Research Article

# Screening, Production and Antibacterial Potential of Biosurfactant from *Bacillus subtilis* Isolated from Oil Contaminated Soil

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**ABSTRACT**

Biosurfactants are surface active metabolites produced by microorganisms and used for various applications. In this present study, biosurfactant producing bacteria were isolated from oil contaminated soil, collected from Manavalakurichy, Kanyakumari District, Tamil Nadu. A to total 12 bacterial strains (MS1-MS12) were isolated and screened for biosurfactant activity. Only 4 strains (MS2, MS5, MS7 and MS11) have biosurfactant activity, which was confirmed by hemolysis and oil spreading test. One potential strain M2 identified as *Bacillus subtilis* was selected for biosurfactant production and the produced biosurfactant was qualitatively assayed by saponification test and thin layer chromatography. Finally, the biosurfactant was tested for antibacterial activity by well diffusion method, and it showed inhibitory against the entire test bacterial pathogens such as *Escherichia coli, Klebsiella pneumoniae, Bacillus cereus* and *Staphylococcus aureus*.

**Keywords:** Biosurfactant, *Bacillus subtilis* oil contaminated soil and antibacterial activity.

## INTRODUCTION

Surfactants are surface active chemical agents, which are used for many purposes in food, agricultural, industrial, cosmetic and pharmaceutical applications.[1] These compounds are amphiphilic agent with both lipophilic and hydrophilic in nature.[2] Most of surfactants are chemically synthesized, mainly from petrochemical origin[3] and cause toxic problems to the environment. To rectify these problems, the alternative source for the surfactant is biological origin i.e. biosurfactant.

Biosurfactants are surface active chemical metabolites produced by microorganisms such as bacteria and fungi. They have compensation over their chemicals counterparts because they are biodegradable, have low toxicity, effective at high temperatures or pH values and have better environmental compatibility.[4-6] Biosurfactants constitute a diverse group of surface active molecules such as glycolipids, lipoproteins, fatty acids, neutral lipids, phospholipids and polymeric structures.[7,8]

Biosurfactants have diverse applications includes biocontrol agent in agricultural field, health and beauty products in the cosmetic industries, etc.[9,10] They possess antibacterial, antifungal and antiviral properties; and they have anti-adhesive action against several pathogenic microorganisms.[11,12]

The most prevalent biosurfactant producers, belong to the genera are *Achromobacter, Acinetobacter, Aeromonas, Alkaligenes, Arthrobacter, Bacillus, Corynebacterium, Flavobacterium, Klebsiella, Micrococcus, Moraxella sp, Proteobacteria, Pseudomonas* and *Streptococcus sp.*[13] *Bacillus sp* is an omnipresent bacterium commonly recovered from air, water, soil and decomposing plant residue.[14] These genera have been found efficient producers of biosurfactants in hydrocarbons rich culture medium.[15] This present study was undertaken to isolate and identify biosurfactant producing bacteria from oil contaminated soil.

## MATERIALS AND METHODS

### Sample collection

Oil contaminated soil samples were collected from two different places of Manavalakurichy, Kanyakumari District, Tamil Nadu, India. An about 5-10 g of each sample collected in a sterilized container and brought to the laboratory immediately.

### Isolation of bacterial strains

The collected samples were subjected serial dilution using sterile saline water. An aliquot of 0.1 ml from $10^{-3}$ to $10^{-5}$ dilutions were taken and spread evenly over the surface of Mineral salt medium (MSM) with the help of clean L-rod. The medium consist of $NaNO_3$ (0.5 g/l), $K_2HPO_4$ (0.5 g/l), $KH_2PO_4$ (0.5 g/l), $MgSO_4.7H_2O$ (0.5 g/l), KCl (0.1g/l), $FeSO_4$ (0.01 g/l) and Agar 15 g/l with 1% of petrol as carbon source.[16]

The inoculated plates were incubated at room temperature for 48-72 hours.

The morphologically different bacterial colonies were selected and sub-cultured in mineral salt medium for further use.

**Screening of biosurfactant production**

***Blood haemolysis test***

Blood agar was prepared using mineral salt medium with sheep blood. A loopfull of fresh bacterial cultures were streaked on the surface of blood agar plates and incubated at 30°C for 48-72 hrs. After incubation, the plates were observed for the presence of haemolysis activity around the colonies indicated the presence of biosurfactant production.[17,18]

***Oil spreading assay***

30 ml of distilled water was taken in a Petri dish and 10 µl of crude oil was added to the surface of water to form a thin oil layer. Then, 10 µl of culture filtrate was gently placed on the centre of the oil layer. In this assay, the presence of biosurfactant would displace the oil and form a clear zone and the diameter of the zone was measured after 30 s, which correlates to the surfactant activity.[19]

**Characterization and identification of potential strain**

The potential biosurfactant producing bacteria was identified by cultural, morphological and biochemical characteristics. Gram staining, motility test, spore staining, indole production test, methyl red test, Voges-Proskauer test, citrate test, triple sugar iron test, urease test, starch hydrolysis, casein hydrolysis and sugar fermentation tests were performed to identify the potential bacterial strains as per given in Bergey's manual of systematic bacteriology.

**Production of biosurfactant**

Production of biosurfactant using the potential strain was carried out in 250 ml of conical flask with 100 ml of mineral salt medium (MSM) with 2% of petrol as carbon source. The medium was inoculated with 1 ml of seed culture and incubated in orbital shaker incubator at 37°C with shaking at 120 rpm for 72 hrs.[20]

**Extraction of biosurfactant**

After suitable incubation, the bacterial cells were removed by centrifugation (9000 rpm at 4°C for 30 min). The supernatant was adjusted to pH 2 using sulphuric acid (1M) prior to biosurfactant extraction using equal volume of chloroform-methanol (2:1) mixture. The organic phase was separated and the solvent was allowed evaporated to concentrate the crude biosurfactant.[21]

**Qualitative analysis**

***Saponification test***

A small amount of crude biosurfactant was taken in tube; to that 1-2 ml of 2% sodium hydroxide solution was added and mixed well. Positive result was observed by the formation of soapy solution.

***Thin Layer Chromatography***

A portion of the crude biosurfactant was separated on a thin layer of silica gel plate with the solvent mixture includes chloroform, methanol, acetic acid and water (25:15:4:2).

The lipid components were detected as brown spots on the plate after spraying with chromosulfuric acid and carbohydrate compounds were detected as red spots on the plate after spraying with α-naphtol and sulfuric acid.[22]

**Antibacterial activity**

Antibacterial activity of the biosurfactant was performed by agar well diffusion method against four bacterial pathogens includes *Escherichia coli, Klebsiella pneumoniae, Bacillus cereus* and *Staphylococcus aureus.* Fresh bacterial culture of 0.1 ml was spread on nutrient agar plate using sterile cotton swab and allowed to dry for few min. Wells of 6 mm in diameter were punched off into medium with sterile cork borer and filled with 50µl of biosurfactant suspension prepared in methanol (1mg/ml). All the plates were kept in a refrigerator to allow pre-diffusion of extract for 30 min and further incubated at $37^0$C for 24 hrs. The antimicrobial activities were evaluated by measuring the zone of inhibition.

## RESULTS

**Isolation and screening of biosurfactant producing bacteria**

A total of 12 morphologically different bacterial colonies were isolated from the collected soil samples, which were codes as MS1-MS12. The entire isolated bacterial colonies were screened for biosurfactant activity and it was found that, only 5 strains were positive in primary screening through hemolytic activity in blood agar plate.

The biosurfactant activities of the positive strains were further confirmed by oil spreading assay. In this test, 4 strains showed positive result, which was confirmed by the formation of zone by displacing the oil.

Among the 4 positive strains, the best one (MS2) was selected for further study, which produced a good haemolytic activity as well as high zone (4.6 mm in diameter) in oil spreading test (Table 1).

**Table 1:** Screening of biosurfactant activity

| Strain code | Hemolysis activity | Oil spreading assay |
|---|---|---|
| MS1 | - | - |
| MS2* | + | + |
| MS3 | + | - |
| MS4 | - | - |
| MS5 | + | + |
| MS6 | - | - |
| MS7 | + | + |
| MS8 | - | - |
| MS9 | - | - |
| MS10 | - | - |
| MS11 | + | + |
| MS12 | - | - |

'+' biosurfactant positive; '-'biosurfactant negative; *best strain

**Characterization and identification of potential strain**

The potential strain MS2 was identified as *Bacillus subtilis* based cultural, morphological and biochemical characteristics (Table 2).

**Table 2:** Characterization and identification of potential strain

| Characterization | Result/Inference |
|---|---|
| Gram reaction | +ve |
| Morphology | rod |
| Arrangements | alienated bacilli |
| Motility test | +ve |
| Spore staining | -ve |
| Catalase test | +ve |
| Oxidase test | +ve |
| Indole test | -ve |
| Methyl red test | +ve |
| Voges-Proskauer test | +ve |
| Citrate test | +ve |
| Urease test | -ve |
| Triple sugar iron test | Acid |
| $H_2S$ production | -ve |
| Starch hydrolysis | +ve |
| Casein hydrolysis | -ve |
| Glucose (Fermentation) | +ve |
| Sucrose | +ve |
| Lactose | +ve |
| Dextrose | +ve |
| Arabinose | +ve |
| **Probable identity** | ***Bacillus subtilis*** |

**Biosurfactant production, extraction and confirmation**

Production of biosurfactant was carried by *Bacillus subtilis* strain.

The biosurfactant was extracted from the whole cell-free culture broth by centrifugation and concentrated by adjusting with sulphuric acid.

Qualitative analysis of biosurfactant was performed by saponification test and thin layer chromatography (TLC).

The crude biosurfactant of *Bacillus subtilis* showed positive for saponification test by forming soapy solution and the lipid components were detected as brown spots in TLC after spraying with chromosulfuric acid with a RF value of 0.97.

**Antibacterial activity**

The biosurfactant produced by *B.subtilis* shows inhibitory activity against entire test organisms (Table 3). This result showed that, highest activity against *E.coli* (14 mm) followed by *K. pneumoniae* (12 mm) and less activity against *B.cereus* (10 mm).

**Table 3:** Antibacterial activity of biosurfactant

| Test organisms | Zone of inhibition (mm) |
|---|---|
| *Escherichia coli* | 14 |
| *Klebsiella pneumoniae* | 12 |
| *Bacillus cereus* | 10 |
| *Staphylococcus aureus* | 11 |

## DISCUSSION

A total of 12 bacterial cultures were obtained from oil contaminated soil collected from Manavalakurichy, Kanyakumari District, Tamil Nadu. Among the 12 isolated strains, only 4 strains have the ability to produce biosurfactant, which was confirmed by blood hemolysis and oil spreading test. Among many methods for screening of biosurfactant activity, blood agar method is often used for a preliminary screening of microorganisms for the ability to produce biosurfactants on hydrophilic media.[23] In this study, the isolate (MS2) lyses the blood cells and exhibit a colorless, transparent zone around the colonies, the result suggest that the potency of biosurfactant production by the strain. Based on the results, the strain MS2 was selected as potential strain for biosurfactant production.

The potential biosurfactant producer MS2 was identified as *Bacillus subtilis* by studying cultural, morphological and biochemical characteristics. *Bacillus* and *Pseudomonas* species were efficient biosurfactant producers,[20] Suganya[24] has isolated biosurfactant producing *Bacillus* and *Pseudomonas* strains from soil samples collected from polluted sites. The strain *Bacillus* sp showed high salt tolerance and their successful production of biosurfactant in a vast pH and temperature, also a potential candidate for microbial enhanced oil recovery.[22]

The qualitative analysis of biosurfactant produced by *B.subtilis* was performed by saponification, it confirms the presence of lipids substances and brown spots in TLC after spraying with chromosulfuric acid confirmed the bacterial metabolites as biosurfactant.

Finally, the antibacterial activity of the biosurfactant produced by *B.subtilis* was carried out by agar well diffusion method against two gram positive and two gram negative bacterial pathogens. In this study, the entire bacterial pathogens such as *E.coli, K.pneumoniae, B.cereus* and *Staph.aureus* were inhibited by the biosurfactant. Also it was observed that, Gram (-) bacteria were more susceptible than Gram (+) bacteria.

Das[25] found that, surfactin produced by *B. circulans* had good antimicrobial activity against most gram positive bacteria than gram negative bacteria.

The lipopeptide biosurfactant produced by *B.licheniformis* exhibited interesting antimicrobial activities against *B.subtilis, B. thuringiensis, B.cereus, Staph. aureus (25*

*mm), P.aeruginosa, E.coli, S.typhi, P.vulgaris* and *K.pneumoniae.*[26]

## CONCLUSION

It was concluded that the bacterial strain *Bacillus subtilis* have the ability to produce potential biosurfactant and it is gain more important in future for various industrial and pharmaceutical applications.

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