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# Isolation and Screening of Potential Biosurfactant Producing Bacteria from Phyllosphere and Soils of Different Contaminated Areas

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**AUTHORS CONTRIBUTION**

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**ABSTRACT**

Biosurfactants are amphipathic compounds with surface activity and emulsification ability. In addition, these compounds are reported to induce the production of plant growth promoting substances, hence, plays a vital role in plant growth and development. A plethora of microorganisms capable of producing biosurfactant compounds which helps to improve the soil quality and nutrient uptake by plants have been reported. Developing microbial inoculants producing biosurfactants to cleanse contaminated agricultural soils assumes greater significance in the wake of heavy metal and oil pollution due to anthropogenic activities. Therefore, the present study was carried out to isolate and screen potential biosurfactant-producing bacteria from contaminated soils and phyllosphere of different plants grown in oil and heavy metal contaminated soils. In the present study a total of 95 bacterial isolates were isolated by leaf imprint method and soil enrichment culture technique from phyllosphere and soil, respectively. Further, potential isolates were screened for biosurfactant production through qualitative assay such as oil spreading test, drop collapse test and penetration assay. Among the 95 bacterial isolates, 63 isolates were able to produce biosurfactants. These promising isolates were subjected to quantitative assay like emulsification index, bacterial adhesion to hydro carbons (BATH) assay and surface tension. The isolate BPB-47 isolated from phyllosphere (*Ficus ingens)* of Raichur petroleum contaminated area showed highest reduction in surface tension (27.15 mN/m), whereas the highest emulsification index were recorded by BSB-24 (72 %) and BPB-4 (72 %) isolated from petroleum contaminated soil of Raichur and phyllosphere (*Psidium guajava*) of Peenya heavy metal contaminated area, respectively. The isolate BSB-22 (78.67%) which was isolated from fly ash contaminated soil of Shaktinagar, Raichur district of Karnataka showed highest bacterial adhesion to hydrocarbons compared to all other isolates.

*Keywords* : Biosurfactant, Phyllosphere, Leaf imprint, Surface tension

PHYLLOSPHERE is a unique, dynamic ecosystem consisting of diverse microflora *viz.,* bacteria, fungi, yeast and algae. Among the diverse microbial community, bacteria are predominant on leaves and play a pivotal role on the homeostasis of plants offering promotion of plant growth (Sivakumar *et al*., 2020). Leaves are home to a wide variety of bacteria and can be covered by up to 5 per cent bacterial biomass. Phyllosphere is subjected to pronounced cyclic and non-cyclic variation and also accounts 60 per cent of the biomass across all taxa on earth making it key habitat for microorganisms. The epiphytic bacteria faces the vagrant effects of high UV exposure, cycle of desiccation and hydration, rapid temperature fluctuation, low and heterogeneous nutrient availability (Lindow and Brandl, 2003 and Nair *et al*., 2017). Also the cuticular waxes deposited as 'limiting skin' on leaf surface acts as barrier for the availability of water and nutrients (Schreiber, 2010). The harsh environment and deplorable situations question the

very existence of microorganisms on the phyllosphere. The epiphytic bacteria have inherently developed certain characters to redeem themselves from adverse conditions. One such riveting phenomenon is the production of biosurfactant molecules.

Surfactants are compounds that lower the surface tension (or interfacial tension) between two liquids, between a gas and a liquid, or between a liquid and a solid. Surfactants may act as detergents, wetting agents, emulsifiers, foaming agents or dispersants. Chemically synthesized surfactants are known to possess powerful bactericidal properties and are capable to modify the physical and chemical properties of the soils (Tobe *et al*., 2015). Long-term treatment with surfactants causes pollution of soil and water, which affects growth, development and metabolism of soil microorganisms and plants. They solubilize nonpolar plant substances such as waxy critical or the lipoidal part of the cell wall that facilitate the rapid absorption of the toxic as well as beneficial chemicals. They enter into the intercellular spaces and affect plant growth systems. Growing public awareness about the environmental hazards and risks associated with chemical surfactants has stimulated the search for ecofriendly, natural substitutes of chemical surfactants. Recently, biosurfactants have received much attention in numerous environmental and industrial applications, because of their unique properties such as high surface activity, non-toxic nature, environmentally friendly, biodegradable and tolerance of extreme temperatures, pH and salinity. These properties allow biosurfactants to be a preferable alternative to chemical surfactants. As a result, there is an intensive search for microbes which are capable of producing biosurfactants (Tian *et al*., 2016).

Biosurfactants are amphipathic compounds produced on living surfaces, mostly on microbial cell surfaces or excreted extracellular hydrophobic and hydrophilic moieties that confer the ability to accumulate between fluid phases, thus reducing surface and interfacial tension at the surface and interface respectively (Md, 2012). The biosurfactants accumulate at the interface between two immiscible fluids or between a fluid and a solid. By reducing surface (liquid-air) and interfacial (liquid-liquid) tension they reduce the repulsive forces between two dissimilar phases and allow these two phases to mix and interact more easily (Pacwa-Plociniczak *et al*., 2011). Biosurfactant can enhance the contact between water and leaf surfaces. Water relations are important especially, in the phyllosphere ecology. These biosurfactants increase the wettability of the leaf, to enhance diffusion of nutrients across the waxy cuticle. Changes in leaf wettability may bring profound alterations in the abundance and distribution of microorganisms (Bunster *et al.*, 1989).

When the plant perceives the biosurfactants, there will be induction of early signalling events. This in turn activates intricate network of phytohormones such as salicyclic acid or jasmonic acid, regulate late defense-related responses. By this activation of defense related signals, Induced systemic resistance (ISR) is triggered (Crouzet *et al*., 2020 and Lohithkumar & Krishna Naik, 2021). Biosurfactant producing bacteria are found to have plant growth promoting (PGP) traits such as IAA synthesis, siderophore formation, phosphate solubilization, HCN production and antagonistic activities against some phytopathogens. These biosurfactant producing microorganisms help in alleviating environmental stress and thereby increasing plant productivity (Bhuyan-Pawar *et al*., 2015). Considering the role of biosurfactants in plant growth promotion, the present study was taken up to survey phyllo sphere and soil habitat in the hydrocarbon and oil contaminated sites and isolate biosurfactant producing plant growth promoting bacteria from these environments.

## Material and Methods

### Isolation of Biosurfactant Producing Phyllosphere Bacteria by Leaf Imprinting Method

The leaf samples were washed gently with running tap water to remove the dirt. An intact individual leaf was placed onto tryptic soy agar (TSA) enriched with 1 per cent of crude oil and was pressed with the

smooth end of a sterile glass rod until a clear imprint of the entire leaf was obtained on the agar surface and incubated at 37°C for 2 days.

**Isolation of Biosurfactant Producing Soil Bacteria by Enrichment Culture Technique**

The homogenized soil sample of five gram each was suspended in a 100-mL conical flask containing 50 mL sterile water and incubated in a rotary shaker at 30°C (180 rpm) for 2 hour, followed by allowing it to stand for 30 min at room temperature. Subsequently, supernatant of 0.5 mL was transferred into 50 mL of Luria - Bertani (LB) broth and incubated in a rotary shaker at 37°C with shaking (180 rpm) for 24 h to enrich microbial population. Then one millilitre sample of the enriched medium was added to 50 mL of minimal salt medium containing 2 per cent (v/v) crude oil as the sole carbon source and incubated at 37°C with shaking (180 rpm) for 7 days. The incubated suspensions were serially diluted up to $10^{-7}$. Then, an aliquot of 100 µL of each dilution was spread on LB agar plates and incubated at 30°C for 48 h. Morphologically distinct colonies were selected and purified by streaking on the agar plates.

**Screening of Bacterial Isolates for Biosurfactant Production**

The bacterial isolates were screened for biosurfactant production by growing the cultures in 100 ml Erlenmeyer flask containing 50 mL of mineral salt broth. A loopful of bacterial culture was inoculated into a flask containing 50 mL of sterilized medium and incubated in a shaker at 30°C for 7 days at 200 rpm. After 7 days of incubation, culture broth was centrifuged at 6000 rpm and 4°C for 15 minutes and the supernatant was filtered through 0.45µm pore size filter paper (Millipore). The cell free supernatant and the bacterial cell (pellet) were used for qualitative and quantitative assays.

**Qualitative Assay**

For preliminary screening of biosurfactant producing bacteria, qualitative tests like oil spreading test, drop collapse test and penetration assay were performed.

*Oil spreading test* : Oil spreading experiment was performed following the method described by (Morikawa *et al.*, 2000). The oil spreading test was carried out by adding 20 ml of distilled water to a petri plate followed by addition of 20 µl of crude oil to the surface of the water. Then 10 µl of cell free supernatant was carefully pipetted onto the centre of the oil surface. Formation of clear zone by the displacement of oil indicates the presence of biosurfactant in supernatant.

*Drop collapse test* : A drop of the culture supernatant was placed carefully on an oil coated glass slide and observed after one minute. If the drop of supernatant collapsed and spread on the oil coated surface, it indicates the presence of biosurfactant. A Triton X-100 solution and distilled water were used as positive and negative control, respectively (Bodour and Miller-Maier, 1998).

*Penetration assay* : The cavities of a 96 well micro titre plate were filled with 150 µl of a hydrophobic paste consisting of oil and silica gel. The paste was covered with 10 µl of oil. Then, the supernatant of the culture was colored by adding 10 µl of a safranin to 90 µl of the supernatant. The colored supernatant was placed on the surface of the paste (walter *et al*., 2010). If biosurfactant is present, the hydrophilic liquid will break through the oil film barrier into the paste. The silica is entering the hydrophilic phase and the upper phase will change from clear red to cloudy white within 15 minutes.

**Quantitative Screening**

*Emulsification index test* : Emulsifiction assay was carried out in a 10 mL testube by homogenizing equal volume of 2ml crude oil and 2 ml of cell free supernatant by vortexing at 1000 rpm for 2 minutes and the test tubes were kept undisturbed for 24 h at room temperature. The height of the stable emulsion layer was measured after 24h and emulsification index was calculated by using the formula (Cooper and Goldenberg, 1987).

$$\text{Emulsification Index } (E_{24}) = \frac{\text{Height of the emulsion}}{\text{Height of the total layer}} \times 100$$

**Bacterial Adhesion to Hydrocarbons (BATH)**

The cell pellets were washed twice and suspended in a phosphate buffer salt solution and diluted using the same buffer solution to an optical density (OD) of 0.5 at 600 nm. To the cell suspension (2 ml) in test tubes 100 µl of crude oil was added and vortexed for 3 min. After vortexing, crude oil and aqueous phase were allowed to separate for 1 h. Optical density of the aqueous phase was then measured at 600 nm using a UV visible spectrophotometer (Thermo scientific, Biomate 3S, China). From the OD values, percentage of cells attached to crude oil was calculated using the following formula :

$$\text{Per cent bacterial cell adherence} = \frac{(1\text{-}OD_{\text{shaken with oil}})}{(OD_{\text{original}})} \times 100$$

and the hydrophobicity is expressed as per cent of bacterial cell adherence

**Surface Tension**

The bacterial isolates were grown in 50 mL of minimal salt broth and incubated for 7 days. After incubation cell free supernatant was obtained by centrifugation at 6000 rpm and 4°C for 15 minutes. This liquid is subjected to surface tension measurement using stalagmometer. The liquid (cell free supernatant) was sucked into the clean stalagmometer upto mark A, then it was allowed to fall down due to gravity. The number of drops were recorded when the liquid passes from mark A to B. The procedure was repeated three times to obtain the mean value. The density of the liquid as well as distilled water was measured. The surface tension of the biosurfactant was calculated using the below formula (Walter *et al.*, 2010).

$$\sigma_L = \frac{\sigma_w \times N_w \times \rho_L}{N_{L \times PW}}$$

Where, $\sigma_L$ is the surface tension of the liquid under investigation, $\sigma_W$ is the surface tension of water, $N_L$ is the number of drops of the liquid, $N_W$ is the number of drops of water, $\rho_L$ is the density of the liquid and $\rho_W$ is the density of water.

**Statastical Analysis**

The data obtained from laboratory experiments were statistically analyzed using completely randomized design (CRD). The statistical analysis was done by using WASP: 2.0 (Web Agri Stat Package 2).

## Results and Discussion

**Sample Collection and Isolation of Biosurfactant Producing Bacteria**

A total of 95 morphologically distinct and separate colonies were isolated from phyllosphere and soil samples that were collected from different contaminated areas and were purified. Among 95 isolates, 54 isolates were from phyllosphere and 41 isolates were from soil. The cultures were numbered from 1 to 54 for phyllosphere (Table 1a) samples and 1 to 41 (Table 1b) for soil samples with prefix BPB and BSB, respectively. Results obtained in the present study confirm with the study by Kurniati *et al.,* 2019 who isolated 19 biosurfactant-producing bacteria from hydrocarbon contaminated soils. Charan and Patel, 2017 isolated 58 bio surfactant producing microorganism from the petroleum contaminated soil in Gujarat. The isolates obtained were further used to screen for their biosurfactant producing potential.

**Screening of Bacterial Isolates for Biosurfactant Production**

Biosurfactants are structurally very diverse group of biomolecules, hence a single method for detection of biosufactant producing bacteria would be insufficient (Satpute *et al.*, 2008). Therefore, combinations of various screening methods were attempted. Qualitative and quantitative tests were carried out for the 95 bacterial isolates to screen their biosurfactant producing potential. Qualitative screening experiments include the oil-spreading test, drop collapse test and penetration assay and quantitative screening experiment include the BATH assay, emulsification assay and surface tension measurement.

TABLE 1A

Sampling location, sources of phylloshere and bacteria isolated

| Location | Sample | Bacteria isolated code |
|---|---|---|
| Peenya, | Leaf Mango (*Mangifera indica*) | BPB-1, BPB-2 and BPB-3 |
| Bangalore | Leaf Guava (*Psidium guajava*) | BPB-4, BPB-5, BPB-6, BPB-7, BPB-8 and BPB-9 |
| | Leaf Jackfruit (*Artocarpus heterophyllus*) | BPB-10, BPB-11, BPB-12and BPB-13 |
| | Leaf Calotropis (*Calotropis procera*) | BPB-14, BPB-15, BPB-16, BPB-17 and BPB-18 |
| | Leaf Singapore cherry (*Muntingia calatura*) | BPB-19, BPB-20, BPB-21, BPB-22and BPB-23 |
| | Leaf Crossandra (*Crossandra infundibuliformis*) | BPB-24 |
| | *Leaf* African tulip (*Spathodia companulata)* | BPB-25 and BPB-26 |
| | Leaf *Chrysanthemum (Chrysanthemum morifolium)* | BPB-27, BPB-28, BPB-29, BPB-30, BPB-31 and BPB-32 |
| Shaktinagar, | Leaf Gokulakanta (*Hypgrophila auriculata*) | BPB-33, BPB-34, BPB-35and BPB-36 |
| Raichur | Leaf Custard apple (*Annona reticulata*) | BPB-37 |
| | Leaf Tasmanian blue gum (*Eucalyptus globulus*) | BPB-44 and BPB-45 |
| | Leaf Indian tulip (*Thespesia populnea*) | BPB-46, BPB-47, BPB-48 and BPB-49 |
| | Leaf Common mullein (*Verbascum thapsus*) | BPB-52, BPB-53 and BPB-54 |
| Raichur | Leaf Calotrope (*Calotropis procera*) | BPB-38, BPB-39, BPB-40 andBPB-41 |
| | Leaf Weeping fig (*Ficus benjamina*) | BPB-42 and BPB-43 |
| | Leaf Red leaved fig (*Ficus ingens*) | BPB-50 and BPB-51 |

*Note* : BPB- biosurfactant producing phyllosphere bacteria

TABLE 1B

Sampling location, sources of contaminated soil and bacteria isolated

| Location | Sample | Bacteria isolated code |
|---|---|---|
| Peenya, | Heavy metal contaminated soil | BSB-1, BSB-2, BSB-3 and BSB-4 |
| Bangalore | Petrol contaminated soil | BSB-5, BSB-6, BSB-7 BSB-8 and BSB-37 |
| | Agro chemicals contaminated soil | BSB-9, BSB-10, BSB-11 BSB-12, BSB-38 and BSB-38 |
| | Agro chemicals contaminated soil | BSB-13, BSB-14 and BSB-40 |
| | Paint oil contaminated soil | BSB-15, BSB-16, BSB-17, BSB-18 and BSB-41 |
| Shaktinagar, | Fly ash contaminated soil | BSB-19 and BSB-20 |
| Raichur | Fly ash contaminated soil | BSB-21 and BSB-22 |
| | Fly ash contaminated soil | BSB-27 and BSB-28 |
| | Fly ash contaminated soil | BSB-29, BSB-30 and BSB-31 |
| | Fly ash contaminated soil | BSB-34, BSB-35 and BSB-36 |
| Raichur | Petrolium contaminated soil | BSB-23 and BSB-24 |
| | Petrolium contaminated soil | BSB-25 and BSB-26 |
| | Petrolium contaminated soil | BSB-32 and BSB-33 |

*Note* : BSB - biosurfactant producing soil bacteria

**Oil Spreading Test**

The oil spreading test was used to observe clear zone during the addition of cell free supernatant samples on oil-water surface. Oil spreading test or sometimes referred to as an oil displacement assay has advantage that it can detect biosurfactants with low activity and quantity (Plaza *et al*., 2006). The oil spreading test results were stated positively when a clear zone is formed on the supernatant droplets on the oil layer. Out of the 95 bacterial isolates, 62 isolates showed a clear zone of oil displacement indicating positive results for oil displacement test as indicated in (Table 2 & Plate 1B). The clear zone was formed

TABLE 2

Qualitative screening results of biosurfactant producing bacteria

| Isolates | Qualitative screening | | |
|---|---|---|---|
| | Oil spreading test | Penetration assay | Drop collapse test |
| Negative control | - | - | - |
| Positive control | + | + | + |
| BPB-1 | + | + | + |
| BPB-2 | - | - | - |
| BPB-3 | + | + | + |
| BPB-4 | + | + | + |
| BPB-5 | - | - | - |
| BPB-6 | + | + | + |
| BPB-7 | + | + | + |
| BPB-8 | + | + | + |
| BPB-9 | - | - | - |
| BPB-10 | - | - | - |
| BPB-11 | - | - | - |
| BPB-12 | + | + | + |
| BPB-13 | - | - | - |
| BPB-14 | + | - | + |
| BPB-15 | + | + | + |
| BPB-16 | - | - | - |
| BPB-17 | + | + | + |
| BPB-18 | - | - | - |
| BPB-19 | + | + | + |
| BPB-20 | + | + | + |
| BPB-21 | - | - | - |
| BPB-22 | + | + | + |
| BPB-23 | - | - | - |
| BPB-24 | + | + | + |
| BPB-25 | - | - | - |
| BPB-26 | + | + | + |
| BPB-27 | - | - | - |
| BPB-28 | + | + | + |
| BPB-29 | + | + | + |
| BPB-30 | - | - | - |
| BPB-31 | - | - | - |
| BPB-32 | - | - | - |
| BPB-33 | + | + | + |
| BPB-34 | + | + | + |
| BPB-35 | + | + | + |
| BPB-36 | + | + | + |
| BPB-37 | + | + | + |
| BPB-38 | - | - | - |
| BPB-39 | + | + | + |
| BPB-40 | + | + | + |
| BPB-41 | + | + | + |
| BPB-42 | + | + | - |
| BPB-43 | + | + | - |
| BPB-44 | + | + | + |
| BPB-45 | + | - | + |
| BPB-46 | + | + | + |
| BPB-47 | + | + | + |
| BPB-48 | + | + | + |
| BPB-49 | + | + | + |
| BPB-50 | + | + | + |
| BPB-51 | + | + | + |
| BPB-52 | + | + | + |
| BPB-53 | + | + | + |
| BPB-54 | + | + | + |
| BSB-1 | - | - | - |
| BSB-2 | + | + | + |
| BSB-3 | - | - | - |
| BSB-4 | - | - | - |
| BSB-5 | - | - | - |
| BSB-6 | + | + | + |
| BSB-7 | + | + | + |
| BSB-8 | + | + | + |
| BSB-9 | + | + | + |
| BSB-10 | - | - | - |
| BSB-11 | + | + | + |
| BSB-12 | + | + | + |

| Isolates | Qualitative screening | | |
|---|---|---|---|
| | Oil spreading test | Drop collapse test | Penetration assay |
| BSB-13 | + | + | - |
| BSB-14 | + | + | + |
| BSB-15 | + | + | + |
| BSB-16 | + | - | + |
| BSB-17 | + | + | + |
| BSB-18 | + | + | + |
| BSB-19 | - | - | - |
| BSB-20 | + | + | + |
| BSB-21 | + | + | + |
| BSB-22 | + | + | + |
| BSB-23 | + | + | + |
| BSB-24 | + | + | + |
| BSB-25 | - | - | - |
| BSB-26 | - | - | - |
| BSB-27 | - | - | - |
| BSB-28 | + | + | + |
| BSB-29 | + | + | + |
| BSB-30 | + | + | + |
| BSB-31 | + | + | + |
| BSB-32 | + | + | + |
| BSB-33 | - | - | - |
| BSB-34 | + | + | + |
| BSB-35 | - | - | - |
| BSB-36 | - | - | - |
| BSB-37 | - | - | - |
| BSB-38 | - | - | - |
| BSB-39 | - | - | - |
| BSB-40 | + | + | + |
| BSB-41 | - | - | - |

*Note*: BPB- biosurfactant producing phyllospheric bacteria, BSB- biosurfactant producing soil bacteria

because of the hydrophobic part of the oil and hydrophilic in biosurfactants, that causes pressure between the hydrophobic and hydrophilic parts. This condition causes interface tension to decrease, the oil layer breaks and a clear zone is formed. The results are in accordance with the observation recorded by Bhat *et al*., (2015) who isolated 75 isolates of *Pseudomonas* spp. from rhizospheric soil and screened for oil displacement test, all the isolates showed clearzone of oil displacement and confirmed the production of biosurfactant. Similarly, El-Gebaly, 2020 isolated 28 bacterial isolates from contaminated soil and screened for biosurfactant production, 40 per cent of isolates showed positive oil spreading activity.

**Drop Collapse Assay**

The drop collapse assay relies on destabilization of liquid droplets by biosurfactants. Drop collapse test determines the surface and wetting activities (Youssef *et al.*, 2004). So, if the droplets are flat in shape, the reactions are positive while if the droplets are spherical in shape, the reactions are negative. Among the 95 bacterial isolates, 57 isolates were able to collapse the drop as the drops turned flat and remaining 38 isolates were negative for drop collapse test as they turned to spherical shape (Table 2 & Plate 1A). In the present study 57 isolates were able to collapse the drop, this might be due to the presence of the biosurfactant, which leads to reduced interfacial tension between the liquid drop and the hydrophobic surface there by the droplets on the film would collapse. The present work corroborates with the findings of Saminathan and Rajendran (2014) who recorded the strongly positive isolates for the drop collapse test, indicating good biosurfactant production potential. Thavasi *et al*. (2011) isolated and screened the 105 strains biosurfactant producing bacteria and subjected for drop collapse test. Among the 105 stains, 82 strains were positive for drop collapse test. They also proved the concept of use of hydrophobic substrates as an effective screening tool for the isolation of biosurfactant producing bacteria. Satpute *et al*. (2010) reported that the drop collapse and oil spread tests can be used together for primary screening of biosurfactant-producing isolates due to their high sensitivity. Biosurfactants that can displace the thin oil layer in the oil spreading test will also spread the supernatant drops on the oil-coated glass slide. However, in this study, not all isolates produced biosurfactants that can collapse the drops of the supernatant on the oil coated glass slide. One of the reasons might be due to the relatively low sensitivity of drop collapse test compared to the oil

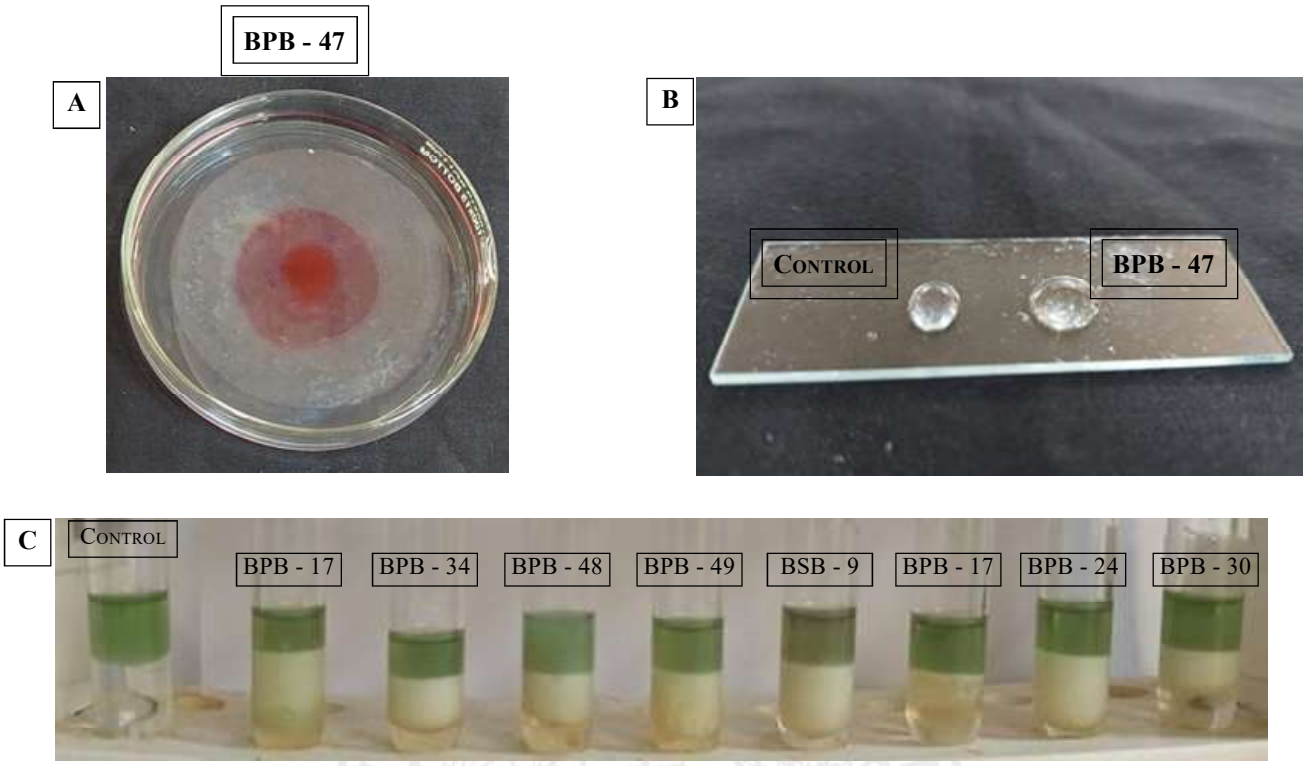


Plate 1 : Screening of biosurfactant producing organisms; A) Zone formation by biosurfactant producing bacteria in oil spreading test; B) Collapsed droplets on the hydrophobic surface in drop collapse assay; C) Stable emulsion formation with hydrophobic substrate in emulsification index test by biosurfactant producing bacteria

spreading technique since a significant concentration of biosurfactant must be present to collapse the aqueous drops (Walter *et al.*, 2010).

**Penetration Assay**

The penetration assay relies on the phenomenon of contacting two insoluble phases which leads to a change in colour when silica gel enters the hydrophilic phase from the hydrophobic paste much more quickly if biosurfactants are present (Sumathi and Yogananth, 2016). Among the 95 isolates evaluated, 58 isolates were positive for penetration assay. Nishanth *et al*. (2010) reported that isolates BPB7 and BPB13 have the penetration ability between two different phases which resulted in mixing of two distinct phases within 15 minutes.

Based on the results obtained by oil spreading, drop collapse and penetration assay, out of 95 bacterial isolates, 63 isolates were selected for further quantitative screening.

**Emulsification Index Test**

Emulsification is a process of mixing two heterogeneous solutions, in which one phase has smaller droplets dispersed in the other phase solution. The addition of biosurfactants to an immiscible matrix leads to formation of an inter mediate layer between aqueous and oil phases, thus reduce the interfacial tension of interphases. Subsequently, the interfacial mass exchange will occur in the surface and lead to the solubilization of dispersed organic compounds into the aqueous solution through micelles (Kaczorek *et al.,* 2018). Emulsifying activity is the one of the most important functions of biosurfactant to enhance contact between oil and water. It was presumed that if the cell free culture broth contains biosurfactant then it would emulsify the hydrocarbons present. E24 is a parameter to measure the emulsifying ability. Among the 63 isolates, 41 isolates emulsified the hydrocarbons present in the biosurfactant.

TABLE 3

Quantitative screening of biosurfactant producing bacteria

| Isolates | Quantitative screening | | |
|---|---|---|---|
| | Emulsification assay (%) | BATH assay (%) | Surface tension (mN/m) |
| Control | 0.00 (1.00) p | 0.17 (1.08) H | 83.763 abcde |
| BPB - 1 | 0.00 (1.00) p | 61.37 (7.90) gh | 58.725 yzA |
| BPB - 3 | 12 .00 (3.60) m | 20.75 (4.66) v | 83.322 abcde |
| BPB - 4 | 72.00 (8.54) a | 22.60 (4.86) u | 73.937 jklm |
| BPB - 6 | 32.00 (5.74) i | 35.51 (6.04) opq | 62.792 tuvwxy |
| BPB - 7 | 0.00 (1.00) p | 35.91 (6.08) op | 70.515 mnopq |
| BPB - 8 | 36.00 (6.08) h | 5.46 (2.54) D | 68.832 nopqr |
| BPB - 12 | 0.00 (1.00) p | 46.60 (6.90) lm | 61.012 vwxyzA |
| BPB - 14 | 8.00 (3.00) n | 4.80 (2.41) D | 52.025 B |
| BPB - 15 | 0.00 (1.00) p | 65.54 (8.16) ef | 87.27 a |
| BPB - 17 | 64.00 (8.06) b | 56.79 (7.60) ij | 33.646 D |
| BPB - 19 | 0.00 (1.00) p | 45.19 (6.80) m | 72.251 lmnop |
| BPB - 20 | 0.00 (1.00) p | 0.17 (1.08) H | 67.719 opqrst |
| BPB - 22 | 0.00 (1.00) p | 0.76 (1.33) G | 74.111 jklm |
| BPB - 24 | 0.00 (1.00) p | 48.56 (7.04) l | 80.546 defgh |
| BPB - 26 | 0.00 (1.00) p | 6.56 (2.75) C | 63.336 stuvwxy |
| BPB - 28 | 0.00 (1.00) p | 5.68 (2.59) CD | 74.966 ijklm |
| BPB - 29 | 48.00 (6.99) f | 67.23 (8.26) de | 62.355 uvwxy |
| BPB - 33 | 0.00 (1.00) p | 71.00 (8.48) c | 67.133 qrstu |
| BPB - 34 | 64.00 (8.06) b | 5.32 (2.51) D | 44.85 C |
| BPB - 35 | 0.00 (1.00) p | 33.66 (5.89) q | 64.978 rstuvw |
| BPB - 36 | 0.00 (1.00) p | 15.83 (4.10) xy | 70.434 mnopq |
| BPB - 37 | 36.00 (6.08) h | 42.27 (6.58) n | 76.668 hijkl |
| BPB - 39 | 20.00 (4.58) p | 27.83 (5.37) rs | 72.673 lmno |
| BPB - 40 | 0.00 (1.00) p | 0.00 (1.00) H | 65.297 rstuv |
| BPB - 41 | 0.00 (1.00) p | 20.04 (4.59) v | 73.657 klmn |
| BPB - 42 | 0.00 (1.00) p | 28.94 (5.47) r | 77.771 fghijk |
| BPB - 43 | 12.00 (3.60) m | 16.83 (4.22) wx | 86.153 abc |
| BPB - 44 | 28.00 (5.38) j | 10.11 (3.33) A | 71.042 mnopq |
| BPB - 45 | 0.00 (1.00) p | 33.92 (5.91) pq | 83.342 abcde |
| BPB - 46 | 24.00 (5.00) k | 2.10 (1.76) F | 80.47 defgh |
| BPB - 47 | 32.00 (5.74) i | 47.94 (7.00) l | 27.154 E |
| BPB - 48 | 56.00 (7.50) d | 48.96 (7.07) l | 33.008 D |
| BPB - 49 | 64.00 (8.06) b | 78.40 (8.91) a | 29.272 DE |
| BPB - 50 | 0.00 (1.00) p | 69.79 (8.41) cd | 78.164 fghijk |
| BPB - 51 | 8.00 (3.00) n | 17.98 (4.36) w | 78.036 fghijk |
| BPB - 52 | 52.00 (7.27) e | 63.00 (8.00) fg | 61.928 vwxyz |

| Isolates | Quantitative screening | | |
|---|---|---|---|
| | Emulsification assay (%) | BATH assay (%) | Surface tension (mN/m) |
| BPB - 53 | 20.00 (4.58) $^{l}$ | 23.09 (4.91) $^{tu}$ | 63.346 $^{stuvwxy}$ |
| BPB - 54 | 24.00 (5.00) $^{k}$ | 14.13 (3.89) $^{z}$ | 68.255 $^{opqrs}$ |
| BSB - 2 | 24.00 (5.00) $^{k}$ | 24.63 (5.06) $^{t}$ | 81.833 $^{bcdefg}$ |
| BSB - 6 | 40.00 (6.40) $^{g}$ | 0.19 (1.09) $^{H}$ | 71.957 $^{lmnopq}$ |
| BSB - 7 | 20.00 (4.58) $^{l}$ | 14.86 (3.98) $^{yz}$ | 81.197 $^{cdefgh}$ |
| BSB - 8 | 28.00 (5.38) $^{j}$ | 5.29 (2.51) $^{D}$ | 79.609 $^{defghi}$ |
| BSB - 9 | 48.00 (6.99) $^{f}$ | 55.48 (7.52) $^{j}$ | 58.947 $^{xyzA}$ |
| BSB - 11 | 24.00 (5.00) $^{k}$ | 58.30 (7.70) $^{ij}$ | 72.206 $^{lmnopq}$ |
| BSB - 12 | 0.00 (1.00) $^{p}$ | 0.00 (1.00) $^{H}$ | 73.903 $^{jklm}$ |
| BSB - 13 | 0.00 (1.00)$^{p}$ | 13.49 (3.81) $^{z}$ | 78.934 $^{efghij}$ |
| BSB - 14 | 56.00 (7.54) $^{d}$ | 52.91 (7.34) $^{k}$ | 57.137 $^{zA}$ |
| BSB - 15 | 60.00 (7.80) $^{c}$ | 5.22 (2.50) $^{D}$ | 63.914 $^{rstuvwx}$ |
| BSB - 16 | 20.00 (4.58) $^{l}$ | 26.55 (5.25) $^{s}$ | 60.809 $^{vwxyzA}$ |
| BSB - 17 | 20.00 (4.58) $^{l}$ | 37.04 (6.17) $^{o}$ | 67.532 $^{pqrst}$ |
| BSB - 18 | 60.00 (7.80) $^{c}$ | 74.78 (8.70) $^{b}$ | 56.149 $^{AB}$ |
| BSB - 20 | 56.00 (7.54) $^{d}$ | 58.97 (7.74) $^{hi}$ | 60.036 $^{wxyzA}$ |
| BSB - 21 | 64.00 (8.06) $^{b}$ | 0.38 (1.17) $^{GH}$ | 76.781 $^{ghijkl}$ |
| BSB - 22 | 28.00 (5.38) $^{j}$ | 78.67 (8.93) $^{a}$ | 74.879 $^{ijklm}$ |
| BSB - 23 | 20.00 (4.58) $^{l}$ | 29.21 (5.50) $^{r}$ | 82.491 $^{abcdef}$ |
| BSB - 24 | 72.00 (8.54) $^{a}$ | 55.58 (7.52) $^{j}$ | 33.391 $^{D}$ |
| BSB - 28 | 0.00 (1.00) $^{p}$ | 0.20 (1.09) $^{H}$ | 84.565 $^{abcd}$ |
| BSB - 29 | 4.00 (2.23) $^{o}$ | 2.52 (1.88) $^{EF}$ | 83.309 $^{abcde}$ |
| BSB - 30 | 60.00 (7.80) $^{c}$ | 24.09 (5.01) $^{tu}$ | 80.586 $^{defgh}$ |
| BSB - 31 | 4.00 (2.23) $^{o}$ | 67.18 (8.26) $^{de}$ | 86.747 $^{ab}$ |
| BSB - 32 | 0.00 (1.00) $^{p}$ | 22.89 (4.89) $^{tu}$ | 67.785 $^{opqrst}$ |
| BSB - 34 | 32.00 (5.74) $^{i}$ | 2.75 (1.94) $^{E}$ | 79.93 $^{defghi}$ |
| BSB - 40 | 24.00 (5.00) $^{k}$ | 8.90 (3.15) $^{B}$ | 76.267 $^{hijkl}$ |

*Note* : BPB- biosurfactant producing phyllospheric bacteria, BSB- biosurfactant producing soil bacteria.Values followed by the same letter in each column are not significantly different from each other as determined by DMRT ($p>0.05$). The percentage values were transformed by square root transformation ($\sqrt{X+1}$) and then analyzed

The highest emulsification index was recorded by the isolate BSB-24 (72%) and BPB-4 (72%) whereas lowest emulsification index was exhibited by the isolate BSB-29 (4%) and BSB-31 (4%) while 23 isolates could not emulsify the hydrocarbons in the biosurfactant (Table 3 & Plate-1C). The lower values of emulsification index indicate that the isolates produce a low amount of biosurfactant. The present study is in accordance with work reported by Ndibe *et al*. (2018) who reported that 54.5 per cent of the biosurfactant producing isolates that were able to emulsify crude oil. Another study by (Budsabun, 2015) reported that *Serratia marcescens* BS-03 isolated from oil contaminated soil exhibited the highest emulsification activity with the highest emulsification index.

**Bacterial Adhesion to Hydrocarbons (BATH)**

The adherence of cell with hydrophobic compounds like crude oil is considered as one of the method to screen bacteria for biosurfactant production, because cells attach themselves with oil droplets by producing surface active compounds called biosurfactants. The decrease in the turbidity of the aqueous phase correlates with the hydrophobicity of the cells (Sumathi and Yogananth, 2016). All the isolates were able to show cell adherence with hydrocarbons except the isolate BPB-40. However, the significantly higher cell adherence was observed with BSB-22 (78.67%) followed by BPB-49 (78.40%) whereas, least cell adherence was noticed with BPB-20 (0.17%) and results obtained are represented in Table 3. In the present study, the positive cell hydrophobicity was reported as an indication of biosurfactant production by the isolates. The results are in accordance with the study conducted by Thavasi *et al*. (2011) who showed that among 105 isolates, 91 (86.6%) bacterial isolates were positive for the BATH assay, which indicated the affinity of the bacterial cells towards hydrophobic substrate.

**Reduction of Surface Tension**

Bacterial strains were evaluated for surface tension reduction to confirm the production of biosurfactant. The surface tension of the supernatant (cell-free broth) was measured and compared with the control (uninoculated broth). The result revealed significant reduction of surface tension by BPB-47 (27.15 mN/m) followed by BPB-49 (29.27 mN/m), which was lowest as compared to all other isolates. Whereas, surface tension of control was found to be highest recorded (83.763mN/m). The reduction in surface tension could be due to the presence of excreted extracellular hydrophobic and hydrophilic moieties of biosurfactant in cell free supernatant. Therefore, the biosurfactant is successful in reducing the surface tension of the medium. The results of the present study are similar to the results obtained by Kumar *et al*. (2017) who isolated 24 bacteria from rhizospheric soil collected from different areas of Telangana and Andhra Pradesh and documented that the surface tension reduction of cell free culture broth ranged from 60.39 to 27.96mN/m. An experiment conducted by Burch *et al*., 2011 clearly indicates that the surface tension of individual droplets of water is lowered due to the production of syringafactin by *Pseudomonas syringae* on leaves. The resultant spreading of water droplets across the leaf expands the zones of colonization for the bacteria that produced the surfactant and apparently increases their access to local, but dispersed nutrient-rich colonization sites on the leaf.

Biosurfactants possess both hydrophilic and hydrophobic moieties in their structure which confers their ability to accumulate between various phases. Many members of the bacterial community are capable of producing biosurfactants on leaves and in soil. The present study reported the screening of 95 bacterial strains isolated from contaminated sites. Six different methods were used to screen bacterial isolates for biosurfactant production and it was found that qualitative tests like drop collapse, oil spreading and penetration assay are reliable methods to screen large number of samples for biosurfactant production. After screening all the isolates for biosurfactant production using qualitative assay, out of 95 isolates 63 isolates shown biosurfactant production, these isolates were further subjected to quantitative assays like emulsification, BATH and surface tension. Out of sixty three isolates, five best performing isolates each were selected from contaminated phyllosphere and soil based on surface tension reduction and oil spreading activity. Since these isolates have proved their efficiency in reducing the surface tension, rigorous screening of these isolates for plant growth promoting traits assumes greater significance for inoculant development offering a viable option for bioremediation of contaminated soils and enhanced crop productivity.

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