of isolates:** The isolated microbial cells were observed by Gram staining method with the help of a compound microscope (Unicon GE-50). Bacilli-like bacterial cell morphology was observed. Biochemical analyses such as Gram staining, Glucose fermentation, Colonial characteristics, Lipid hydrolysis, Voges Proskauer, Starch hydrolysis, Methyl red test, Catalase activity, Indole production, Spores, Casein hydrolysis and Gelatin hydrolysis were performed following the standard protocol of Barrow and Feltham (1993).

**Confirmation of bacteria using screening media:** On Starch agar (30 g in1000 ml), Casein agar (35 g in 1000 ml) and *Bacillus agar* medium (49.22 g in 1000 ml), *Bacillus* sp. was screened as per the protocol of Holt *et al.* (1994). Bacterial isolates were streaked over differential media and then cultured for 24 hr at 37 $^0$C. Hi- Chrome Bacillus agar medium was inoculated and incubated with bacterial colonies which exhibited obvious zones on Starch agar and Casein agar and used for additional research based on appearance and pigment synthesis.

**Production of biosurfactant:** Minimal Salt Medium was used to inoculate bacterial isolates, and diesel (1% w/v)was added as a carbon source, thereafter incubated for 7 days at 37 $^0$C. The culture medium was centrifuged at 8000 revolutions per minute for 10 min, and the supernatant was extracted following the procedure of of Faisal *et al*. (2023) with diminutive modifications.

## Screening of Biosurfactants

**Hemolysis efficacy:** Blood agar plates (5% sheep blood) were streaked with isolated bacteria and retained at 37 $^0$C for 24 hrs as per the procedure of Carrilo *et al.* (1996). The results were recorded based on the hemolytic activity and zone of hemolysis.

**Oil dispersing analysis:** Following the protocol outlined by Morikawa *et al.* (2000), a thin layer of oil was prepared by adding 500 µl of oil onto the surface of 30 ml of distilled water. Subsequently, 10 µl of the supernatant from the bacterial culture was carefully pipetted onto the center of the oil-coated film layer. Diameter of the clearing zones were measured to observe any displacement of the oil, indicating the presence of biosurfactant activity. Distilled water served as a negative control in this experiment.

**Emulsification test:** One ml of cell-free supernatant was added to 500 µl of oil, which was then vortexed at great speed for 3 min. It was then left undisturbed at room temperature for 24 hr as Van Dyke *et al*. (1993). The height of the liquid and the height of stable emulsion layer were measured after 24 hr. Distilled water was used as a negative control.

**Bacterial adhesion to hydrocarbon assay:** The cell culture was centrifuged at 10,000 rpm for 10 min, as specified in the procedure by Rosenberg *et al.* (1980). The cell pellets were collected and washed twice with phosphate buffer and resuspended in phosphate buffer (pH 7.0). The optical density of this dispersion was read at 600 nm wavelength. One ml of a cell solution was mixed with 50 µl of oil and vortexed for approximately 3 min. The hydrocarbons and aqueous phases were left for 2 hrs to separate. A UV spectrophotometer set at 600 nm was used to read the optical density.

**Drop collapse analysis:** To provide a uniform oil coating, sterile petri plates were coated with oil. At the center of Petri plate, a 5 µl of supernatants were placed following the procedure by Jain *et al.* (1991). After 15 min, the reactions were visually examined with the a magnifying glass. Distilled water was retained as a negative control.

**Identification of bacteria by molecular methods:** Bacterial genomic DNA was purified and utilized as a template for 16S rRNA gene amplification by phenol-chloroform method of (Cheng and Jiang, 2006). The 16S rRNA was amplified using the particular primers 16SF (5'- AGAGTTTGATCMTGGCTCA G -3') and 16SR (5'-ACGGYTACCTTGTTACGACTT-3'). Purified amplified 16S rRNA strands were forwarded to Chromus Biotech in Bangalore for sequence analysis. The collected 16S rRNA gene sequence was submitted to the Basic Local Alignment Search Tool (BLAST). The MEGA10 program was used for molecular evolutionary analysis. The partial 16S rRNA gene sequence of bacterial strain was deposited in the NCBI GenBank database for accession number.

**Biosurfactant extraction:** The acid precipitation method illustrated by Bezza and Chirwa (2016) was used to extract the biosurfactant. The MSM broth culture was centrifuged at 10,000 rpm for 15 minutes to extract the biosurfactant. The cell-free supernatant was precipitated with 6N HCl (pH 2.0) and preserved at 4 $^0$C in the refrigerator overnight. The precipitated cell-free supernatant was centrifuged for 15 min at 10,000 rpm (Remi R-8C), the pellet was recovered and the pH was maintained at 7.0 by adding deionized water. The crude biosurfactant was extracted with methanol and chloroform mixture and left undisturbed for 30 min to separate a bottom layer containing biosurfactant and dried by vaporization at 37$^o$C for an hour.

## Analytical Techniques

**UV-visible spectroscopy analysis:** UV-visible analysis was done as specified in the procedure by Chandrasekharan and BeMiller (1980) with a span of 200–800 nm was used to analyze the collected biosurfactants using an LT291 UV-vis spectrometer.

**Thin-layerchromatography:** Thin-layer chromatography was

performed for the preliminary evaluation of extracted biosurfactant (Cao *et al*., 2009). Commercial surfactin from Merck, Mumbai, was employed as a positive control.

**Fourier transform infrared spectroscopy:** The FTIR spectrum analysis was conducted using a Shimadzu FTIR-8200 instrument from Japan. An attenuated total reflection (ATR) accessory with a diamond ATR crystal was employed for the analysis. The analysis was performed with a resolution of 4 $cm^{-1}$ across the spectral range of 4000–550 $cm^{-1}$. The biosurfactant samples were placed under the infrared beam for analysis. Commercial surfactin sourced from Merck Mumbai served as a reference for comparative analysis.

**High-performance thin-layer chromatography:** To obtain more precise data, an improved version of TLC named high-performance thin-layer chromatography was implemented. Using a solvent system of TOLUENE: ETHYL ACETATE + FORMIC ACID (20:3, v/v), HPTLC was carried out on silica 60 plates (10.0 x 10.0 cm, Merck, Germany) as explained by Srivastava (2011). An ATS4 automatic sampling instrument (CAMAG Linomat 5, Switzerland) was used to detect samples. The plates were stained after development by immersing them in a Phosphomolybdic acid reagent solution, heated to 60°C, and evaluated. Photos were taken for documentation.

**GC/MS analysis spectrometry:** A gas chromatograph machine (Agilent) was loaded with 1µl of biosurfactant sample. Helium as a carrier gas, was used at a steady flow rate of 1.5 ml $min^{-1}$, and the operating temperature of GC injector was 260 $^0$C. The temperature of the ion source was synchronized at 280 $^0$C. The GC ran for a total of 90.67 min, as planned by the GC-MS analyzer. Summits in chromatographs were identified by comparison with mass spectral standards.

## Applications

**Antibacterial assay:** The antibacterial activity of biosurfactant was evaluated by well-diffusion method (Balouiri *et al*., 2016), *Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae* and *Staphylococcus aureus* bacterial cultures were dispersed in Muller Hinton agar media. Using a sterile stainless cork borer, an agar well measuring 5 mm diameter was created. Each well received 10 µl of the extract. An ampicillin disc was used as a positive control, and the plates were incubated at 37 $^0$C for 24 hr with DMSO as a negative control. The antibacterial activity was determined by measuring the diameter of zone of inhibition.

**Plant growth promotion:** After surface sterilization with 1% sodium hypochlorite, the seeds of *Vigna radiata* were washed thrice with sterilized water. Twelve sterile seeds were arranged in a Petri dish with filter paper soaked in 10 ml of biosurfactant solution. The dish was incubated for 7 days at 30 $^0$C, thereafter the root and shoot lengths were measured.

**Biocompatibility assay:** A 96-well plate was seeded with 200 µl of cell suspension (L929-Mouse embryo fibroblast cell lines NCCS, Pune) without the test agent and incubated for 24 hr. The biosurfactant (12.5, 25, 50, 100 and 200 µg) was applied to the cell lines. The plates were incubated for 24 hr at 37 $^0$C in an environment containing 5% $CO_2$. Spent media was removed at the end of the incubation time, and MTT reagent was added at a final concentration of 0.5 mg $ml^{-1}$ of total volume. To protect the plates from light exposure, aluminum foil was wrapped around them. Plates were then incubated for further 3 hr as explained by Skladanowski *et al.* (2016). Following the removal of MTT reagent, 100 µl of DMSO solubilization solution was applied. A gyratory shaker was used to accelerate the dissolution. The absorbance was read at 570 nm on a spectrophotometer. The medium control consisted of a medium free of cells. A medium containing cells but devoid of the experimental biosurfactant was used as a negative control. Cells treated with 1µM $ml^{-1}$ of doxorubicin served as a positive control of the medium.

**Wound healing activity:** The cell lines (L929 –Mouse embryo fibroblast cell line NCCS, Pune) were cultured in 10% FBS-supplemented DMEM with high glucose medium. The cells were seeded on a 12-well tissue culture plate at a density of 0.25 million cells per well. A fresh 200 µl pipette tip was used to gently scrape the monolayer along the center of the well. The unattached cells were eliminated from the wells by washing them twice with the medium. The cells were treated with biosurfactant prepared in fresh basal media and incubated at 37 $^0$C with 5% $CO_2$ in an incubator for 48 hrs (Jonkman *et al*., 2014). Cell images were taken at different time intervals (0, 24, 48 hrs).

**Statical analyses :** Analysis of variance (ANOVA) was employed to ascertain the statistical parameters pertinent to the

**Table 1:** Morphological and biochemical analyses of bacterial isolates obtained from oil-contaminated soil

| Bacterial Isolates | Gram Staining | Casein Hydrolysis | Glucose Fermentation | Catalase Activity | Lipid Hydrolysis | Starch Hydrolysis | Indole production | Methyl Red text | Gelatin Hydrolysis | Voges Proskouw | Spores |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $B_1$ | +ve | +ve | +ve | +ve | +ve | +ve | -ve | +ve | +ve | +ve | +ve |
| $B_2$ | +ve | +ve | +ve | +ve | +ve | +ve | -ve | +ve | +ve | +ve | +ve |
| $B_3$ | +ve | +ve | +ve | +ve | +ve | +ve | -ve | +ve | +ve | +ve | +ve |
| $B_4$ | +ve | +ve | +ve | +ve | +ve | +ve | -ve | +ve | +ve | +ve | +ve |
| $B_5$ | +ve | +ve | +ve | +ve | +ve | +ve | -ve | +ve | +ve | +ve | +ve |

**Table 2:** Oil spreading assay of biosurfactant isolated from bacterial isolates

| Bacterial | Diameter of clearing zone | Interpretation |
|---|---|---|
| $B_1$ | 39± 0.3 | Positive |
| $B_2$ | 36 ± 0.35 | Positive |
| $B_3$ | 25± 0.27 | Positive |
| $B_4$ | 17±0.17 | Positive |
| $B_5$ | 0 | Negative |

Values are mean ± S.D.

**Table 3:** Emulsification index of biosurfactant from bacterial isolates on petrol and coconut oil

| Bacterial isolates | Emulsification ability test | Height of emulsion (mm) | Emulsification Index (%) |
|---|---|---|---|
| $B_1$ | petrol | 19 ± 0.56 | 38 |
| | coconut oil | 22± 0.54 | 44 |
| $B_2$ | petrol | 22± 0.57 | 44 |
| | coconut oil | 20± 0.49 | 40 |
| $B_3$ | petrol | 21± 0.45 | 42 |
| | coconut oil | 18± 0.35 | 36 |
| $B_4$ | petrol | 17± 0.30 | 34 |
| | coconut oil | 18± 0.33 | 36 |
| $B_5$ | petrol | 19± 0.37 | 40 |
| | coconut oil | 18± 0.32 | 36 |

Values are mean ± S.D.

**Table 4:** Cell hydrophobicity of biosurfactant from selected bacterial isolates

| Bacterial isolates | OD of aqueous phase of hydrocarbons | OD of bacterial suspension | Cell adherence (%) |
|---|---|---|---|
| $B_1$ | 0.34 | 0.993 | 65 |
| $B_2$ | 0.372 | 0.935 | 60 |
| $B_3$ | 0.387 | 0.938 | 58 |
| $B_4$ | 0.261 | 0.402 | 35 |
| $B_5$ | 0.189 | 0.278 | 32 |

**Table 5:** Drop collapse assay of biosurfactant extracted from bacterial isolates

| Bacterial isolates | Drop Collapse Assay |
|---|---|
| $B_1$ | +++ |
| $B_2$ | +++ |
| $B_3$ | +++ |
| $B_4$ | + |
| $B_5$ | - |

optimization of culture conditions. A significance criterion of $p < 0.05$ was adopted for determining statistical significance. The data were expressed as mean ± S.D. from independent experiments

## Results and Discussion

Five bacterial strains ($B_1$, $B_2$, $B_3$, $B_4$ and $B_5$) were recovered from contaminated soil collected from different districts of Kerala and Tamil Nadu states (India). The analysis revealed that the samples collected from Kalamassery, district of Kerala showed the maximum bacterial colony population ($10.^6x10^5$ CFU $ml^{-1}$). The lowest bacterial colony number was found in the Madukkarai district of Tamil Nadu ($1x\ 10^3$ CFU $ml^{-1}$). Following biochemical analysis and screening media, the bacterial strains were identified as *Bacillus* sp. for confirmation 16S rRNA gene sequencing was done. Cellular morphology revealed that all the isolates were rod-shaped, Gram-positive endospore-forming bacteria. All the bacterial isolates showed positive biochemical tests (Table 1). The morphological and cellular properties of *Bacillus* sp., have been extensively documented by Basha *et al.* (2021). They have described the morphological features including rod-shaped appearance under the microscope and the presence of endospores. Nayarisseri *et al.* (2018) shed light on various aspects of *Bacillus* morphology and cellular characteristics. Biochemical analyses such as casein hydrolysis, starch hydrolysis, and glucose fermentation documented by Nayarisseri *et al.* (2018) showed similar positive results.

All bacterial isolates showed hemolytic activity while bacterial strains $B_1$ and $B_2$ showed the highest level of hemolysis zone (2 cm). Paul (2018) formerly reported that biosurfactants produced by *Bacillus* sp and *Pseudomonas* sp can induce the lysis of erythrocytes and form a clear zone. Erythrocytes are lysed by the organisms that produce biosurfactants, resulting in a translucent zone across all sides of the colony. According to Barakat *et al.* (2017), the concentration of biosurfactants is proportional to the transparent zone of hemolysis. In oil spreading test, the diameter of the zone and the presence of biosurfactants are directly correlated. Except for isolate B5, all the strains were positive (Table 2). According to Basha *et al.* (2021), the isolates can be screened for biosurfactants effectively based on the oil spreading positive outcomes. According to Paul (2018), the effectiveness of the oil-spreading assay is used to gauge the generation of biosurfactants. Isolate $B_1$ showed the highest oil-displacing activity. The biosurfactants in the cell-free supernatant emulsified with the hydrocarbons present in the test solution.

The emulsification test was carried out using organic solvent (petrol and coconut oil). In petrol, the bacterial isolate $B_2$ displayed a higher emulsification index of 44% while the bacterial isolate $B_1$ showed a higher emulsification index of 44%, with coconut oil (Table 3). Basha *et al.* (2021) explored the emulsification potential of surfactin derived from *Bacillus subtilis* MTCC 2422 against both kerosene and petrol, observing emulsification indices of 68% and 291.4%, respectively. Similarly, Singh *et al.* (2022) demonstrated that a biosurfactant produced by *Bacillus* sp. exhibited emulsifying activity of 63.1% with oil. Cell hydrophobicity is a good indicator of biosurfactant synthesis.

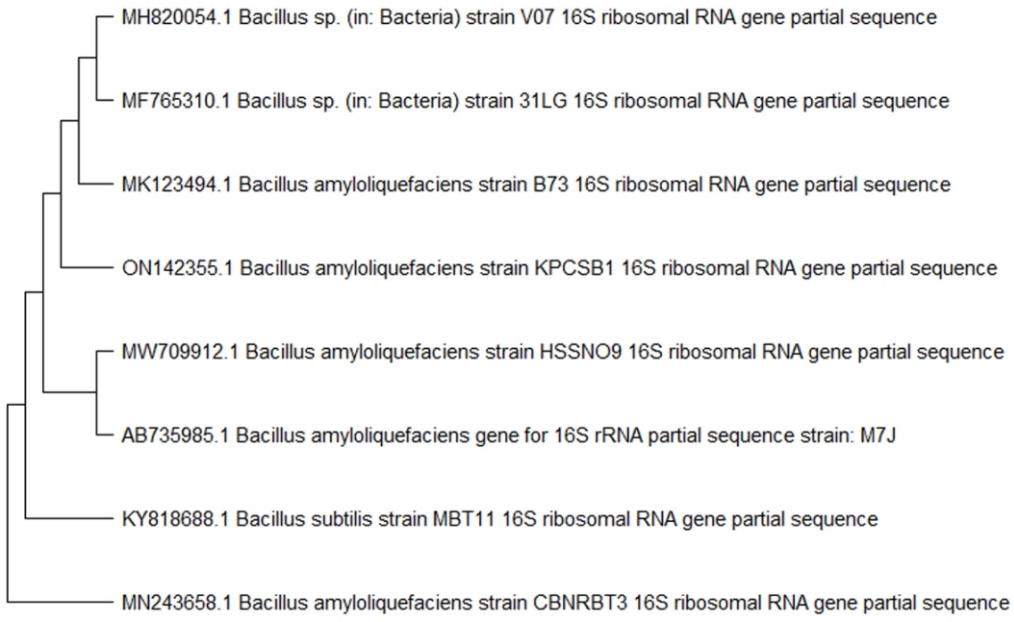



**Fig.1:** Phylogenetic tree of isolate *Bacillus amyloliquefaciens* based on 16S rRNA sequence constructed by MEGA 10 software.

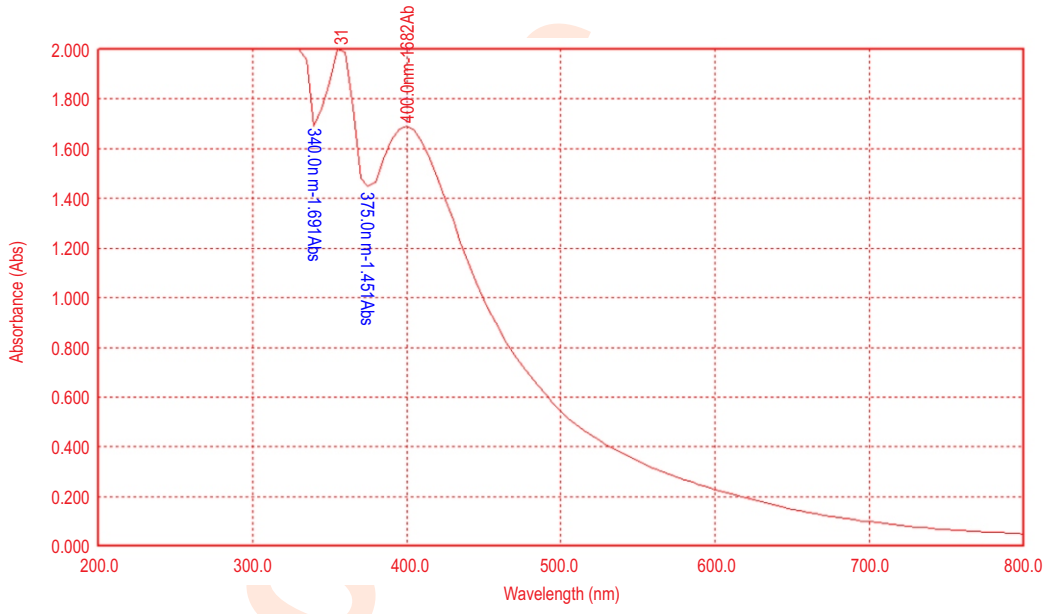



**Fig. 2:** UV analysis of biosurfactant.

The capacity of bacterial cells to cling to hydrocarbons was evaluated by BATH assay. Cell adherence was found maximum in isolate B1 (Table 4). The results of the BATH assay obtained, in this study consistent with the reports of Nayarisseri *et al.* (2018). who reported in their study that, *Staphylococcus* sp exhibited 84.77 % adherence, while *Bacillus* sp. displayed the maximum adherence of 94.23% whereas *E. coli* demonstrated the lowest level of adherence at 60.15 percent. The results of this study showed that *Bacillus* sp exhibits the strongest cell adhesion among other bacteria. According to Faisal *et al.* (2023), *Pseudomonas* sp. exhibited an attachment capacity for oil droplets ranging from 5.66 to 88.33%. The drop collapse assay is

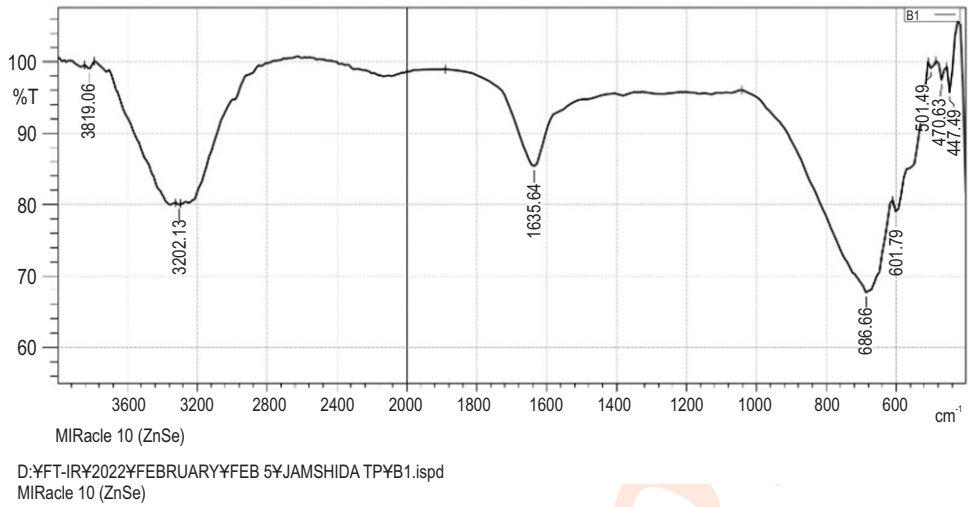



**Fig. 3:** FTIR analysis of biosurfactant.

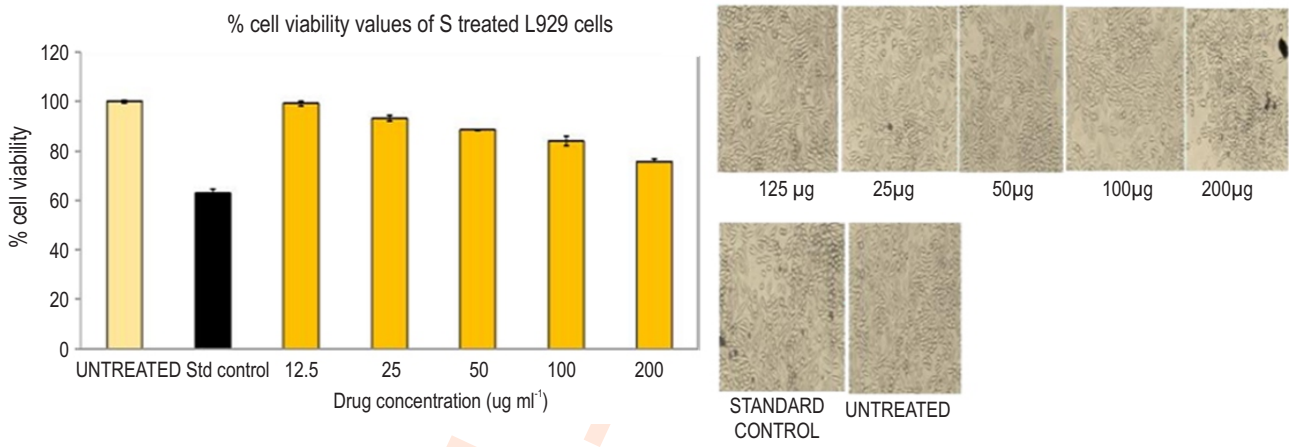



**Fig. 4:** Biocompatibility assay of biosurfactant.

predicated on a bacterial extract containing biosurfactant disrupting liquid drop. Except for isolate B5, each bacterial strain produced favorable results (Table 5). According to Korayem *et al.* (2015) collapsed drops verified the presence of biosurfactant in the sample. In agreement with Faisal *et al.* (2023) biosurfactant generated by *Pseudomonas* sp. also produced positive results in the drop collapse assay. Nayarisseri *et al.* (2018) elucidated the phenomenon wherein a Bacillus droplet collapsed within 58 seconds, thereby providing corroborative evidence for the outcomes observed in the present study. Following preliminary identification and screening, DNA from the selected bacterial isolates were extracted as well and 16S rRNA was amplified with PCR. The MEGA 10 software (Maximum Likelihood Tree) was used to create the phylogenetic tree, which had a distance of 0.1. Phylogenetic tree and molecular characterization revealed that the bacteria produced biosurfactant as *Bacillus amyloliquefaciens* (Fig. 1). *Bacillus amyloliquefaciens* was allocated the NCBI accession number MN243658. Guzman (2020) documented the presence of *Bacillus* sp. in oil-contaminated soil and on the basis of molecular analysis the bacteria was identified as *Bacillus pseudomycoides.* UV-VIS spectroscopy confirms the presence of specific types of functional groups.

The maximum peak for *Bacillus amyloliquefaciens* was visible in the isolated biosurfactant at 400 nm (Fig. 2). UV absorption at 355 nm showed an azo chromophore with an n-* transition. The peak of absorption at 375 nm denotes the presence of arene groups with n-* transitions, such as anthracene. Noudeh *et al.* (2010) confirmed the identity of the product as a lipopeptide produced by *Bacillus licheniformis,*

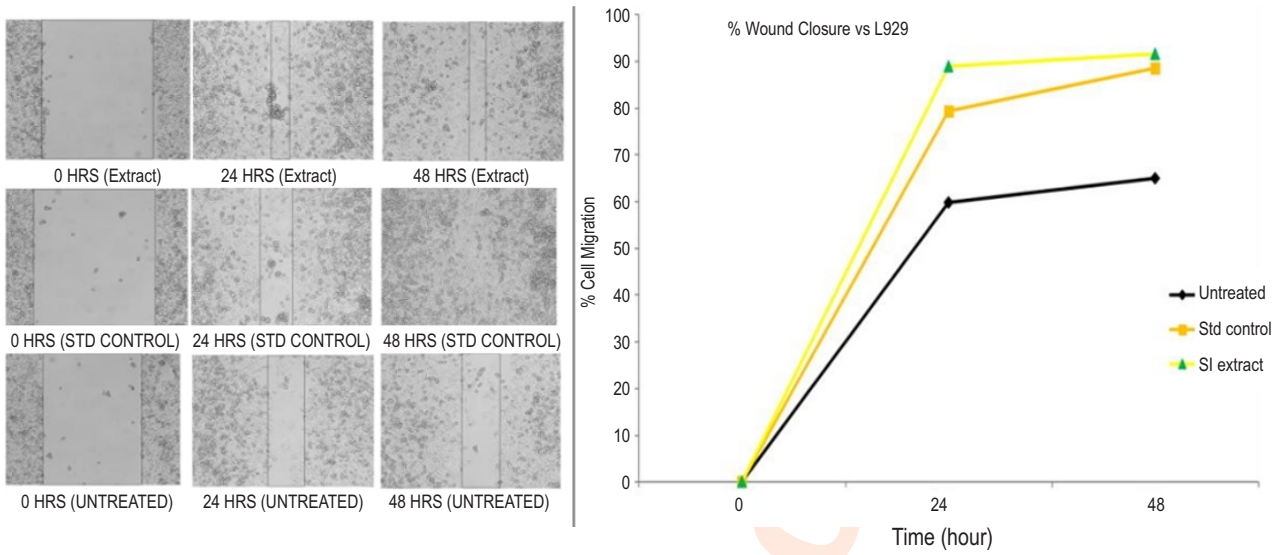



**Fig. 5:** Wound healing activity of biosurfactant.

**Table 6:** Major functional compounds of biosurfactant identified by GC-MS

| Retention time | Functional compounds | Molecular formulae |
|---|---|---|
| 3.3741 | Quinoxaline-2-ethanamine, 6-chloro-N,N-diethyl- | $C_{14}H_{18}ClN_3O$ |
| 5.4151 | Methylene cyclopropane carboxylic acid | $C_5H_6O_2$ |
| 5.9489 | Oxirane, (methoxymethyl)- | $C_4H_8O_2$ |
| 6.1212 | Oxime-, methoxy-phenyl-_ | $C_8H_9NO_2$ |
| 8.162 | Ethylene glycol mono isobutyl ether | $C_6H_{14}O_2$ |
| 8.1688 | Propanoic acid, anhydride | $C_6H_{10}O_3$ |
| 8.1847 | N-Dimethyl amino methyl-tert-butyl- | $C_{10}H_{24}NP$ |
| 11.7987 | 2-Propanamine, N-methyl-N-nitroso- | $C_4H_{10}N_2O$ |
| 13.3161 | 5-Oxotetrahydrofuran-2-carboxylic acid, ethyl ester | $C_7H_{10}O_4$ |
| 21.4879 | Cyclopentane, 1,1,3,4-tetramethyl-, trans- | $C_9H_{18}$ |

**Table 7:** Effect of biosurfactant on the growth (cm) of *Vigna radiata* seedlings

| Treatments | Shoot length | Root length |
|---|---|---|
| Biosurfactant solution | 4.20 ± 0.56 | 1.60 ± 0.83 |
| Control (Water) | 3.10 ± 0.28 | 1.00± 0.46 |

Values are mean ± S.D.

based on the UV spectrum revealing peptide bonds at 339 nm. On the TLC plate, the results indicated the existence of a biosurfactant with a lipopeptide origin. The Rf value of biosurfactant was 0.56 in the TLC chromatogram. Lyu *et al.* (2018) verified that ninhydrin reagent produces a red spot on a TLC plate on the lipopeptide biosurfactant produces by *Bacillus* sp. Halophilic *Bacillus* sp. produced biosurfactant native to the lipopeptide category with an Rf value of 0.68, as confirmed by Donio *et al.* (2013).

The maximum peak of the biosurfactant recovered at 3819 $cm^{-1}$ corresponded to-OH groups (Fig. 3). The spectrum showed a broad absorbance peak centered around 3302 $cm^{-1}$, which is a typical feature of the stretching bond N-H (Barakat *et al.,* 2017). At wave number 1635 $cm^{-1}$, the peak of C=C alkene group detected, showed the presence of biosurfactant. The presence of C-Cl group is shown by the absorption frequencies at 686 $cm^{-1}$ and 601 $cm^{-1}$ (Faisal *et al.,* 2023).

In the previous investigation by Zhou *et al.* (2015), identical functional groups were observed with absorption frequencies of 3432, 1650, and 1558 $cm^{-1}$. According to this research, these bonds indicate that the biosurfactant produced by

*Bacillus* sp. is of cyclic lipopeptide nature. The results of the HPTLC further supported TLC findings. The HPTLC results showed that the bands were separated, with an Rf value of 0.5. Our findings were in line with those of Ohadi *et al.* (2018), who reported HPTLC chromatogram with an Rf value of 0.56 to identify lipopeptide biosurfactant produced by *Acinetobacter junii* $B_6$. For the biosurfactant component separation, Pendse and Aruna (2020) demonstrated HPTLC using a similar mobile phase of toluene and ethyl acetate by Al-Wahaibi *et al.* (2014) elucidated HPTLC as a method for the qualitative identification of biosurfactants. GCMS analyses showed main peaks at distinct retention time of 12.03, 10.62, 9.81 and 6.12. The biosurfactant analyzed in this study exhibited notable concentrations of fatty acid constituents, specifically carboxylic acid and methyl ester. These findings align closely with the results reported by Faisal *et al.* (2023), thereby reinforcing the consistency and validity of the observed chemical composition across independent investigations. This suggests that the presence of carboxylic acids and methyl esters in the biosurfactant is likely a characteristic feature rather than an anomaly, which can be important for further research and practical applications of the biosurfactant.

The component quinoxaline possesses a variety of intriguing biological properties, including antimicrobial, anticancer and antiprotozoal properties. Pharmaceutical drugs and biopolymers are madeofcarboxylic acid. The production of detergents, lubricants, and pain relievers use oxirane. While producing chemicals and coupling agents for metal household cleaners, ethylene glycol monobutyl ether serves as an intermediary. Brinda *et al.* (2023) pointed out the lipopeptide nature of biosurfactant produced by *Bacillus* sp. by GCMS spectrum. According to Lyu *et al.* (2018), more than ten significant derivatives were found in the biosurfactant of *Bacillus* sp. Lv 13 produced, with peaks between 9.45 and 27.67. The elucidation of lipopeptide-based biosurfactant production by *Bacillus velezensis* KLP2016, as demonstrated by Meena *et al.* (2021), provides further insight into the biochemical pathways and genetic determinants involved in the biosurfactant synthesis in *Bacillus* sp. The biosurfactants produced by *Bacillus velezensis* KLP2016 likely consist of peptide sequences derived from amino acids, coupled with fatty acid chains. A study conducted by Faisal *et al*. (2023) provides evidence indicating that the biosurfactant produced by *Bacillus amyloliquefaciens* possess structural features consistent with a lipopeptide.

Biosurfactants were tested for antibacterial activity against *Pseudomonas aeruginosa, Escherichia coli*, *Klebsiella pneumoniae*, and *Staphylococcus aureus* by well-diffusion method. Gram-negative and Gram-positive bacteria were both effectively inhibited by the biosurfactant produced by *Bacillus amyloliquefaciens.* The biosurfactant produced by *Bacillus subtilis* exhibited antibacterial efficacy against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa,* and *Salmonella enterica* typhi, as reported by Yuliani *et al.* (2018). The findings reported by Zampolli (2022) highlight the inhibitory effects of lipopeptide surfactants produced from *Bacillus subtilis* and *Rhodococcus* sp. on the growth of various bacterial species, namely *Mycoplasma* spp., *Escherichiacoli* and *Staphylococcus aureus*. This observation suggests that the lipopeptide surfactants possess antimicrobial properties, effectively suppressing the proliferation of these pathogenic bacteria. Such inhibitory effects are likely attributed to the ability of lipopeptide surfactants to disrupt bacterial cell membranes, leading to cell lysis and ultimately inhibiting bacterial growth. Moreover, the study also revealed that the application of the biosurfactant solution resulted in accelerated growth of green gram plants compared to the control group (Table 7). This outcome suggests a positive influence of the biosurfactant solution on plant growth and development. The enhanced growth of green gram plants may be attributed to several factors associated with biosurfactants, including improved soil moisture retention, enhanced nutrient uptake, and stimulation of root growth. Additionally, the antimicrobial properties of the biosurfactant may contribute to a healthier soil microbiome, promoting favourable conditions for plant growth. According to Marchut-Mikolajczyk *et al.* (2021), microbial surfactants may indirectly enhance plant growth by making available nutrients to microorganisms residing in the rhizosphere. Chopra *et al.* (2020) reported that biosurfactants increase the availability of various important micronutrients, which in turn improves the germination process of seeds in hydrocarbon-contaminated soil. As demonstrated in Fig. 4, biosurfactant showed minimal toxicity up to 200 µg $ml^{-1}$, however, in the case of control, 100% cell viability was achieved. Cytotoxicity MTT assay, revealed that the test chemical was not harmful to mouse fibroblasts, as the cell survival was 70% at maximum dose (200 µg $ml^{-1}$). Rodriguez-Lopez *et al.* (2020) observed cell viability in mouse fibroblast cells treated with varying concentrations of biosurfactant derived from *Bacillus* sp. Their findings indicated 90% cell viability, suggesting minimal toxicity. Kumari *et al.* (2023) assessed the cytotoxicity of the lipopeptide biosurfactant on HEA 293 cells using MTT assay, revealing negligible cytotoxic effects. The amphiphilic nature of lipopeptides allows them to interact with cell membranes without disrupting their integrity significantly. This property is crucial in maintaining cell viability and minimizing cytotoxic effects. Some lipopeptides have been reported to possess bioactive properties that can stimulate cell proliferation and viability. Lipopeptide biosurfactants are often metabolically degraded and cleared from the cellular environment. This process reduces their persistence within cells and tissues, minimizing the potential for cytotoxicity associated with prolonged exposure.

In wound healing activity untreated cells showed a wound closure percentage of 59% and 64% after 24 and 48 hrs, respectively. However, after 24 and 48 hrs, 88% and 91% wound closure percentage were seen in a treated cell line (Fig. 5). This outcome showed that the presence of biosurfactant enhanced the rate of cell migration approaching the wound scratch region. According to Muthukumar *et al.* (2023), this could be affected by promoting the mechanisms of inflammation, proliferation, and remodeling of wound-healing process. According to Sana *et al.*

(2017), there has been a discernible healing activity as evidenced by the development of keratinocytes, thick epidermal layer, and connective tissue regeneration at the wound site.

The isolated *Bacillus amyloliquefaciens* (MN 243658) exhibited significant potential across diverse sectors including the pharmaceutical industry, agriculture and cosmetic industries. Surfactin, derived from this strain, holds promise for bioremediation efforts aimed at contaminated sites. Its antimicrobial activity and wound-healing properties make it particularly valuable in pharmaceutical applications. However, further research is necessary to develop strategies for enhancing large-scale production and to explore additional vital applications of this biosurfactant.

## Acknowledgment

The authors wish to applaud the members of the Centre for Bioscience and Nanoscience Research for their support during the study.

**Authors' contribution: C.M. Brinda:** Carried out the research as well as wrote the manuscript; **J. Jesteena:** Facilitated analysis; **R. Ragunathan:** Directed and monitored the project.

**Funding:** No grant was received from any funding organizations for this study.

**Research content:** The research content of the manuscript is original and has not been published elsewhere.

**Ethical approval:** Not applicable.

**Conflict of interest:** The authors have no conflict of interest.

**Data availability:** Not applicable.

**Consent to publish:** All authors agree to publish the paper in the *Journal of Environmental Biology.*

## References

Al-Wahaibi, Y., S. Joshi, S. Al-Bahry, A. Elshafie, A. Al-Bemani and B. Shibulal: Biosurfactant production by *Bacillus subtilis* B30 and its application in enhancing oil recovery. *Colloids Surf. B.,* **114**, 324-333 (2014).

Araujo, J.S., J.C. Rocha, M.A.O. Filho, V. T. Ribeirol, L.T.C. Vasconcelos, N.K. Araujo, E.L.B. Neto and E. Santos: Production of rhamnolipids by *Pseudomonas aeruginosa* AP029-GLVIIA and application on bioremediation and as a fungicide. *Biosci. Biotechnol. Res. Asia.,* **17**, 467-477 (2020).

Badmus, S.O., H.K. Amusa, T.A. Oyehan and T.A. Saleh: Environmental risks and toxicity of surfactants: overview of analysis, assessment, and remediation techniques. *Environ. Sci. Pollut. Res. Int.,* **28**, 62085-62104 (2021).

Balouiri, M. N., M. Sadiki and S. K.Ibnsouda: Methods for *in vitro* evaluating antimicrobial activity: A review. *J Pharm Anal.*, **6**, 71-79 (2016).

Banat, I. M., S.K. Satpute, S.S. Cameotra, R. Patil and N.V. Nyayanit: Cost effective technologies and renewable substrates for biosurfactants production. *Front. microbiol.*, **5**, 697(2014).

Barakat, K.M., S.W.M. Hassan and O.M. Darwesh: Biosurfactant production by haloalkaliphilic *Bacillus* strains isolated from Red Sea, *Egypt. J. Aquat. Res.,* **43**, 205-211 (2017).

Barrow, G.I. and R.K.A. Feltham: Cowan and Steel's Manual for the Identification of Medical Bacteria. 3rd Edn., Cambridge University Press, Cambridge, 352 pages (1993).

Basha, S., A. Noorjahan, M. Karuppaiya, S. Mahesh and B. Aiyamperumal: Screening and characterization of biosurfactant producing novel marine bacteria *Bacillus tequilensis* KM15 and its application for plant growth promotion. *Nat. Volat. Essent. Oils,* **5**, 810472-10488 (2021).

Bezza, F.A. and E.M.N. Chirwa: Biosurfactant-enhanced bioremediation of aged polycyclic aromatic hydrocarbons (PAHs) in creosote contaminated soil. *J. Chemosphere,* **144**, 635-644 (2016).

Bjerk, T.R., P. Severino, S. Jain, C. Marques, A.M. Silva, T. Pashirova and E.B. Souto: Biosurfactants: properties and applications in drug delivery, biotechnology and ecotoxicology. *Bioengine. (Basel.),* **8**, 115-122 (2021).

Brinda, C.M., R. Ragunathan and J. Johney: Diversity and distribution of potential biosurfactant producing *Bacillus* sp MN 243657, GCMS analysis and its antimicrobial study. *Biosci. Biotechnol. Res. Asia,* **20**, 271-291 (2023).

Cao, X.H., Z.Y. Liao, C.L. Wang, M.F Lu and G.W. Huang: Purification and antitumor activity of a lipopeptide biosurfactant produced by *Bacillus natto* TK-1. *Biotechnol. Appl. Biochem*., **52**, 97-106 (2009)

Carrillo, P.G., C. Mardaraz, S.I. Pitta-Alvarez and A.M. Giuliett: Isolation and selection of biosurfactant producing bacteria. *J. Microbiol. Biotechnol.*, **12**, 82 – 84 (1996).

Chandrasekharan, E.V. and J.N. BeMiller: Constituent analysis of glycosaminoglycans. In: Methods in Carbohydrates Chemistry (Ed.: R.L. Whistler). 2nd Edn., Academic Press, New York, pp. 95-96 (1980).

Cheng, H.R. and N. Jiang: Extremely rapid extraction of DNA from bacteria and yeasts. *Biot. Lett.,* **28**, 55-59 (2006).

Chopra, A., S. Bobate, P. Rahi, A. Banpurkar, P.B. Mazumder and S. Satpute: *Pseudomonas aeruginosa* RTE4: A tea *Rhizobacterium* with potential for plant growth promotion and biosurfactant production. *Front. Bioeng. Biotechnol.,* **8**, 1-14 (2020).

Donio, M.B.S., S.F.A. Ronica, V.T. Viji, S. Velmurugan, J.A. Jenifer, M. Michaelbabu and T. Citarasu: Isolation and characterization of halophilic *Bacillus* sp. BS3 able to produce pharmacologically important biosurfactants. *Asian Pac. J. Trop. Med.,* **6**, 876-883 (2013).

Faisal, Z.G., M.S. Mahdi and K.H. Alobaidi: Optimization and chemical characterization of biosurfactant produced from a novel *Pseudomonas guguanensis* strain Iraqi ZG.K.M. *Int. J. Microbiol.,* **3**,1-16 (2023).

Falk, N.A.: Surfactants as antimicrobials: A brief overview of microbial Interfacial chemistry and surfactant antimicrobial activity. *J. Surfact. Deterg.,* **5**,1119-1127 (2019).

Guzman, J.P.M.: Isolation, screening, and characterization of biosurfactant-producing *Bacillus* spp. from soil and their potential biofilm inhibitory activities against *Pseudomonas aeruginosa. J. Microbiol. Biotechnol. Food Sci.,* **10**, 245-248 (2020).

Holt, J.G., N.R. Krieg, P.H. Sneath, J.T. Staley and S.T. Williams: Gram-positive cocci. In: Bergey's Manual of Determinative Microbiology (Ed.: W.R. Hensyl). 9th Edn., pp. 527-558 (1994).

Jain, D., D. Collins-Thompson and H. Lee: A drop-collapsing test for screening surfactant-producing microorganisms. *J. Microbiol. Methods.,* **13**, 271–279 (1991).

Jonkman, J.E.N., J.A. Cathcart, F. Xu, M. E. Bartolini, J. E. Amon, K.M. Stevens and P. Colarusso: An introduction to the wound healing assay using live-cell microscopy. *Cell Adh. Migr.*, **8**, 440-451 (2014).

Korayem, A.S., A.A. Abdelhafez, M.M. Zaki and E.A. Saleh: Optimization of biosurfactant production by *Streptomyces* isolated from Egyptian arid soil using placket–burman design. *Ann. Agric. Sci.,* **60**, 209–217 (2015).

Kumari, S., T. S. Howlett, R. N. Ehrman, S. Koirala, O. Trashi, I. Trashi, Y. H. Wijesundaraa and J.J. Gassensmith: *In-vivo* biocompatibility of ZIF-8 for slow release via intranasal administration. *Chem. Sci.,* **14**, 5774-5782 (2023).

Lyu, Y., T. Zhang, B. Dou, G. Li, C. Ma and Y. Li: A lipopeptide biosurfactant from *Bacillus* sp. Lv13 and their combined effects on biodesulfurization of dibenzothiophene. *RSC Adv.,* **8**, 38787-38791 (2018).

Marchut-Mikolaiczyk, O., P. Drozdzynski, A. Polewczyk, W. Smulek and T. Antczak: Biosurfactant from endophytic *Bacillus pumilus* 2A: physico-chemical characterization, production and optimization and potential for plant growth promotion. *Microb. Cell. Fact.,* **1**, 20-40 (2021).

Mathew, J.P., G. Alba, J. Marianne, Torrwa and M. Louie: Isolation, screening characterization of biosurfactant producing *Bacillus* sp. from soil and their potential biofilm inhibitory activities against *Pseudomonas aeruginosa. J. Microbiol. Biotechnol. Food Sci.,* **10**, 24-248 (2020).

Meena, A.K., R. Ilavarasan, R. Singh, D. Parashar, M. Motiwale, A. Perumal, N. Srikanth and K.S. Dhiman: Evolution of pharmacological activity with molecular docking of active constituents present in root sand small branches of *Aegle marmelos:* A comparative study using HPLC, GC–MS, LC-MS. *Phytomed. Plus,* **2**, 210-227 (2021).

Morikawa, M., Y. Hirata and T. Imanaka: A study on the structure-function relationship of the lipopeptide biosurfactants. *Biochem. Biophys. Acta.,* **1488**, 211 – 218(2000)

Muthukumar, B., M.S. Nandini, P. Elumalai, M. Balakrishnan, A. Satheeshkumar, M.S. Alsalhi, S. Devanesan, P. Parthipan, A. Rajasekar and T. Malik: Enhancement of cell migration and wound healing by nano-herb ointment formulated with biosurfactant, silver nanoparticles and *Tridax procumbens*. *Front. Microbiol.,* **10**, 10-25 (2023).

Nayarisseri, A., P. Singh and S.K. Singh: Screening, isolation and characterization of biosurfactant producing *Bacillus subtilis* strain ANSKLAB03. *Bioinformation.,* **14**, 304-314 (2018).

Nikunj, D. and J. Tejas: A concise review on surfactants and its significance. *Int. J. Appl. Chem.,* **13**, 663-672 (2017).

Noudeh, G.D., M.H. Moshafi, P.K. Azaeli and F. Akef: Studies on bio-emulsifier production by *Bacillus licheniformis* PTCC 1595. *Afri. J. Biotechnol.,* **9**, 352-356 (2010).

Ohadi, M., G. Dehghannoudeh, H. Forootanfar, M. Shakibaie and M. Rajaee: Investigation of the structural, physico-chemical properties and aggregation behavior of lipopeptide biosurfactant produced by *Acinetobacter junii* B6. *Int. J. Biol. Macromol.,* **112**, 712-719 (2018).

Paul, B.F.: Screening and extraction of biosurfactant producing bacteria from oil contaminated soils. *Int. J. Adv. Res.,* **6**,1873-1878 (2018)

Pendse, A.S and K. Aruna: Physico-chemical and analytical characterization of biosurfactant produced by *Serratia rubidaea* KAP. *Int. J. Biol. Sci.,* **7**, 25-40 (2020).

Rodríguez-Lopez, L., A. Lpez-Prieto, M. Lopez-Alvarez, S. Perez-Davila, J. Serra, P. Gonzalez, J.M. Cruz and A.B. Moldes: Characterization and cytotoxic effect of biosurfactants obtained from different sources. *ACS Omega,* **5**, 31381−31390 (2020).

Rosenberg, M., D. Gutnick and E. Rosenberg: Adherence of bacteria to hydrocarbons—a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol. Lett.,* **9**, 29–33 (1980).

Sana, S., A. Mazumder, S. Datta and D. Biswas: Towards the development of an effective *in vivo* wound healing agent from *Bacillus* sp. derived biosurfactant using *Catla catla* fish fat. *RSC Adv.,* **7**, 13668–13677 (2017).

Sanders, E.R.: Aseptic laboratory techniques: plating methods. *J. Visu. Exp.,* **63**, 3064 (2012).

Sarubbo, L.A., C.S. Maria da Gloria, B.D. Italo Jose, Karen, O.B. Gercyane, B.G. Ribeiro, I.A. Silva and S.M. Twigg: Biosurfactants: production, properties, applications, trends, and general perspectives. *Biochem. Eng. J.,* **181**, 1-19 (2022).

Sing, P., Y. Patil and V. Rale: Biosurfactant production: emerging trends and promising strategies. *Appl. Microbiol.,* **126**, 2-13 (2019).

Singh, P.K., S. Chibber and V.S. Nagoria: Isolation and characterization of bio-surfactant producing bacteria from oil contaminated soil. *Int. J. Curr. Microbiol. App. Sci.,* **11**, 351-360 (2022).

Skladanowski M., P. Golinska, K. Rudnicka and H. Dahm: Evaluation of cytotoxicity, immune compatibility and antibacterial activity of biogenic silver nanoparticles., *Med. Microbiol. Immunol.*, **6**, 1-11 (2016).

Srivastava, M.M.: An overview of HPTLC: A modern analytical technique with excellent potential for automation, optimization, hyphenation and multidimensional applications. In: High-Performance Thin-Layer Chromatography (Ed.: M. Srivastava). Springer-Berlin/Heidelberg, Germany, pp. 3–24 (2011).

Van Dyke, M.I., S.L. Gulley, H.Lee and J.T. Trevors: Evaluation of microbial surfactants for recovery of hydrophobic pollutants from soil. *J. Ind. Microbiol.*,**11**, 163 – 170 (1993).

Yuliani, H., M.S. Perdani, I. Savitri, M. Manurung, M. Sahlan, A. Wijanarko and H. Hermansyah: Antimicrobial activity of biosurfactant derived from *Bacillus subtilis* C19. *Energy Procedia,* **153**, 274–278 (2018).

Zampolli, J., A.D. Giani, A.D. Canito, G. Sello and P.D. Gennaro: Identification of a novel biosurfactant with antimicrobial activity produced by *Rhodococcus opacus* R7. *Microorganisms,* **10**, 1-16 (2022).

Zhou, H., J. Chen, Z. Yang, B. Qin, Y. Li and X. Kong: Biosurfactant production and characterization of *Bacillus* spp. ZG0427 isolated from oil-contaminated soil. *Ann. Microbiol.,* **65**, 2255–2264 (2015).

Zhu, Y. and M.L. Free: Introduction to surfactants. 2nd Edn., Elsevier-Radarweg, Netherlands, pp.1-311 (2020).