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Biosurfactant production from *bacillus* sp. and its application in the medical field

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Abstract

Biosurfactants as the name suggests are surface active compounds produced by the microorganisms. They are amphiphilic molecules which possess both hydrophilic and hydrophobic moieties and exhibit emulsifying activity. Biosurfactants are eco-friendly, biodegradable and non-toxic compounds. The objective of this study was to isolate biosurfactant producing *Bacillus* sp and detect its antimicrobial activity against pathogens. Among 10 isolates 5 cultures showed biosurfactant activity which was identified by screening techniques. The screening techniques carried out were drop collapse test, oil spreading technique, emulsification index, foaming activity and haemolysis test. From the five, highest biosurfactant yielding culture was selected for small scale production. Chloroform: methanol extraction procedure was carried out to precipitate out the biosurfactant from the production medium. The crude biosurfactant was found to be lipopeptide by TLC and it showed antimicrobial activity against *Staphylococcus aureus* and *Shigella boydii*.

Keywords: Biosurfactants, *Bacillus* sp., Chloroform: methanol extraction, TLC, antimicrobial activity.

INTRODUCTION

Biosurfactants are generally produced by bacteria, yeasts and fungi; hence they are called “green surfactants and microbial molecules”. Biosurfactants are less toxic and eco-friendly in nature. They are commercially produced and utilized in various fields such as environment bioremediation, food processing industries, cosmetics and pharmaceuticals[1]. Biosurfactants are amphiphilic compounds, which contains both hydrophobic (non-polar) and hydrophilic (Polar) moieties, that reduces the surface and interfacial tensions of any liquid [2]. They have higher selectivity and better foaming properties. As they are surface

active compounds, they have special properties such as foaming, wetting, emulsification/ de-emulsification, dispersion and coating, which are very useful in biological remediation and physic-chemical technologies of both metal and organic contaminants [3].

Based on molecular weight, biosurfactants are divided into low-molecular weight and high molecular weight compounds. Glycolipids, phospholipids and lipopeptides are low molecular weight compounds whereas proteins, lipopolysaccharides and form high molecular weight compounds. Low molecular weight compounds are effective in lowering surface and interfacial tensions but comparatively high molecular weight compounds are less effective at stabilizing oil-in-water emulsions [4].

Several microorganisms are known for the production of biosurfactants such as *Bacillus subtilis*, *B*. *licheniformis*, *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*, *Nocardia* sp., *Corynebacterium* sp., *Penicillium spiculisporum*, *Torulopsis apicola*, *Saccharomyces cerevisiae*, *Candida lipolytica* etc [5]. The medical purposes of biosurfactant illustrate their role as anti-adhesive coating agents against several pathogens. These coating agents are used as medical insertional materials with synthetic drugs and chemicals which in turn leading to a reduction of a large number of hospital acquired infections. Biosurfactants are widely used therapeutic agents due to their antibacterial, antifungal and antiviral activities [6].

MATERIALS AND METHODS

Collection of soil samples

Ten soil samples were collected from different automobile workshops in and around Coimbatore, Salem and Karur.

Enrichment of the Soil samples

1g of petrol contaminated soil samples were mixed in 100 ml of Mineral Salt Medium (MSM) with 2 ml diesel oil as the carbon sources and was incubated for 72 h at 30^{o}C [7].

Isolation of *Bacillus* sp. from enriched soil sample

1ml of the sample from enrichment medium was serially diluted and plated on Nutrient agar medium. The plates were incubated at 30^{o}C for 24 h. After incubation, morphologically distinct colonies were selected for further studies.

Screening for Biosurfactant producers

The selected bacterial isolates were grown in Mineral salt medium supplemented with 1% diesel oil as carbon source and incubated at 30°C for 48 h in shaker incubator with 150 rpm. The supernatant was collected using centrifugation at 10000 rpm for 30 min at 4°C in cooling centrifuge.

The biosurfactant property of *Bacillus* culture supernatant was assessed by standard technique as follows:

(a) Drop Collapse Test: In a clean glass slide 0.1 ml of diesel oil was spread evenly. The flattening property of diesel oil was observed for a period of 30 sec to 1 min and the result was recorded [8].

(b) Oil Spreading Test: 40 µl of distilled water was added to a petri dish (15 cm diameter) followed by the addition of 20 µl of diesel oil to the surface of water then 10 µl of culture supernatant was dropped onto the oil surface. The diameter of clear zones on the oil surface was measured and the results were recorded.

(c) Emulsification Index: Equal volumes of supernatant with diesel oil (1:1) were mixed well in a glass tube. Then the mixture was vortexed for 2 min and left to stand for 18 to 24 h. The emulsification index was calculated by dividing the height of the emulsion layer by total height x 100.

(d) Foaming Activity: Isolated strains were grown separately in 250 ml Erlenmeyer flasks, each containing 100 ml of nutrient broth medium. The flasks were incubated at 37°C on a shaker incubator (200 rpm) for 72 h. Foam activity is detected as duration of foam stability and foam height.

(e) Blood Hemolysis Test: Fresh isolated colony was taken and streaked on blood agar plate and incubated for 24 h under 30°C. The presence of clear zone indicates the biosurfactant producing bacteria.

Characterization of selected soil isolates

Morphological, biochemical and physiological characterisation of the isolates were performed to identify *Bacillus* sp.

Production of biosurfactants

1% inoculum of positive isolate was transferred aseptically into Minimal Salt medium containing: KNO_3 (0.3%), Na_2HPO_4 (0.22%), KH_2PO_4 (0.014%), NaCl (0.001%), $MgSO_4$ (0.06%), $CaCl_2$ (0.004%), $FeSO_4$ (0.002%) with 2% diesel oil and incubated at 30°C for 72 h at 120 rpm in shaker.

Extraction of Biosurfactant

Cultured supernatant was obtained by centrifugation at 10000 rpm for 30 minutes at 4°C. The pH of supernatant was adjusted to 2 using 1M H_2SO_4 and the precipitate was extracted thrice with Chloroform: Methanol in the ratio of 2:1. The precipitate was collected by centrifugation and evaporated to dryness to remove residual solvents and it was re-dissolved in sterile dist. H_2O.

Identification of crude biosurfactant by Thin Layer Chromatography

Silica plate was prepared and the crude biosurfactant was spotted on the plate. The biosurfactant was separated using the solvent Chloroform: methanol: acetic acid: water in ratio 25: 15: 4: 2. Ninhydrin reagent was sprayed to detect lipopeptide biosurfactant as red spots.

Antimicrobial Activity of Biosurfactant

The medical samples such as *E.coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Shigella boydii*, *and Staphylococcus aureus* were used to determine the antimicrobial activity of biosurfactant.

RESULTS AND DISCUSSION

Isolation of *Bacillus* sp.

10 different *Bacillus* cultures were isolated from the soil samples and screened for biosurfactant production (Figure 1).

Figure 1 *Bacillus* sp.

Screening for biosurfactant

a) Drop collapse test- Depending on the concentration of crude biosurfactant the drop of supernatant collapses to varying degrees on oil coated glass slide (Figure 2).

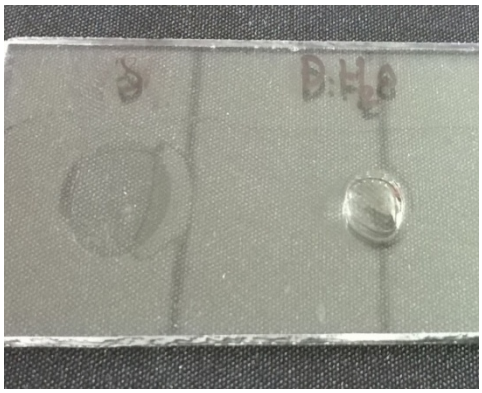

Figure 2 Drop collapse test

b) Oil spreading technique- Clear zone in the oil containing plate indicates the ability to displace oil (Figure 3).

Figure 3 Oil spreading technique

c) Foaming activity- Foam activity is detected as duration of foam stability (Figure 4).

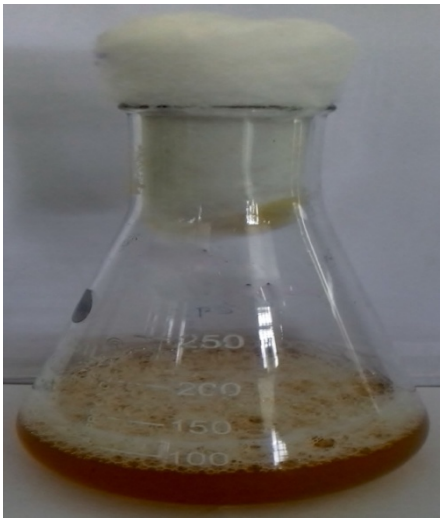

Figure 4 Foaming activity

d) Hemolysis- Presence of clear zone indicates the biosurfactant producing bacteria (Figure 5).

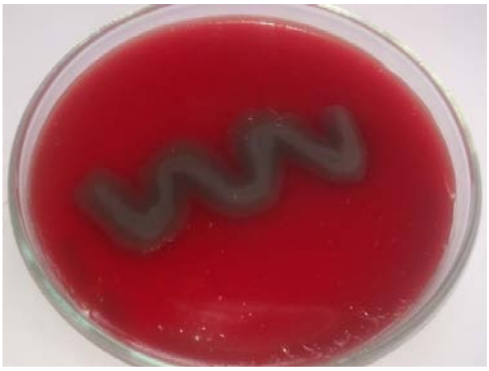

Figure 5 Hemolysis

Production of Biosurfactant

Five isolates which showed positive result in screening for biosurfactant production was inoculated in the production medium (Figure 6).

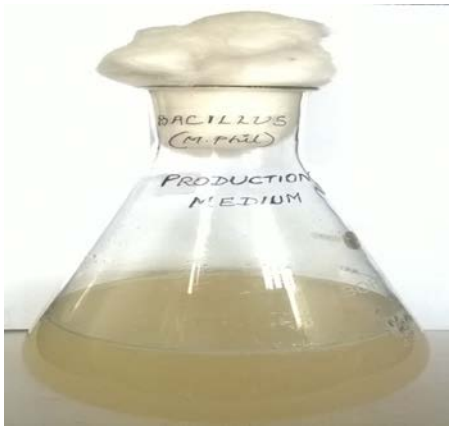

Figure 6 Production medium inoculated with *Bacillus* sp.

Extraction of Biosurfactant

The crude biosurfactant was extracted from culture supernatant by chloroform: Methanol extraction method. The product was obtained in the form of creamy white powder (Figure 7).

Figure 7 Crude Biosurfactant

The weight of the crude extract obtained from 5 different *Bacillus* sp. is given below (Table 1):

Table 1: Weight of crude extract obtained per 100 ml of production medium

***Bacillus* Cultures**	**BS (gm/100 ml)**
A	0.0254
B	0.3356
C	0.0296
D	0.0364
E	0.0144

Among the five positive *Bacillus* sp. culture B showed more production i.e. 0.3356 gm/100 ml compared to the other samples. Thus culture B was selected for more production and application.

Identification of crude biosurfactant Thin Layer Chromatography

The isolated biosurfactant is identified as lipopeptide indicated by the formation of red spot on TLC plate (Figure 8).

Figure 8 Thin layer chromatography

Antimicrobial Activity of Biosurfactant

A clear zone was observed on both plate having *Staphylococcus aureus* and *Shigella boydii* with BS concentration of 20 mg/ 10 ml which indicates the antimicrobial activity of crude biosurfactant from *Bacillus* sp (Figure 9, Table 2).

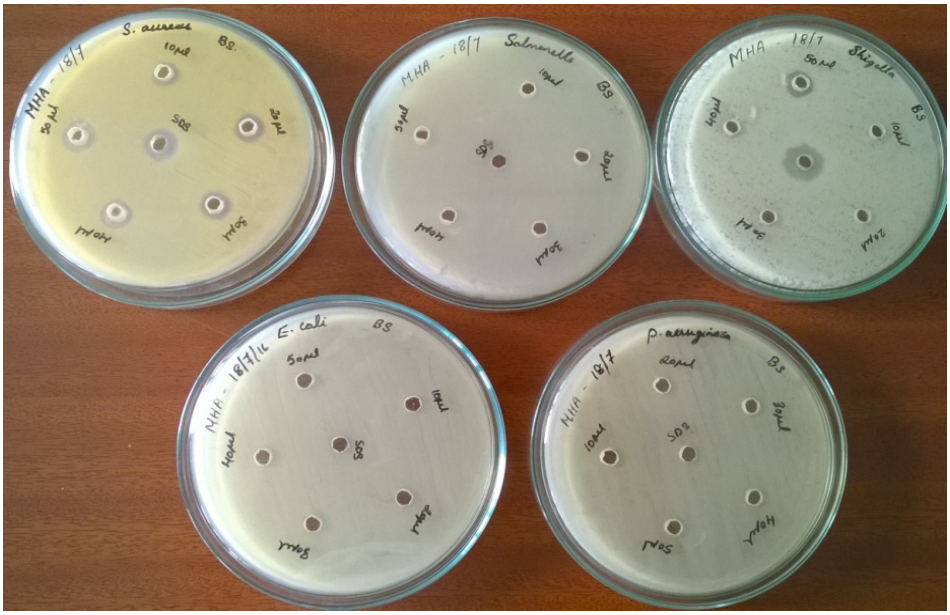

Figure 9 Zone of Inhibition by crude biosurfactant against pathogens

Table 2: Zone of inhibition by Crude BS from *Bacillus* sp. against pathogens

Sl No	Bacterial pathogens	BS (20mg/10ml)					
		SDS	10 µl	20 µl	30 µl	40 µl	50 µl
1	*Staph aureus*	11mm	8mm	9mm	9mm	10mm	11mm
2	*Shigella boydii*	11mm	-	-	-	-	9mm
3	*Salmonella typhi*	-	-	-	-	-	-
4	*E. coli*	-	-	-	-	-	-
5	*P. aeruginosa*	-	-	-	-	-	-

CONCLUSION

Biosurfactant produced from the *Bacillus* sp. isolated from petroleum contaminated soil was found to show antibacterial activity against *Staphylococcus aureus* and *Shigella boydii*. This indicates the applicability of biosurfactants in the medical field. Thus it is a promising alternative to the chemical surfactants used which are toxic. It has the potential to be used as anti-adhesive coating agents for human implants thereby reducing the nosocomial infections and application of synthetic chemicals. Their use is limited due to the high cost of production and extraction and lack of knowledge of toxicity on humans.

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