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ORIGINAL ARTICLE **OPEN ACCESS**

Isolation and Molecular Analysis of Biosurfactant Producing Bacteria Isolated From Rhizosphere Soil

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ABSTRACT

Biosurfactants (BS) are a type of secondary metabolite generated by a wide range of microorganisms that have surface active properties. Biosurfactant producers have demonstrated a significant advantage over chemical surfactants in terms of biodegradability, low toxicity, and environmental friendliness. The objective of this research is to isolate and screen BS-producing bacteria from rhizosphere soil samples, characterise them, and identify the bacteria isolated at the molecular level. Oil spreading technique and drop-collapse test were used to screen the Biosurfactants producing strains. The biosurfactant had exotic antibacterial action against tested bacteria, with S. aureus showing the highest antimicrobial activity. Since chemicals are not ecologically friendly and cause difficulties in the health sector, pharmaceutical firms, and cosmetic industries, it is vital to develop a biological equivalent for surfactants rather than chemicals. The results showed that the biosurfactant had a potential antimicrobial activity increasing impact, suggesting that it might be used with antibiotic formulations to improve antimicrobial efficiency against multidrug-resistant pathogenic microorganisms.

Keywords: Biosurfactants, Rhizosphere soil, Bacteria, Petrol, Oliveoil, PCR.

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INTRODUCTION

Surfactants are a class of industrial chemicals that are found in practically every modern industrial process [1]. The majority of surface-active chemicals in use are chemically produced and are often poisonous and non-biodegradable. Microorganisms produce biosurfactants, which are amphipathic molecules having both hydrophilic and hydrophobic moieties [2]. These are surface active compounds that can reduce surface tension in both aqueous and hydrocarbon mixtures. Surfactants are split into two types based on their molecular weight: low-molecular-weight surfactants and high-molecular-weight polymers (polymeric compounds) [3]. Low-molecular-weight surfactants, such as glycolipids and lipopeptides, have a molecular structure. Low-molecular-weight surfactants, such as glycolipids, lipopeptides, phospholipids, and fatty acids, have both a hydrophobic and a hydrophilic portion in their molecular structure, which can reduce the surface tension of a solution [4-7]. The polymeric compounds, on the other hand, have distinct structures that affect emulsion formation and stability, but they do not require to diminish surface tension [8, 9]. Biosurfactants provide a number of advantages over chemical surfactants, including reduced toxicity, increased biodegradability, increased foaming capacity, and optimal activity at extreme temperatures, pH levels, and salinity [10, 11]. As a result, industrial applications hold a great deal of promise for them [10,11]. As a result, they have a lot of potential in industrial applications. Many scientists agree biosurfactants will eventually replace chemical surfactants in the cosmetics, pharmaceutical, and food-processing sectors, as well as in detergents, healthcare, and environmental applications. Biosurfactants have a lot of potential in the oil industry, particularly in terms of Microbial Enhanced Oil Recovery (MEOR) and crude oil transfer (12-15). Bacteria that produce biosurfactants are common and can be found in a variety of habitats with varying temperatures, pH levels, and salinity. Several bacterial genera, including Bacillus, Pseudomonas, Burkholderia, and Flavobacterium, have been shown to create biosurfactants; nonetheless, the bacteria are regarded as generous biosurfactant producers [16]. Biosurfactant-producing bacteria are common in soil and water

contaminated with hydrocarbons and oilfield generated water, where they make biosurfactants to utilise hydrocarbons as a carbon source [17-19].
Many studies have been published on microbially synthesised surfactants derived from hydrocarbon-contaminated sources, but due to a lack of knowledge in the isolation of microorganisms, the current study investigates the potential of rhizospheric bacteria for the production of biosurfactant using different recovery solvents, as well as the hydrocarbon degradation activity of potential biosurfactant producing microorganisms.

MATERIAL AND METHODS

COLLECTION OF SOIL SAMPLE

Soil sample was collected from an undisturbed site of the Center for Bioscience and Nanoscience Research (CBNR) laboratory, Coimbatore and stored at 4°C.

ISOLATION AND SCREENING OF BIOSURFACTANT PRODUCERS FROM SOIL

Collected soil sample were serially diluted and plated on Nutrient Agar plates containing 1% olive oil for isolating Biosurfactant producers from the soil. And the Colony Forming Unit (CFU) was recorded. Colonies with various morphologies were selected and purified after 24 hours based on Gram staining characteristics, cell shape, and colony morphology. The colonies were inoculated in Nutrient Broth and incubated at 37°C for 48 hours for further studies [20].

PRODUCTION & SCREENING OF BIOSURFACTANT PRODUCERS

Inoculated 1ml of the isolated culture into 100ml Minimal Salt Medium (MSM) containing 2% (v/v) crude oil as the only carbon source in a 250ml conical flask and was incubated in a rotary shaker for 72 hours at 37°C and 160 rpm. Bacterial cells were removed after 3 days of fermentation by centrifugation at 5000 rpm for 20 minutes at 4°C. To find biosurfactant-producing strains, the cell-free supernatant was exposed to several screening procedures [21].

a) Drop-collapse test

2µl of crude oil were coated into the wells of a polystyrene 96 well micro-plate and dried at 22°C for 24 hours. 10µl , 5µl, 2.5µl and 1.25µl of filtered cell-free supernatant, pellet and distilled water to the middle of the oil-coated wells of 1st, 2nd and 3rd columns respectively. The wells of 1st row left with oil alone. And observed after 1–2 minutes when the oil drop was flat and considered positive for biosurfactant production. Those that formed rounded drops got a negative grade, indicating that no biosurfactant was produced [22].

b) Oil spreading test

The oil spreading test was performed by pouring 40ml of distilled water onto a 15cm diameter Petri dish. After that, 20µl of crude oil were poured onto the water's surface, forming a thin coating. Then 10µl of culture supernatant was poured over the crude oil layer's centre. The area of the clear zone on the oil surface was measured and compared to a negative control of 10µl of distilled water [23].

BIOMASS ESTIMATION OF THE BIOSURFACTANT PRODUCED [24]

About 1 ml of biosurfactant producing bacterial culture in the MSM medium was taken and centrifuged at 5000 rpm for 5 minutes. Transferred the supernatant, collected the pellet and weighed.

ANTIBACTERIAL ACTIVITY OF THE BIOSURFACTANT PRODUCED [25]

Using the agar well diffusion technique, the antibacterial activity of the synthesised Biosurfactant is determined. Two Mueller Hinton Agar plates were taken, and the entire agar surface were swabbed with *S. aureus* and *E. coli*. Then, aseptically, 4 wells were punched and 8µl of cell free supernatant, a mixture of 4µl supernatant and 4µl ethyl acetate, 8µl crude oil, and 8µl distilled water were transferred to separate wells. The distilled water in the 4th well was taken as negative control and an antibiotic disc placed at the center was taken as positive control. The agar plates were incubated at 37°C for 24 hours.

IDENTIFICATION OF BIOSURFACTANT-PRODUCING BACTERIA

Morphological and biochemical analysis were used to characterise biosurfactant- producing bacteria, and the 16S rRNA gene was used to identify bacterial isolates up to species level. The bacterial DNA was amplified using the Universal Forward and Reverse primers 27F (5-AGAGTTTGATCCTGGCTCAG3) and 1492R (5-GGTTACCTTGTTACGACTT-3). The amplification reaction was done in a total volume of 20µl consisting of bacterial isolated DNA (2µl), forward primer (2µl), reverse primer (2µl), PCR master mix (8µl) and distilled water (6µl). PCR was performed with initial denaturation at 94°C for 4 minutes, 20 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 15 seconds, extension at 72°C for 30 seconds and with final extension at 72°C for 4 minutes and stored at 4°C. Lane 1 and 2 were loaded with PCR products of

Biosurfactant producing bacterial samples, Lane 3 and 4 were loaded with DNA marker and 16S rRNA of *Bacillus sp*. respectively. The results were observed in UV Spectrophotometer.

RESULTS AND DISCUSSION

ISOLATION AND SCREENING OF BIOSURFACTANT PRODUCERS

The Biosurfactantp prducing bacteria from Rhizosphere soil were isolated on Nutrient Agar plates containing 1% olive oil. The soil had shown culturable populations by Colony Forming Units (Fig. 1).

Fig. 1. Isolation of Biosurfactant producing bacteria from Rhizosphere soil

PRODUCTION & SCREENING OF BIOSURFACTANT PRODUCERS

The pellet and supernatant were separated by centrifugation following biosurfactant-producing bacteria were grown on MSM medium containing 2% crude oil.

a) Drop-collapse test :

The drop-collapse ability of isolated strain cell-free supernatant was good, with the drop flattening out or even vanishing totally (Fig. 2). There was also a sufficiently big and distinct halo zone, indicating substantial biosurfactant synthesis (Table 1).

Table 1. Drop-Collapse Test Scores in Different Concentrations of Samples

Sl. No.	Sample	Concentration			
		10µl	**5µl**	**2.5µl**	**1.25µl**
1	Cell free supernatant	+	+	+	+
2	Pellet	+	-	-	-
3	Distilled Water	-	-	-	-

Flat drops were given a positive (+) score indicating partial to total spreading over the oil surface, whereas rounder drops were given a negative (-) score indicating a lack of biosurfactant synthesis. As a negative control, sterile distilled water was used (-)

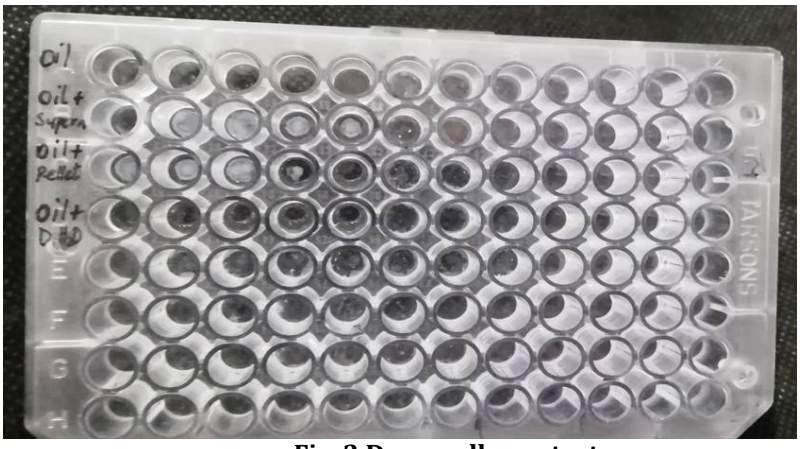

Fig. 2.Drop-collapse test

b) Oil spreading test :

An oil spreading assay was used to further screen the biosurfactant producing microorganisms. The oil spreading assay results were comparable with the Drop-collapse test results, indicating that the positive

bacterial strains tested on agar were likewise positive in the oil spreading assay. The biosurfactant concentration in the culture broth is directly proportional to the oil displacement area in the oil spreading assay. An aggregation of biosurfactants induced oil displacement at the interface between the two immiscible fluids (oil and water). Surface (liquid-air) and interfacial (liquid-liquid) tensions were reduced as a result of the accumulation. The repulsive forces between the two distinct phases eventually dissipated, enabling the two phases to mix and interact more freely. As a result, the oil in the plate inoculated with 10µl culture was displaced from the water surface, resulting in a clear zone. The strain also showed a 12cm^2 major oil displacement area, indicating biosurfactant production.

ANTIBACTERIAL ACTIVITY OF THE BIOSURFACTANT PRODUCED

A partially purified biosurfactant showed antibacterial activity against the bacterial pathogens *E. coli* and *S. aureus* (Fig. 3). In a well diffusion experiment, biosurfactant developed a zone of inhibition against *S. aureus* and *E. coli.* When combined with ethyl alcohol, it showed a maximal zone of inhibition of 13mm against *S. aureus* (Table 2).

Table 2. Antimicrobial Activity of The Biosurfactant Produced by The Isolated Bacteria

Names/Sample	Clear Zone of Inhibition Diameter (mm)	
	Staphylococcus aureus	*Escherichia coli*
S1	9	3
S+Eth	13	Nil
Crude	8	4
DW	5	Nil
Antibiotic disc	10	5

A **B**

Fig. 3. Antimicrobial susceptibility testing of produced Biosurfactant against (A) *Staphylococcus aureus* and (B) *Escherichia coli*

IDENTIFICATION OF BIOSURFACTANT-PRODUCING BACTERIA

The strain was a Gram-positive, spore-forming bacterium with morphological and biochemical characteristics similar to *Bacillus* species (Table 3). The gene amplification was obtained using PCR as the DNA underwent five series of cycles. All of the isolates tested had their 16S rRNA matched to *Bacillussp (*Fig. 4).

Table 3. Morphological and Biochemical Characteristics of Isolated Bacteria

Sl. No.	Test characteristic	Result
1.	Gram staining	+
2.	Spore staining	+
3.	Capsule staining	-
4.	Motility test	+
5.	Voges-Proskauer test	+
6.	Starch hydrolysis	+
7.	Oxidase	-
8.	Catalase	-
9.	Sucrose	+
10.	Maltose	+
11.	Fructose	+

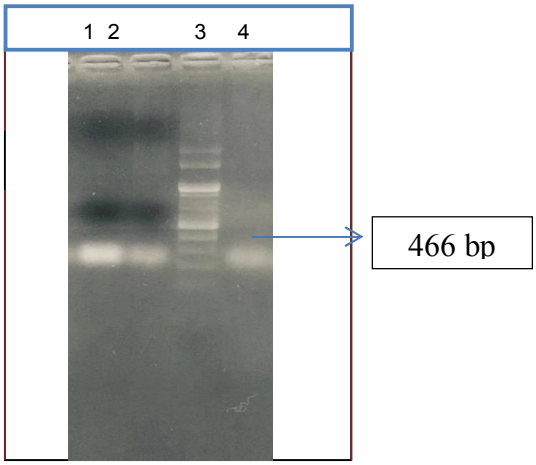

Fig.4. 16 SrRNA gene bands of the Biosurfactant Producing Bacteria
[Lane 1: 16S rRNA of Biosurfactant Producer, Lane 2 :Duplicate, Lane 3 : DNA ladder, Lane 4 : 16S rRNA of *Bacillus sp*. (Control)]

In this study, by using both cultural and molecular methods, it was shown that many isolated bacteria strains from the undisturbed soil (rhizosphere soil) from the CBNR laboratory were able to produce Biosurfactants as well as to degrade oil. The isolated bacteria strain was *Bacillus*sp. which showed high production of biosurfactants. As these bacterium has shown high degradation rates, future studies can be employed to analyze oil recovery, surfactant production in the detergent industries to reduce surface tension as well as it can be potentially used in the environment and the medical fields.

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