International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2015): 78.96 | Impact Factor (2015): 6.391

Isolation, Screening and Production of Biosurfactant by *PSEUDOMONAS AEROGINOSA* SD4 Using Various Hydrocarbon Sources

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Abstract: ***Rising environmental concerns lead to emergence of biosurfactants as a potential alternative to the synthetic surfactants. In this study a total of 65 strains isolated from petrol bunk soil were screened using oil spreading method. Among the isolates four strains TD3, TD4, SD3 and SD4 showing higher Biosurfactant activity were selected, purified and subcultured on Psuedomonas selective agar plate. Of these four strains selected, isolate SD4 had maximum Biosurfactant activity. The isolate SD4 was identified as Pseudomonas aeroginosa strain by morphological, biochemical and with 16s rRNA analysis. Maximum Biosurfactant production was obtained at pH 7 and at incubation time of 72 hours. On analysis with diesel, petrol and kerosene at 2% (v/v), P. aeroginosa SD4 exhibited maximum Biosurfactant production utilizing diesel incorporated into the production medium with highest E24 value (53-86 %) with olive oil, castor oil, coconut oil and sunflower oil respectively. Further the biosurfactant of 80 mg was extracted per ml of medium with diesel source and the Biosurfactant was characterized as rhamnolipid with phenol-sulphuric test and TLC analysis. Thus, this work emphasis the suitability of Pseudomonas aeruginosa SD4 further to be explored in the area of environmental and industrial application.***

Keywords: Biosurfactant, 16s rRNA, *Pseudomonas aeroginosa* , diesel, rhamnolipid

1. Introduction

Surfactants are amphiphilic molecules with both hydrophilic and hydrophobic regions attributing towards reduce in surface tension by the formation of aggregates at interfaces between fluids of different polarities [1]. Naturally occurring surface-active compounds derived from microorganisms are called bio-surfactants. They are structurally diverse group of surface-active molecules and are made up of chemical structure such as glycolipids, lipopeptides fatty acids, polysaccharides-protein complexes, peptides, phopspholipids and neutral lipids [2]. Many bacteria and yeasts such as *Thiobacillus thiooxidane, Aspergillus spp., Arthobacter, P. aeruginosa etc*. produces large quantities of fatty acid and phospholipids during growth on –n-alkanes. *Pseudomonas* species form the largest group of bacteria producing biosurfactants. Many strains of *Pseudomonas* have been reported to produce glycolipids, especially rhamnolipids [3]. Biosurfactants have extensive environmental applications such as bioremediation and dispersion of oil spills, enhanced oil recovery and transfer of crude oil ([4,5].

For an economical biosurfactant production process, it is important to identify the microorganisms that produce biosurfactant and to optimize the cultivation medium and the fermentation process itself. It is estimated that raw materials account for 10 to 30% of the overall production cost of biosurfactants [6].

To reduce the production cost, different routes could be considered with respect to enhance of yield and product accumulation, the improvement of economical processes and the use of cost-free feed stock for growth of microorganism and Biosurfactant production. Optimization of various parameters is one of the means that could be investigated for maximum production of Biosurfactant. Hence this study intended isolate a robust *Psuedomonas* strain from petrol bunk waste soil with suitable screening methods and optimize the cultural conditions for maximum production of rhamnolipid from selected *Psuedomonas* strains.

2. Materials and Methods

2.1 Isolation of Biosurfactant producing bacteria

Various soil samples were collected from petrol bunk in Thalavaipuram, and Srivilliputhur, Virudhunagar (Dist.), Tamilnadu, India in a sterile container. The samples were spread plated on sterilized nutrient. The plates were incubated at 37° C for 24 hours. Morphology of grown colonies were studied and further screened using *Pseudomonas* selective agar. Selected bacterial strains were maintained on agar slants at 4°C for future study.

2.2 Identification analysis of the bacterial isolates

Microscopic examination and biochemical characterization of the isolates were carried on the basis of characters given in Bergey's manual of Systematic Bacteriology [7]. Species identification of the selected strain was done by using 16S rDNA sequencing analysis (Macrogen, South Korea). Overnight culture (1.5ml) of isolates in nutrient broth was centrifuged at 8000 rpm for 10min at room temperature. The cell pellet was used for extraction of total genomic DNA. For amplification of the 16s rRNA gene, universal primers F27 (5-AGAGTTGATCMTGGCTCAG-3) and R1492 (5-TACGGYTACCTTGTTACGACTT-3) were used. DNA sequence data sets were assembled using the Bioedit sequence alignment editor software, version 7.0. sequence

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similarity values were determined using the basic local alignment search tool (BLAST) of the National Centre of Biotechnology Information (NCBI). The cultures were maintained at -50ºC on Nutrient Broth with 40% glycerol. For each experiment an overnight culture was inoculated in fresh Nutrient broth (NB) and further incubated to ensure exponential growth conditions.

2.3 Screening for Biosurfactant (BS) production

2.3.1 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 (olive oil) to the surface of water, 10µl of supernatant of culture broth [8].

2.3.2 Para Film-M test

One drop of Bromophenol blue indicator was added to 2 ml of cell-free supernatant. 10 µl of this sample was carefully placed like a drop on parafilm-M with a micropipette. The shape of this drop on the surface was inspected after 1min. Sodium lauryl sulfate and phosphate buffer (pH 7.0) were used as positive and negative controls respectively. If the drop becomes flat, it indicates the presence of biosurfactant. If it remains in dome shape, it indicates the absence of Biosurfactant [9].

2.3.3 Emulsification Index (E24).

The cultures of selected isolates were suspended in test tube in mineral salt media (MSM) containing 15g $NaNo_3$ (g/L); 1.1g KCl (g/L); 1.1 NaCl (g/L);0.00028g $FeSO_4.7H_2O$ (g/L); 3.4g KH_2PO_4 (g/L); 4.4g K_2HPO_4 (g/L); 0.5g $MgSo_4$ .7 H_2O (g/L) and 0.5g Yeast extract (pH 7.0±0.2).. Emulsification index was determined by adding 2 ml of oil and 2 ml of supernatant in test tube. The tubes were vortexed for 5 minutes and kept for 24 hours at room temperature. The emulsification index (E24) is as the height (mm), multiplied by 100 [10].

2.4 Optimization of production parameters

Various parameters such as incubation period, initial pH and different carbon sources were optimized using MSM medium for maximum Biosurfactant production in triplicates. Initial pH value of the production medium was adjusted at different levels in the range of pH 5 to 10 using 1N HCl and 1N NaOH. Bacterial growth was measured by incubating the cultures at different time intervals viz. up to 120 hours at 37˚C. The different hydrocarbon source such diesel, petrol and kerosene were used for production of biosurfactant. The OD values were measured at 600nm. Then BS assay was analyzed using oil spreading method and emulsification index was carried by using coconut oil, olive oil, castor oil, and sunflower oil.

2.5 Estimation of Biosurfactants production

2.5.1 Phenol-Sulphuric Acid Method

To 1ml of cell free supernatant, 1ml of 5% (v/v) phenol was added. To this mixture, 2.5 ml of concentrated sulphuric acid was added drop by drop, until characteristic colour was developed. Development of orange colour indicated the presence of glycolipids [9].

2.5.2 Orcinol method

The biosurfactant from the samples were estimated by orcinol assay method. In this method, the orcinol assay was used for the direct assessment of the amount of glycolipids in the sample. The 100 µl of each sample was added with 900 µl of a solution containing 0.19% (v/v) orcinol (in 53% H_2SO_4). After heating for 30 min at 80ºC, the samples were cooled at room temperature and the OD values were measured. Control was prepared with distilled water. The rhamnolipid concentrations were calculated from a standard curve prepared with L-rhamnose and expressed as rhamnose equivalents [10].

2.6 Extraction of the BS

The filtrate was centrifuged at 4°C and 8500 rpm for 20 min in order to remove the microbial cells. The obtained supernatant was treated by acidification to pH 2.0 using a 6M HCl, and the acidified supernatant was left overnight at 4°C for the complete precipitation of the biosurfactants. After centrifugation, the precipitate was dissolved in a 0.1M NaHCO3 solution, followed by the biosurfactant extraction step with a solvent having a 2:1 CHCl3–C2H5OH ratio at room temperature (25–27 °C) The organic phase was transferred to a round bottom flask connected to a rotary evaporator in order to remove the solvent at 40°C [11].

2.7 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 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.

3. Results

3.1 Isolation and Screening of Biosurfactant producing bacteria

A total of 65 isolates were screened using oil spreading method. Of these 56 isolates produced biosurfactant activity in olive oil. Based on zone formation only 8 different colony were selected from both soil samples and labeled as TD3,TD5, TD6,TD8 and SD1, SD2, SD3 , SD4 respectively (Table 1). They were further screened using *Pseudomonas* selective agar. Among 8 isolates, TD3, TD5, SD3 and SD4 showed increased Biosurfactant activity with zone of 35-50 mm. Isolates SD4 showed maximum zone of oil displacement (50 mm).

Table 1 Zone of oil displacement of bacterial soil isolates

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Isolates	*Diameter of oil displacement (mm)*
TD3	35±2.3
TD5	38±2.5
TD6	30±2.1
TD8	30±1.7
SD1	25±1.3
SD2	26±1.1
SD3	42±2.7
SD4	50±3.4

3.2 Characterization of selected isolates

The isolates TD3, TD5, SD3and SD4 with higher activity were selected for biochemical analysis were characterized as being a non- lactose fermenting (NLF), Gram negative, motile rod shape organism. D-glucose was fermented with the production of acid and gas, Indole, Methyl Red and Voges Proskauer negative and Citrate, Catalase and oxidase positive. With these characteristics the isolates were identified as *Pseudomonas* sp. confirmed by biochemical test (Table 2).

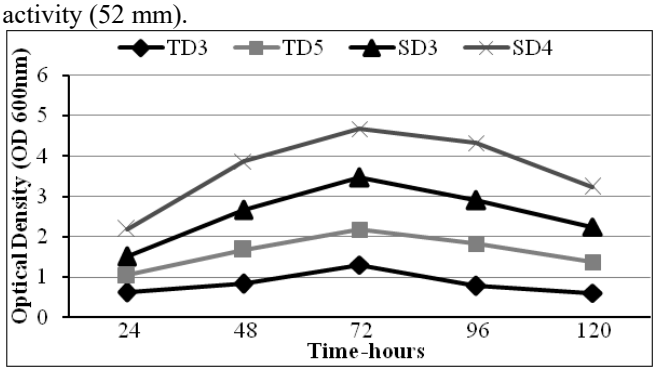
Table 2: Microscopic and Biochemical Characterization of selected Bacterial isolates

Microscopic and Biochemical test	Isolates			
	TD3	TD5	SD3	SD5
Gram staining	-	-	-	-
Indole	-	-	-	-
Methyl red	-	-	-	-
Voges-proskauer	-	-	-	-
Citrate	+	+	+	+
Oxidase	+	+	+	+
Glucose	+	+	+	+
Sucrose	-	-	-	-
Lactose	-	-	-	-

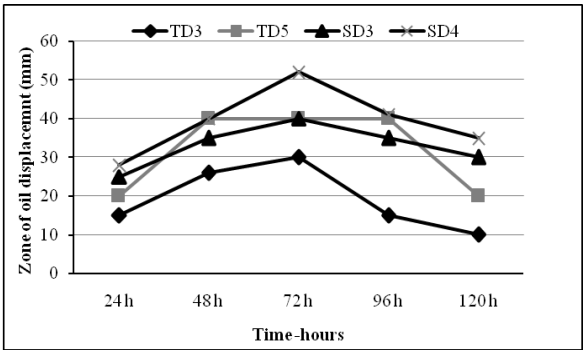
3.3 Optimization of Biosurfactant Production

3.3.1 Effect of incubation period on Biosurfactant Production

The cultures were incubated at different time intervals viz. 24 h, 48 h, 72 h, 96 h and 120 h at 37˚C and analyzed for bacterial growth and biosurfactant activity. In the present investigation, the amount of biosurfactant production and growth rate increase by increasing incubation period up to 72 h for all selected *Pseudomonas* strains. Incubation beyond the optimum time showed rapid decline in growth and Biosurfactant production (Figure 1). Among the selected strains *Pseudomonas* SD4 has maximum BS activity (52 mm).



(a)



(b)

Figure 1: Effect of incubation period on (a) Growth and (b) Biosurfactant Production of *Pseudomonas* sps.

3.3.2 Effect of pH on Biosurfactant Production

The growth of selected strains and Biosurfactant production at various pH was shown in Figure 2. All the selected *Psuedomonas* sp. showed higher growth rate at pH 7 and the reduction in growth was observed at extreme acidic and alkaline. Hence pH 7 was selected as optimum pH for production.

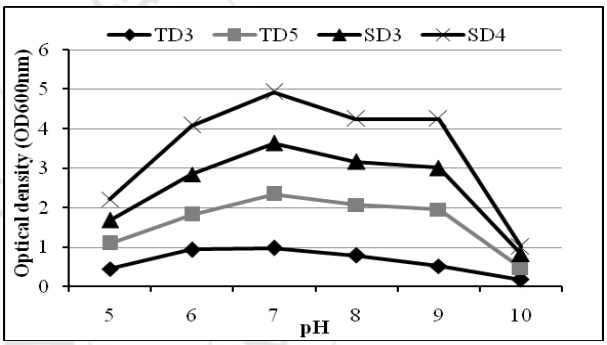


Figure 2: Effect of pH on Biosurfactant Production of *Pseudomonas* sps.

3.3.3 Effect of hydrocarbon on Biosurfactant Production

The carbon sources play a major role in Biosurfactant production. The ability of *P. aeruginosa* to utilize various hydrocarbons was analyzed with diesel, petrol and kerosene at 2% (v/v). Among these hydrocarbon sources tested, *P. aeruginosa* SD4 exhibited maximum BS production while utilizing diesel as source with the highest zone formation (30-65 mm) tested in Olive oil, castor oil, coconut oil, and sunflower oil respectively (Table 3).

Table 3: Zone of oil displacement on various vegetable oils with Biosurfactant produced using different hydrocarbon sources

Hydrocarbon sources	Isolates	Zone of oil displacement (mm)			
		A	B	C	D
Kerosene	TD3	10	20	32	10
	TD5	20	18	43	20
	SD3	20	35	42	20
	SD4	25	40	45	40
Petrol	TD3	10	20	20	18
	TD5	20	30	24	20
	SD3	22	35	27	28
	SD4	26	45	30	35
Diesel	TD3	10	20	30	13
	TD5	10	29	36	18

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	SD3	15	40	42	25
	SD4	30	53	65	46

A- Olive oil, B- Castor oil, C- coconut oil, D- Sunflower oil

And also it was able to utilize kerosene and petrol sources effectively compared with other strains. All the four isolates of *Pseudomonas sp.* have the ability to emulsifying oils. The highest E24 value was observed with diesel as hydrocarbon source by *Pseudomonas sp.* SD4 were 53.6, 76.2, 85.5 and 66.7 with olive oil, castor oil, coconut oil and sunflower oil respectively (Table 4).

Table 4: Emulsification activity (E24%) on various vegetable oils with Biosurfactant produced using different hydrocarbon sources

Hydrocarbon sources	Isolates	A	B	C	D
Kerosene	TD3	29.4	30.5	66.3	30.2
	TD5	33.2	20.2	65.6	34.5
	SD3	33.5	35.8	65.3	33.5
	SD4	38.8	46.6	67.5	44.3
Petrol	TD3	23.3	26.2	46.2	26.3
	TD5	33.7	29.4	49.7	27.5
	SD3	36.4	33.6	53.6	38.8
	SD4	39.2	48.7	56.4	45.4
Diesel	TD3	33.4	33.5	66.5	28.4
	TD5	33.6	38.4	69.1	29.2
	SD3	46.5	46.4	73.5	33.5
	SD4	53.6	76.2	85.5	66.7

A- Olive oil, B- Castor oil, C- coconut oil, D- Sunflower oil

3.4 Extraction of the BS

In the present investigation, the recovery of biosurfactant produced by *Pseudomonas* sp. from complex fermentation broth was carried out. Biosurfactant was precipitated and extracted with a solvent system $CHCl_3$:C_2H_5OH in a ratio 2:1. The organic phase was evaporated in a rotary evaporator to give a white powder. *Pseudomonas* sp. SD4 produced highest biosurfactant of 80 mg was extracted per ml of medium (80.3 mg/ml) in diesel source. While SD3 produced highest biosurfactant of 60.7 mg was extracted per ml of medium with petrol source. The biosurfactant was separated by an simple and reliable method without loss of its activity.

3.5 Characterization of Biosurfactant

The type of biosurfactant was identified by phenol-sulphuric test was performed with cell-free supernatant of *Pseudomonas* sp. The selected isolates had shown orange colour indicated that the biosurfactant obtained was glycolipid in nature. The Thin layer chromatography for *Pseudomonas* sp. SD4 extracts obtained from different hydrocarbon source was performed. In the present search, *Pseudomonas* sp. SD4 had shown yellow spots when sprayed with an anthrone reagent in TLC plate which further indicates the presence of rhamnolipid (Figure 3).

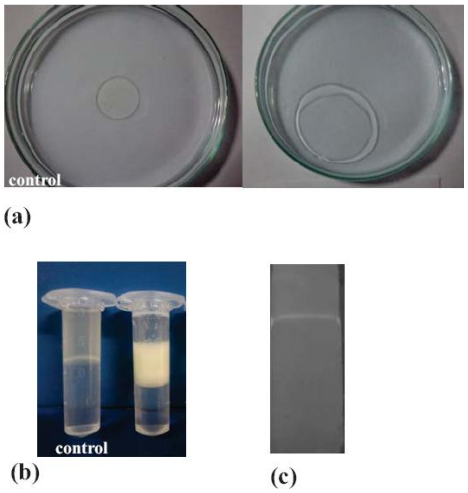

Figure 3: Biosurfactant activity of culture supernatant of *Pseudomonas aeruginosa* SD4 in MSM medium (a) Oil spreading technique (b) Emulsification activity with sterile MSM medium was taken as control and (c) TLC analysis.

3.6 Nucleotide sequence

The 16S rDNA gene of *Pseudomonas* sp. SD4 was sequenced at Macrogen, Korea. The BLAST result of sequenced 16S rRNA gene showed that the isolate exhibit 99% similarity to *Pseudomonas aeruginosa* and the sequence has been deposited in GenBank under the accession no. KX082774.

4. Discussion

Microbes are distinguished for secretion of Biosurfactant a surface active agent which were initially proposed to function as emulsifiers of biodegradable hydrocarbons. The main functions of bio-surfactant in microbial cells are emulsification of water insoluble substrates such as hydrocarbons and facilitate its transport into the cell to stimulate the growth [12]. Oil contaminated environment contain large amount of hydrocarbons. As bacteria uses wide range of hydrocarbon waste, there is an extensive interest in the search for biosurfactant producing novel bacterial species. In this aspect work was carried out for isolating, screening and identification of efficient biosurfactant producing bacteria from natural environment. Habitats that are enriched with petrochemical waste are the best sources in which we can isolate Biosurfactant producing bacteria.

Similarly, out of 20 isolates screened, only *Pseudomonas aeruginosa* UKMP14T was isolated from the petroleum contaminated soil showed positive result by the oil-spreading technique [13]. *Pseudomonas aeruginosa and Saccharomyces cerevisiae* were isolated from the petroleum contaminated soil samples and analyzed by oil spreading method [14]. *Pseudomonas fluorescens* showed oil displacement test positive for mustard oil [15]. Again these four isolates were tested for biosurfactant production by parafilm-M test, where flat drops were shown by TD3, TD5, SD3 and SD4 taking sodium laryl sulphate as positive control and negative as negative control. Three isolates were tested for biosurfactant production by parafilm-M test,

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where a flat drop was shown by NDYS-4 [9]. Further the results suggested that the oil-spreading technique and para Film-M test as more sensitive than the other methods for biosurfactant detection in the supernatant from a culture medium. However, the maximum Biosurfactant production by *P. aeruginosa* F32 was observed during its stationary phase at 96 h [16]. The maximum biosurfactant production by *Klebsiella pneumonia* IVN51 was recorded in 48-120 h [15]. Hence pH 7 was selected as optimum pH for production. *P. aeruginosa* UKMP14T produced biosurfactant at a wide range of pH from 6.5 to 9.0 [13]. Medium pH was tested from 5.0 to 8.0 and highest biosurfactant production observed by the isolate *P. aeruginosa* PBSC1 was 5.13 g/l at pH 7 [17]. However, medium pH was tested from 5.0 to 11.0 and optimum pH was found as 10 for *P. aeruginosa* [18]. The result revealed that strains of *Pseudomonas* can grow and produced Biosurfactant at wide pH range. Similarly, the increased emulsifying properties of *Bacillus subtitilis and P. aeroginosa* was observed with diesel oil as carbon source [8]. Hydrocarbon added to the fermentation medium seems to induce Biosurfactant production. In *P. aeruginosa* PBSC1 the crude motor oil enhanced the Biosurfactant production (4.99 g/l) [18]. *Pseudomonas* sp. (L15) produces 73.5 mg/ml glycolipid biosurfactant whereas *Bacillus spp*. L4 produces 7.1 mg/ml glycolipid [19]. *Pseudomonas aeruginosa* strain SP4 exposed orange colour that shown the presence of glycolipid biosurfactant [20]. NDYS-4 isolate had shown orange colour when phenol and sulphuric acid were added. The orange colour specified that the biosurfactant obtained was glycolipid in nature [14]. Surfactin from *Bacillus subtilis* was identified by red color spot where as rhamnolipid from *Pseudomonas aeruginosa* was identified by yellow color spot [8, 22].

5. Conclusion

Many microorganisms had been explored that were capable of producing Biosurfactant. But need for newly isolated Biosurfactant producing strain was still remained a focus of research. In this study robust Biosurfactant producing bacteria was isolated from oil spilled soil. The bacteria characterized based on 16 rDNA analysis and was identified as *Pseudomonas aeroginosa*. *Pseudomonas aeroginosa* SD4 showed potential utilization of petrol, diesel, kerosene and other vegetable oils as substrate and its production efficiency was increased by optimization of culture conditions. Hence the research conducted in our laboratory has revealed that *Pseudomonas aeroginosa* SD4 that uses both hydrocarbon and vegetable oil effectively can be a potential candidate for bioremediation and oil recovery. Further it can be also used as Bioemulsifier in food biopreservation.

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