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Comparative Study for Biosurfactant Production by Using *Bacillus subtilis* and *Pseudomonas aeruginosa*

T. Priya and G. Usharani

Department of Microbiology, Annamalai University, Annamalai Nagar-608 002 Tamil Nadu, India

Abstract: Biosurfactants are amphiphilic compounds produced by various bacteria and fungi which reduce surface and interfacial tension. Oil contaminated soil were collected from four different automobile shop in Mayiladuthurai, Tamilnadu, India. *Bacillus subtilis* and *Pseudomonas aeruginosa* were isolated and identified from these four samples and these were screened for biosurfactants production using vegetable oil, kerosene, petrol and diesel by oil spreading technique and emulsification stability test and the best one of *Bacillus subtilis* and *Pseudomonas aeruginosa* were used for biosurfactants production using vegetable oil, kerosene, petrol and diesel as source. The isolated biosurfactants were identified using TLC method. Among four different oils; diesel is the best source for the production of biosurfatant in both *Bacillus subtilis* and *Pseudomonas aeruginosa* and *Pseudomonas aeruginosa* have higher activity than *Bacillus subtilis*.

Key words: Biosurfactant · *Bacillus subtilis* · *Pseudomonas aeruginosa* and TLC

INTRODUCTION

Biosurfactants are amphiphilic compounds produced on living surfaces, mostly microbial cell surfaces or excreted extracellularly and contain hydrophobic and hydrophilic moieties that reduce surface tension and interfacial tension between individual molecules at the surface and interface respectively [1]. Biosurfactants are produced by different microorganisms such as bacteria, fungi and yeast. Biosurfactants have several advantages, including low toxicity, high biodegradability, low irritancy and compatibility with human skin [2]. Biosurfactants have gained importance in the fields of enhanced oil recovery, environment bioremediation, food processing and pharmaceuticals [3].

Biosurfactant producing microorganisms were naturally present in the oil contaminated soil. Oil contaminated environment contain large amount of hydrocarbons. Hydrocarbons are composed of complex chemical structure i.e., aliphatic and aromatic hydrocarbons. Microorganisms exhibit emulsifying activity by producing biosurfactants and utilize the hydrocarbons as substrate often mineralizing them or converting them into harmless products.

Among the different classes of biosurfactants, rhamnolipid and surfactin are best studied biosurfactants. Rhamnolipid is one of the type of glycolipids, in which one or two molecules of rhamnose are linked to one or two molecules of β-hydroxydecanoic acid while the OH group of one of the acids is involved in glycosidic linkage with the reducing end of the rhamnose disaccharide, the OH group of the second acid is involved in ester formation [1]. Rhamolipid is produced by *Pseudomonas aeruginosa,* a gram-negative, motile, non-spore forming bacteria. Surfactin is a cyclic lipopeptide commonly used as an antibiotic [4]. Surfactin's structure consists of a peptide loop of seven aminoacids (L-asparagine, glycine, two L-leucine, L-valine and two D-Leucines) and anhydrophobic fatty acid chain thirteen to fifteen carbon long [5]. In the various course of studies of its properties, surface was found to exhibit effective characteristics like antibacterial, antiviral, antifungal, antimycoplasma and hemolytic activities [6]. Surfactin is produced by *Bacillus subtilis,* a gram-positive, motile, spore forming bacteria.

The present study focused on the biosurfactant production by *Bacillus subtilis* and *Pseudomonas aeruginosa* isolated from oil contaminated soil using four different oils as substrate and the biosurfactant production was screened by oil spreading technique and emulsification stability test. Isolated biosurfactants were identified by using TLC method.

Correspoding Author: T. Priya Department of Microbiology, Annamalai University, Annamalai Nagar-608 002 Tamil Nadu, India

MATERIALS AND METHODS

Isolation and Identification of *Bacillus Subtilis* and *Pseudomonas Aeruginosa*: Oil contaminated soils were collected from four automobile shops in Mayiladuthurai.

Serial dilution technique using nutrient agar was performed to isolate bacteria from soil. Staining techniques and biochemical tests [7] were performed to identify *Bacillus subtilis* and *Pseudomonas aeruginosa*.

Screening for Biosurfactant Activity: Biosurfactant activity of isolated bacteria was detected by using oil spreading technique and emulsification stability test in four different oils namely vegetable oil, Kerosene, petrol, diesel.

Oil Spreading Technique: The 50 ml of distilled water was added to a large Petri dish (15 cm diameter) followed by the addition of 20µl of oil to the surface of water, 10µl of supernatant of culture broth [8].

Emulsification Stability (E24) Test: E24 of culture samples was determined by adding 2 ml of oil to the same amount of culture, mixing with a vortex for 2 min and leaving to stand for 24 hours. The E24 index is given as percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm) [9].

Optimization of Growth: Bacterial growth was optimized using different parameters like pH, temperature, potassium di-hydrogen phosphate and Magnesium sulphate.

Biosurfactant Production: Bacteria were inoculated on the mineral salt agar medium [10]. Colonies grown on this media were inoculated in mineral broth which contain 2% of oil (vegetable oil, kerosene, petrol, diesel) and it was incubated in an in an optimized condition for 24 to 48 hours in a shaker operating at 120 rpm / min. biosurfactants was isolated using acid precipitation method at pH 2 using HCl [11].

Analytical Method

Thin Layer Chromatography: Preliminary characterization of the biosurfactant was done by TLC method. A portion of the crude biosurfactant was separated on a silica gel plate using $CHCl_3$:CH_3OH:H_2O (70:10:0.5, v/v/v) as developing solvent system with different color developing reagents. Ninhydrin reagent (0.5 g ninhydrin in 100 mL anhydrous acetone) was used to detect lipopeptide biosurfactant as red spots and anthrone reagent (1 g anthrone in 5 mL sulfuric acid mixed with 95 mL ethanol)-to detect glycolipid biosurfactant as yellow spots [12].

RESULT AND DISCUSSION

Isolation and Identification of *Bacillus Subtilis* and *Pseudomonas Aeruginosa*: *Bacillus subtilis and Pseudomonas aeruginosa* were isolated from four different oil contaminated soil. *Bacillus subtilis* is a gram positive, motile, endospore forming bacteria and it hydrolyses starch, casein and gelatin (Table1). *Pseudomonas aeruginosa* is a gram negative, rod shaped, motile bacteria and its biochemical characters were shown in Table 2.

Screening for Biosurfactant Activity

Oil Spreading Technique: In oil spreading technique, all four isolates of *Bacillus subtilis* (BS1, BS2, BS3 and BS4) *and Pseudomonas aeruginosa* (PS1, PS2, PS3 and PS4) produces biosurfactants activity in vegetable oil, kerosene, petrol and diesel. Among four *Bacillus subtilis* isolates BS3 produces the higher zone formation of 6mm, 17 mm, 16mm and 20 mm in vegetable oil, kerosene, petrol and diesel respectively. Similarly PS3 produces higher zone formation of 8mm, 15mm, 18mm and 22mm in vegetable oil, kerosene, petrol and diesel respectively. Both *BS3* and PS3 shows higher biosurfactant activity in diesel and PS3 have more activity than BS3 (Table 3).

Table 1: Characteristics of *Bacillus subtilis*

S. No	Test	Result
1	Gram Staining	Grampositive rod
2	Endospore staining	Positive
3	Starch Hydrolysis	Positive
4	Casein Hydrolysis	Positive
5	Gelatin Hydrolysis	positive

Table 2: Characteristics of Pseudomonas aeruginosa

S. No	Test	Result
1	Gram staining	Negative
2	Indole	Negative
3	Methyl red	Negative
4	Voges proskauer	Negative
5	Citrate	Positive
6	TSI	K/K
7	Glucose	Positive
8	Maltose	Positive
9	Lactose	Negative
10	Sucrose	Negative

Table 3: Oil Spreading Technique for *Bacillus subtils* (BS) and *Pseudomonas aeruginosa* (PS)

	E24 Value (%)							
Sample	Vegetable oil		Kerosene		Petrol		Diesel	
	BS	PS	BS	PS	BS	PS	BS	PS
1	34	40	43	42	45	48	50	56
2	32	41	43	45	48	52	51	59
3	45	48	52	55	55	58	60	68
4	40	42	49	43	50	50	53	56

Table 4: Emulsification stability test for *Bacillus subtils* (BS) and *Pseudomonas aeruginosa* (PS)

	Zone Formation (mm)							
Sample	Vegetableoil		Kerosene		Petrol		Diesel	
	BS	PS	BS	PS	BS	PS	BS	PS
1	5	4	12	11	14	15	15	17
2	4	5	11	13	13	16	16	19
3	6	8	17	15	16	18	20	22
4	5	4	12	14	14	17	17	18

Table 5: Analysis of biosurfactants using Thin Layer Chromatography

	Rf Value	
Carbon source	BS	PS
Vegetable Oil	0.48	0.64
Kerosene	0.51	0.63
Petrol	0.52	0.68
Diesel	0.58	0.72

BS-Bacillus subtilis

PS-Pseudomonas aeruginosa

Emulsification Stability (E24) Test: All the four isolates of *Bacillus subtilis* and *Pseudomonas aeruginosa* have the ability of emulsifying oils. The highest E24 value was observed in BS3 are 45, 52, 55 and 60 mm in vegetable oil, kerosene, petrol and diesel respectively. Similarly the highest E24 value was observed in PS3 are 48, 55, 58 and 68 in vegetable oil, kerosene, petrol and diesel respectively. Among the four oil the higher E24 value was observed in diesel and PS3 shows the better E24 value than the BS3 (Table 4).

As BS3 and PS3 shown higher surfactant activity, they have taken for Biosurfactant production using four different oils (Table 5).

Process Optimization for Biosurfactant Production

Optimization of pH: The pH ranges from 6, 6.5, 7, 7.5, 8 and 8.5 were used for the optimization of pH for the growth of BS3 and PS3. Both the organism have higher growth rate at pH 7 and PS3 shows more growth than BS3 (Fig. 1).

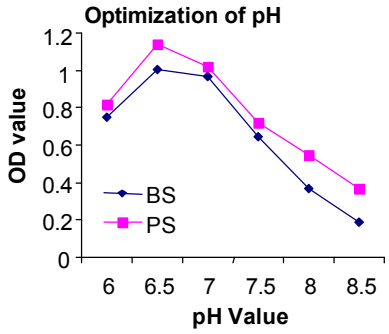

Fig. 1: BS-*Bacillus subtilis*
PS-*Pseudomonas aeruginosa*

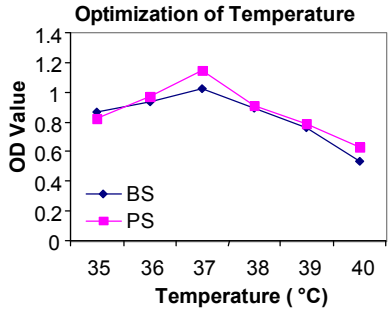

Fig. 2: BS-*Bacillus subtilis*
PS-*Pseudomonas aeruginosa*

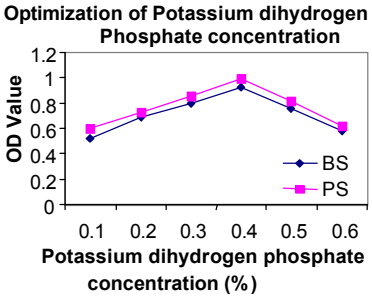

Fig 3: BS-*Bacillus subtilis*
PS-*Pseudomonas aeruginosa*

Optimization of Temperature: The temperature ranges from 35°C to 40°C for the optimization. At 37°C BS3 and PS3 shows higher growth rate and the PS3 shows better growth rate than BS3 at 37°C (Fig. 2).

Optimization of Potassium Di-hydrogen Phosphate: Potassium di-hydrogen phosphate is used in the concentration of 0.15% to 0.6% is used for optimization. The maximum growth was observed at 0.4% concentration in both BS3 and PS3. PS3 shows better growth than BS3 (Fig. 3).

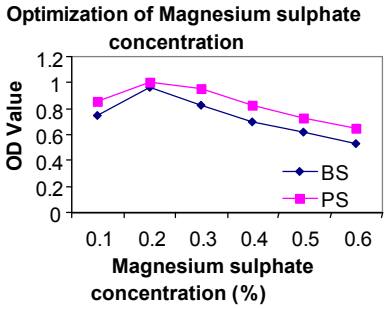

Fig. 4: BS-*Bacillus subtilis*
PS-*Pseudomonas aeruginosa*

Optimization of Magnesium SulphateL: The Magnesium sulphate concentration ranges from 0.1% to 0.6% is used for optimization. In both organisms better growth was observed at 0.2% and the best result shown in PS3 than BS3 (Fig. 4).

Analysis of Biosurfactant: Biosurfactants isolated by acid precipitation method at pH2 was identified by TLC method. Surfactin from *Bacillus subtilis* was identified by red color spot where as rhamnolipid from *Pseudomonas aeruginosa* was identified by yellow color spot. The R_f value of surfactin are 0.48, 0.51, 0.52, 0.58 in oil, kerosene, petrol and diesel respectively and R_f value of rhamnolipid are 0.64, 0.63, 0.68, 0.72 oil, kerosene, petrol and diesel respectively.

These results conclude both the organisms' uses diesel as the best carbon source for biosurfactant production. Comparatively *Pseudomonas* aeruginosa have higher biosurfactant activity than the *Bacillus subtilis*.

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