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**ORIGINAL RESEARCH ARTICLE**

# Isolation and Screening of Biosurfactant Producing Microorganisms from Hydrocarbon Contaminated Soils from Automobile Workshop

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## ABSTRACT

Biosurfactants are amphiphilic compounds produced on living surfaces, mostly on microbial cell surfaces. These molecules reduce surface tension between aqueous solutions and hydrocarbon mixtures. They are biodegradable non- toxic and eco-friendly materials. In the present study, isolation and screening of microorganisms from oil contaminated soil samples from four different automobile workshops for their biosurfactant producing ability was investigated. The biosurfactant producing ability of the microorganisms were investigated by Oil spreading technique, Haemolytic activity, Drop collapse test, Emulsification Index and Methylene Blue Agar plate method. The best isolate was identified as *Pseudomonas aeruginosa* based on standard microbiological techniques. The bacterial isolates were grown in Mineral Salt Medium (MSM) with addition of 1% (V/V) oil as carbon source. The biosurfactants produced by the isolate was extracted using chloroform: methanol extraction method. The crude biosurfactant was further characterized by Thin layer chromatography (TLC) and Fourier Transform Infrared Spectroscopy (FT-IR). The studies confirmed the presence of rhamnose sugar moiety in the glycolipid chain. Hence, the biosurfactant confirmed as Rhamnolipid. The large scale production of Rhamnolipid was studied using useful industrial waste samples.

**Key words:** Biosurfactants, *Pseudomonas aeruginosa*, Soil samples, Rhamnolipid.

## 1. INTRODUCTION

Naturally occurring surface - active compounds derived from microorganisms are called biosurfactants. The biosurfactants are complex molecules covering a wide range of chemical types including peptides, fatty acids, phospholipids, glycolipids, antibiotics, lipopeptides, etc., 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 [2].

Biosurfactant producing microorganisms were naturally present in the oil contaminated soil and aquatic environment. Oil contamination is results from oil refineries and oil spill. 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.

The most prevalent bacterial hydrocarbon degraders and surfactant producers, belong to the genera are *Pseudomonas, Achromobacter, Flavobacterium, Micrococcus, Bacillus, Arthrobacter, Klebsiella, Acinetobacter, Aeromonas, Alkaligenes, Streptococcus* sp., *Corynebacterium* sp., *Moraxella* sp., and *Proteobacteria* [4]. Among the different classes of biosurfactants rhamnolipid and surfactin are best studied. Rhamnolipid is mostly produced by *Pseudomonas aeruginosa* [5].

Biosurfactants are structurally diverse group of surface active molecules. They are categorized mainly by their chemical composition and microbial origin. Biosurfactants can be divided into low-molecular-mass molecules, which

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efficiently lower surface and interfacial tension and high molecular mass polymers, which are more effective as emulsun-stabilizing agents. The major classes of low mass surfactants include glycolipids, lipopeptides and phospholipids high mass surfactants include polymeric and particulate surfactants [7]. Most biosurfactants are either anionic or neutral and the hydrophobic moiety is based on long chain fatty acids or fatty acid derivatives, whereas hydrophilic portion can be carbohydrate, amino acid, phosphate or cyclic peptide [8].

Among the different classes of biosurfactant, 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 [2]. Rhamolipid is produced by *Pseudomonas aeruginosa*, a Gram-negative, motile, non-spore forming bacteria.

The rhamnolipids were produced when hydrocarbons such as glycerol, glucose, or peptone was the substrate. Best production was obtained with hydrocarbons or glycerol [9]. On the other hand *Pseudomonas* sp. can use the various renewal resources, especially agroindustrial wastes, as the potential carbon sources. This leads to the greater possibility for economical productions and reduced pollution caused by those wastes [3]. One of the important points in the biotechnologically process is to obtained maximum metabolite production with a low cost substrate [10]. The rhamnolipids produced by *Pseudomonas* species from different carbon sources have been extensively studied.

As alternative surfactants, biosurfactants have outstanding advantages, such as high bio-degradability, low toxicity, environmental compatibility, high selectivity, and specific activity at extreme temperatures, pH, and salinity, among others. Biosurfactants have been shown to have a variety of applications, including enhancing crude oil recovery from oil reservoirs, mobilizing heavy crude oil transport in pipelines, and cleaning oil sludge from oil storage facilities. They are also used in soil/sand bioremediation, remediation of organics and metals, and as emulsifiers in agriculture and medicine in biological control [1, 11]. In the present study, biosurfactant producing organisms *Pseudomonas aeruginosa* was isolated from oil contaminated soil samples from Mayiladuturai, Nagappatinum District, Tamil Nadu, India. The preliminary screening test such as hemolytic assay, oil spreading techniques, drop collapse test, emulsification index and methylene blue agar plate method was carried out for biosurfactant activity. The characterization of biosurfactant was confirmed by TLC and FT-IR. The biosurfactant was identified as rhamnolipid.

## 2. MATERIALS AND METHODS

### 2.1 Sampling

Soil samples were collected from oil spilled surfaces of four different automobile workshops in Mayiladuthurai, Nagappatinum District, Tamil Nadu, India. The samples were collected in sterile polythene bags and were taken to the laboratory for analysis.

### 2.2 Isolation and enumeration of bacterial isolates from the sample

Five g of the oil spilled soil samples were inoculated in 50 ml of Nutrient broth and incubated at 25˚C for 72 hrs. After incubation the medium was serially diluted from $10^{-1}$ to $10^{-6}$ in sterile water. From the dilutions ($10^{-1}$ to $10^{-6}$), 1 ml was transferred to sterile Petri dish and over that 20 ml of Nutrient agar was poured. The plates were then inverted and incubated at 25˚C for 48 hrs. Control and replica plates were maintained.

### 2.3 Bacteriological isolation techniques

After incubation, the different discrete colonies formed on the plate that had between 30 and 300 colony forming unit (CFU) were streaked on nutrient agar slant and incubated at ambient temperature (37˚C) for 24 hrs to obtain their pure cultures. These pure cultures, were sub-cultured on nutrient agar slant, incubated at 37˚C for 24 hrs and stored at 4˚C for bio -surfactants production screening.

### 2.4 Screening for Biosurfactant activity

Cultures were inoculated in the Mineral salt medium and it was incubated for 48 hrs. After incubation period, cultures were centrifuged and the supernatants were collected for screening of biosurfactant activity.

#### 2.4.1 Oil spreading technique

Each of the bacterial species was screened for biosurfactants production using the oil spreading techniques [12]. The procedure is as follows: the bacterial isolates were streaked on nutrient agar

slant and incubated for 24 hrs at 37. After 24 hrs, two loops of culture were inoculated in 50 ml of nutrient broth in a 50 ml Erlenmeyer flask and incubated at 37°C for 48 hrs. A fter the inoculum development, 50 ml of distilled water was added to a large Petri dish (25 cm in diameter) followed by the addition of 20 µl of crude oil to the surface of the distilled water and 20 µl of the supernatant of the cultures isolated from the soil.

### 2.4.2 Emulsification index ($E_{24}$ %)

Emulsification index ($E_{24}$) of culture samples was determined by adding 2 ml of oil to the same amount of culture, mixing with a vortex for a 2 min and leaving to stand for 24 hrs. The $E_{24}$ index is given as percentage of height of emulsified layer (cm) divided by total height of the liquid column (cm) [6].

$$E_{24} = \frac{\text{Height of emulsion formed}}{\text{Total height of the solution}} \times 100$$

### 2.4.3 Drop collapse method

Screening of biosurfactant production was performed using the qualitative drop - collapse test described by [13]. Crude oil was used in this test. Two microlitres of oil was applied to the well regions delimited on the covers of 96 - well micro titer plates and these were left to equilibrate for 24 hrs. Five micro liters of the 48 hrs culture, before and after centrifugation at 12,000 g for 5 min to remove cells, was transferred to the oil-coated well regions and drop size was observed after 1 min with the aid of a magnifying glass. The result was considered positive for biosurfactant production when the drop was flat and those cultures that gave rounded drops were scored as negative, indicative of the lack of biosurfactant production [14].

### 2.4.4 Haemolytic activity

The fresh single colonies from the isolated cultures were taken and streaked on Blood agar plates. The plates were incubated for 48-72 hrs at 37ºC. The bacterial colonies were then observed for the presence of clear zone around the colonies. This clear zone indicates the presence of biosurfactant producing organisms. Results were recorded based on the type of clear zone observed i.e. α-Haemolysis when the colony was surrounded by greenish zone, β- Haemolysis when the colony was surrounded by a clear white zone and γ- Haemolysis when there was no change in the medium surrounding the colony [15].

### 2.4.5 Blue agar plate (BAP) method

Mineral salt agar medium supplemented with glucose as carbon source (2%) and Cetyl Trimethyl Ammonium Bromide (CTAB: 0.5 mg/mL) and methylene blue (MB: 0.2 mg/mL) were used for the detection of anionic biosurfactant [22]. Thirty microlitre of cell free supernatant was loaded into the each well prepared in methylene blue agar plate using cork borer (4 mm). The plate was then incubated at 37°C for 48 - 72 hrs. A dark blue halo zone around the culture was considered positive for anionic biosurfactant production.

### 2.5 Biosurfactant production and extraction

Isolates were grown in 500 ml Erlenmeyer flasks containing 100 ml mineral salt medium adjusted to $P^H$ 7.0 was used as culture medium. The flasks were incubated at 37°C on a shaker incubator for 7 days. To extract the biosurfactant, the bacterial cells were removed by centrifugation and the remaining supernatant liquid was filtered through a 0.22mm pore size filter (milipore).

The cell free supernatant was acidified by using 6N HCl and the pH was adjusted to 2 and keeping it at 4°C overnight. The precipitate thus obtained was pelleted by centrifugation for 20min, dried and weighted.

For further purification the crude surfactant was dissolved in distilled water at $P^H$ 7.0 and dried at 60°C. The dry product was extracted with Chloroform: Methanol (65:15) filtered and the solvent evaporated. The composition of the basal mineral salt medium used in this study was the following Mineral Salt medium (g/l): Glucose, 40; $NH_4HPO_2$, 0.39; $Na_2HPO_4$, 5.67; $KH_2PO_4$, 4.08; $FeSO_4.7H_2O$, 0.015; $MnSO_4.H_2O$, 0.002; $MgSO_4.7H_2O$, 0.197; $CaCl_2.2H_2O$, 0.001; Distilled water, 1000 ml; pH, 7.

### 2.6 Dry weight of biosurfactants

Sterile petriplate was taken and the weight of the plate was measured. The biosurfactant extracted were poured on to the plates and then the plates were placed in the Hot air oven for drying at 100ºC for 30min. After drying the plates were weighted. The dry weight of the biosurfactants was calculated by the following formula:

**Dry weight of biosurfactant**
**= (Weight of the plate after drying**
**– Weight of the empty plate)**

### 2.7 Characterization of biosurfactants

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. Anthrone reagent (1 g anthrone in 5 mL sulfuric acid mixed with 95 mL ethanol) was sprayed to detect presence of sugar as yellow spots [23].

### 2.8 Determination of Carbohydrate

Carbohydrate was estimated by Anthrone test. To the crude surfactant, anthrone (2 g anthrone in 1L $H_2SO_4$) reagent was added and the formation bluish green colored complex indicates the presence of Carbohydrate. Total carbohydrate was drawn using glucose as substrate.

### 2.9 Determination of lipid

#### 2.9.1 Solubility and Saponification test

Small amount of crude biosurfactant was taken in three test tubes. In these tubes, water, alcohol and chloroform were added and their solubility was tested. Lipids are insoluble in water and soluble in alcohol and chloroform.

Two ml of 2% NaOH solution was added to the small amount of biosurfactant and shaken well. Formation of soap indicates the presence of lipid [29].

### 2.10 Characterization of biosurfactant: (FT-IR)

Fourier transform infrared spectroscopy (FT-IR) is most useful for identifying types of chemical bonds (functional groups), therefore can be used to elucidate some components of an unknown mixture. One milligram of freeze-dried partially purified biosurfactant was ground with 100 mg of KBr and pressed with 7500 kg for 30 seconds to obtain translucent pellets. Infrared absorption spectra were recorded on an AVATAR-NICOLAT FT-IR system with a spectral resolution and wave number accuracy of 4 and 0.01$cm^{-1}$, respectively. All measurements consisted of 500 scans, and a KBr pellet was used as background reference [24].

## 3. RESULTS

In the present study, thirty Bacterial isolates were isolated from four different automobile workshop oil contaminated soil samples by standard plate and dilution techniques. Four soil samples designated as $S_1$, $S_2$, $S_3$, S4 (**Table 1**). The $S_3$ soil samples showed highest bacterial count so that soil sample was selected for further study (**Table 2**). Screening for biosurfactant producing potential strain was done by using Oil spreading technique, Haemolytic test, Blue agar test, Drop collapsing and Emulsification index. These strains were designated as PF5G, BS4W, PF7G, MC7Y, SB10O, FB3V, PA2G, RC8O, AB9W, NB11W, MB12W, SC15Y, BS14W, RD16W, MC13Y, PB3A, PB4E PB6B, PB13A, PB18D, PB23A, GG8C, GD11C, GC18A, GG21B, MS1A, RC4S MC7B, PF9A, MW5G (**Table 3**).

### 3.1 Screening for Biosurfactant production

Thirty different isolates were tested for the ability to show the clear zone by displacing the oil around the colony indicating the biosurfactant production. The strains such as PF5G, BS4W, F7G, MC7Y, PA2G, RC8O, AB9W, BS14W, PB3A, PB6B, PB13A, PB18D, PB23A, GG8C GD11C, GC18A, GG21B, PF9A and MW5G showed clear zone. The strain namely, SB10O, FB3V, NB11W, MB12W, SC15Y, RD16W, MC13Y, PB4E, MS1A, RC4S and MC7B showed no clear zone when compared with control (**Fig 1**).

The haemolytic activity was observed in the strain and the result showed clear zone around the colonies. The isolates namely PF5G, BS4W, PF7G, MC7Y, SB10O, FB3V, PA2G SC15Y, BS14W, PB3A, PB4E, PB6B, PB13A, PB18D, PB23A, GG8C, GD11C GC18A, GG21B, RC4S, PF9A showed clear zone. Whereas, PF5G, RC8O AB9W, NB11W, MB12W, RD16W, MC13Y, MS1A, MC7B and PF9A strains showed no clear zone around the colonies (**Fig 2**).

The flat drop appearance in the slide confirmed the positive result for drop collapse test and the result revealed that the strains PF5G, PF7G, PA2G, PB3A, PB4E, PB6B, PB13A, PB18D, PB23A, GG8C, GD11C, GC18A, and GG21B showed positive results. The negative result for drop collapse test as shown by BS4W, MC7Y, SB10O, FB3V, RC8O, AB9W, NB11W, MB12W, SC15Y, BS14W, RD16W, MC13Y, MS1A, RC4S, MC7B, PF9A and MW5G respectively (**Fig 3**).

The dark blue halos were observed on the methylene blue agar plate indicates the positive activity of biosurfactant production and this was positive for the following isolates recorded by PF5G, PF7G, PA2G, RC8O PB3A, PB6B, PB13A, PB23A, GD11C, GC18A and GG21B he negative result for Methylene blue agar test was recorded with the BS4W, MC7Y,SB10O, FB3V, AB9W, NB11W, MB12W, SC15Y, BS14W, RD16W, MC13Y, PB4E, PB18D, GG8C, MS1A, RC4S, MC7B, PF9A and MW5G isolates (**Table 3 & Fig 4**).

Emulsification Index ($E_{24}$) determined the productivity of bioemulsifier. Out of thirty, fifteen

isolates (PF5G, BS4W, PF7G, MC7Y, PA2G, BS14W, PB3A, PB4E, PB6B, PB13A, PB18D, B23A, GD11C, GC18A, WGG21B)showed above 40% 0f emulisificaton index $E_{24}$%.All the other isolates showed below 30%.(SB10O,FB3V RC8O, AB9W, NB11W, MB12W, SC15Y, BS14W, RD16W, MC13Y, , MS1A, RC4S, MC7B, PF9A, MW5G) Diesel and olive oil were the best substrates and Refinery oil was least for emulsification activity (**Table 5**).

Among the thirty isolates obtained from oil contaminated soil samples, PA2G was selected as potent strain based on the screening activity and further used for production and characterization. The isolate PA2G was identified as *Pseudomonas aeruginosa* based on microscopic and biochemical analysis according to Bergey's manual of determinative bacteriology (**Table 4**).

The isolates were further tested for maximum biosurfactant production by inoculating into the MSM medium with different substrates. PA2G showed maximum biosurfactant producing ability (**Table 3**).

**Table 1: History of sites and characteristics of soils used for isolation of hydrocarbon-utilizing bacteria**

| Samples | Soil characteristics and history |
|---|---|
| $S_1$ | Murugan Lorry Service, Kaveri nagar, contaminated for over 25 years. Mayiladuthurai |
| $S_2$ | Guruchandra Car Mechanic Workshop, Koranad, contaminated for over 20 years. Mayiladuthurai |
| $S_3$ | Bala Valli Automobile Workshop, Koranad, contaminated for over 20 years. Mayiladuthurai. |
| $S_4$ | R.M.S Maruthi automobile workshop mahadhana street, contaminated for over 10 years. |

**Table 2: Enumeration of isolated bacteria from oil contaminated soils by serial dilution technique**

| Soil Sample | Number of colonies | | | |
|---|---|---|---|---|
| | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ |
| $S_1$ | 326 | 112 | 65 | 19 |
| $S_2$ | 298 | 121 | 46 | 12 |
| $S_3$ | 361 | 100 | 57 | 11 |
| $S_4$ | 306 | 132 | 59 | 18 |

**Table 3: Screening result 30 isolates from oil contaminated soil samples**

| Test isolated | Hemolytic Assay | Methylene blue Agar plate | Drop Collapsing test | Oil spreading | Emulsification Index (%) |
|---|---|---|---|---|---|
| PF5G | + | + | + | + | 45.6 |
| BS4W | + | - | - | + | 52.2 |
| PF7G | + | + | + | + | 62.4 |
| MC7Y | + | - | - | + | 56.3 |
| SB10O | + | - | - | - | 23.2 |
| FB3V | + | - | - | - | 28.3 |
| PA2G | + | + | + | + | 65.3 |
| RC8O | - | + | - | + | 15.6 |
| AB9W | - | - | - | + | 22.3 |
| NB11W | - | - | - | - | 23.5 |
| MB12W | - | - | - | - | 30.3 |
| SC15Y | + | - | - | - | 25.3 |
| BS14W | + | - | - | + | 59.2 |
| RD16W | - | - | - | - | 24.3 |
| MC13Y | - | - | - | - | 26.3 |
| PB3A | + | + | + | + | 62.5 |
| PB4E | + | - | - | - | 51.2 |
| PB6B | + | + | + | + | 45.3 |
| PB13A | + | + | + | + | 48.8 |
| PB18D | + | - | - | + | 42.0 |
| PB23A | + | + | + | + | 58.4 |
| GG8C | + | - | - | + | 37.5 |
| GD11C | + | + | + | + | 40.8 |
| GC18A | + | + | + | + | 52.5 |
| GG21B | + | + | + | + | 47.2 |
| MS1A | - | - | - | - | 23.7 |
| RC4S | + | - | - | - | 26.2 |
| MC7B | - | - | - | - | 20.2 |
| PF9A | + | - | - | + | 38.9 |
| MW5G | - | - | - | + | 25.9 |

**Table 4: Characteristics and Biochemical test of *Pseudomonas aeruginosa* (PA2G)**

| Characters | Results |
|---|---|
| Shape | Rod |
| Gram staining | Gram negative |
| Endospore staining | Negative |
| Motility | Motile |

| | |
|---|---|
| Cultural morphology | Circular, entire, raised, smooth, florescent green color colonies |
| Indole production | + |
| Methyl red | - |
| Voges proskaeur | + |
| Citrate utilization | + |
| H2s production | - |
| Glucose fermentation | + |
| Lactose fermentation | - |
| Sucrose fermentation | - |
| Coagulase | - |
| Catalase | + |
| Oxidase | + |
| Urease | + |
| Esculin hydrolysis | - |
| Nitrate reduction | + |

**Table 5: Emulsification index test for different hydrocarbon sources**

| Different Hydrocarbon | Emulsification Index (%) PA2G |
|---|---|
| Petrol | 52 |
| Diesel | 68 |
| Crude oil | 48 |
| Olive oil | 60 |
| Refinery oil | 45 |

**Table 6: Determination of Carbohydrate in Biosurfacatant produced from *Pseudomonas aeruginosa* by Anthrone method**

| Carbon source | Carbohydrate concentration (%) |
|---|---|
| Petrol | 47 |
| Diesel | 51 |
| Crude oil | 42 |
| Olive oil | 46 |
| Refinery oil | 40 |

**Table 7: Determination of Lipid in Biosurfacatant produced from *Pseudomonas aeruginosa* (PA2G) by Solubility test and Saponification Test**

| Carbon source | Solubility test | | | Saponification test |
|---|---|---|---|---|
| | Wa | Alcoho | Chloroform | |
| Petrol | - | + | + | + |
| Diesel | - | + | + | + |
| Crude oil | - | + | + | + |
| Olive oil | - | + | + | + |
| Refinery oil | - | + | + | + |

**Table 8: Dry weight of biosurfactant TEIGHT**

| Organism | Plate weight (g) | After drying of biosurfactants in the plate (g) | Dry weight of biosurfactant (g) |
|---|---|---|---|
| *Pseuomonas aeruginosa* | 47.121 | 47.243 | 0.120 |

**Table 10: Characterization of biosurfacant using FT-IR**

| | |
|---|---|
| 539.79 | C-Br |
| 722.12 | -CH=CH-(CiS) |
| 829.49 | Aromatic C-H Bending |
| 922.68 | $CH=CH_2$ |
| 1072.59 | C-O Stretch |
| 1098.93 | C-N Stretch aliphatic amines |
| 1234.67 | C-N Stretch aliphatic amines |
| 1382.55 | $CH_3$ |
| 1400.79 | C-H bending |
| 1459.54 | $CH_2$ |
| 1530.44 | Aromatic C=C stretch |
| 1544.63 | Aromatic ring stretch |
| 1637.82 | C=O Stretch |
| 1654.02 | Carboxylic acid |
| 2853.35 | $CH_2$ |
| 2922.23 | $CH_2$ |
| 2954.64 | C- $CH_3$ |
| 3207.88 | =C-H |
| 3396.29 | Hydrogen –bonded O-H stretches |

**Table 11: Analysis surfactants using Thin Layer Chromatography**

| | Rf Value |
|---|---|
| Carbon source | *Pseudomonas aeruginosa* |
| Petrol | 0.65 |
| Diesel | 0.72 |
| Curde oil | 0.64 |
| Olive oil | 0.68 |
| Refinery oil | 0.62 |

**Fig: 1 Oil spreading techniques**

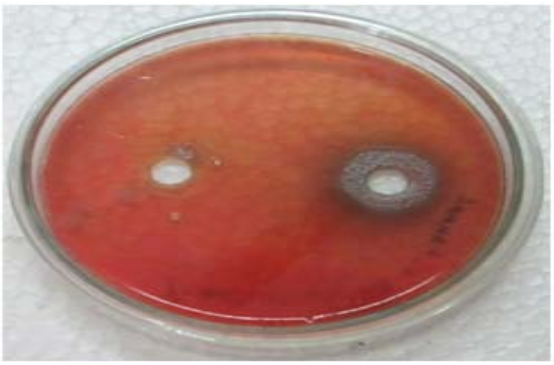

**Fig 2: Heamolysis in Blood Agar**

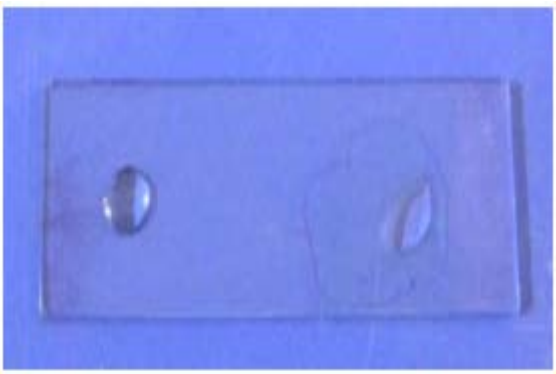

**Fig 3: Drop collapse method**

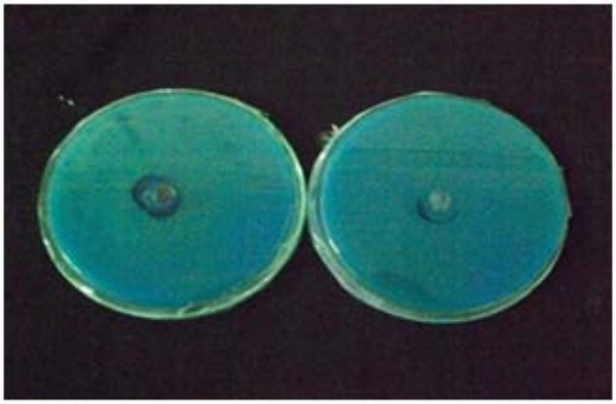

**Fig 4: Methylene blue agar plates**

### 3.2 Extraction and dry weight of biosurfactants

White sediment was obtained from the cultural filtrate while placed in the rotor and then used for the dry weight of the biosurfactants were measured and estimated.

### 3.3 Characterization of biosurfactnats

The biosurfactant produced were characterized by using TLC plates. The sediment obtained was placed in the TLC plate and the plates when sprayed with anthrone reagent it showed yellow sposts in the plates. This shows the presence of rhamnolipid biosurfactants in the organism the TLC plate is shown (Fig -5).The $R_f$ value of the biosurfactant was 0.65, 0.72, 0.64, 0.68, 0.62 in petrol, diesel, crude oil, olive oil, Refinery oil respectively.

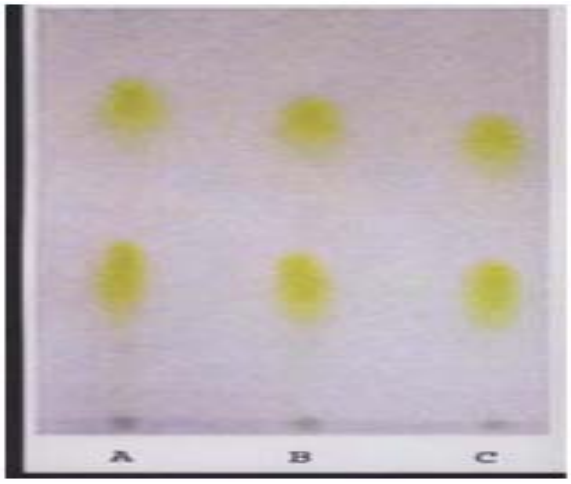


**Fig 5: Thin Layer Chromatography**

### 3.5 Determination of Carbohydrate by Anthrone method

Crude biosurfactant obtained from *pseudomonas aeruginosa* using oils as carbon source were analyzed for the presence carbohydrate using Anthrone method. biosurfactant from PA2G showed positive for carbohydrate analysis. The carbohydrate present in biosurfactant was estimated using standard graph and results were given in (**Table 6**).

### 3.6 Determination of Lipid by Solubility test and Saponification test

Crude biosurfactants produced from was positive for lipid determination. In solubility test all crude surfactants from PA2G were insoluble in water and soluble in water. In Saponification test, biosurfactants from showed positive result (**Table 7**).

### 3.7 FT-IR analysis of biosurfactant

Consistent with this finding is the infrared spectra (IR) analysis of the organic extract of *Pseudomonas aeruginosa* (PA2G) surface active compounds and of the referent rhamnolipids. As seen from (**Fig 6**). In the region 3000-2700 $cm^{-1}$ several C-H stretching bands of $CH_2$ and $CH_3$ groups were also observed. The deformation vibration at 1234 $cm^{-1}$ also confirmed the presence of alkyl groups. Carbonyl stretching band was found at 1654 $cm^{-1}$ a2 and 1072 $cm^{-1}$which is the characteristic peak for ester compound. The wave number 1072 cm-1 indicated the presence of C-O bonds.FTIR results that, the peak observed at 1072.5, 1234.6, 1382.5, and 2853.35, 2922.23, 3207.8 indicated the presence of protein and lipid moieties (Fig 6).

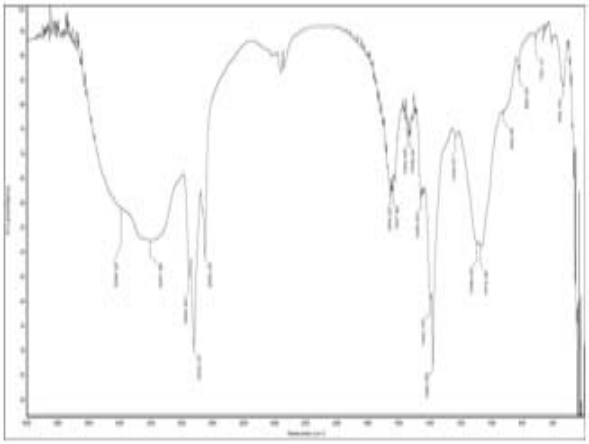

**Fig 6: FTIR spectra analysis of biosurfactant**

## 4. DISSCUSION

The production of surface active compounds by microorganisms had been a subject of interest in recent years. Biosurfactants are becoming important biotechnology products for industrial and medical applications due to their specific modes of action, low toxicity, cleaning oil sludge from oil storage facilities, relatively easy preparation and widespread applicability. They can be used as emulsifiers, de- emulsifiers, wetting and foaming agents, functional food ingredients and as detergents in petroleum petrochemicals, environmental managements, agrochemicals, foods and beverages, cosmetics

and pharmaceuticals and in the mining and metallurgical industries,

For the present study, the isolated bacteria were collected from oil contaminated soil samples from automobile workshop for their biosurfactant production. Biosurfactant production was confirmed by the conventional screening methods including oil spreading technique, hemolytic activity and blue agar plate method. Biosurfactant producing microorganisms are naturally present in the oil and hydrocarbon contaminated soils [17].
In the present study, rhamnolipid production by *Pseudomonas aeruginosa* isolated from four different oil contaminated soil samples. The oils were used as the carbon source for the biosurfactant production. Biosurfactants can be produced with high yield by some microorganisms, especially *Pseudomonas* sp. [3]. [16] reported rhamnolipid production by *Pseudomonas aeruginosa* from industrial wastes (distillery and whey wastes) as substrates, produced rhamnolipid biosurfactants when grown with molasses as the carbon and energy source.

The result on oil spreading was similar to the work done by [18]. Displacement of oil clearly is a sign of extra-cellular surfactant present in the supernatant of culture. Blood agar method is often used for a preliminary screening of microorganism for the ability to produce biosurfactant on hydrophilic media [19]. From in our results twenty one out of thirty isolates showed lysis of the blood cell and exhibit a colorless transparent ring around the colonies. The flat drop appearance in micro titer plate confirmed the positive result for drop collapse test as suggested by [20], proving the use of drop collapse method. Emulsification activity is one of the criteria to determine the potential of biosurfactant. Emulsifying activities ($E_{24}$) determined the productivity of bioemulsifier [21]. In our study the highest Emulsification index ($E_{24}$) index value showed PA2G strain. Blue agar plate method is a semi quantitative agar plate method that is based on the formation of an insoluble ion pair of anionic surfactants with the cationic surfactant CTAB and the basic dye methylene blue [25]. In the present study the PA2G strain was selected as a potent strain for biosurfactant production that strain showed positive result for CTAB method.

The extracted biosurfactant was characterized by TLC. Anthrone reagent was used to detect glycolipid biosurfactant as yellow spot from *Pseudomonas aeruginosa* [23]. The component obtained was rhamnolipid i.e., a glycolipid from *pseudomonas aeruginosa* while sprayed with Anthrone reagent on TLC plate. [12] also reported rhamnolipid from *pseudomonas aeruginosa* in TLC plate.

Biosurfactant isolated in the present study was lipid containing amphiphatic molecules that could be extracted using an appropriate organic solvent system. Various type of organic solvent could be used and they were applicable either singly or in combination for biosurfactant extraction [26]. In the present study, chloroform and methanol (2:1v/v) solvent system was used. Mixtures of solvents were commonly used to facilitate adjustment of the polarity between the solvent as the extraction agent and the biosurfactant to be extracted.

In the present study the Infrared spectrum chart of purified biosurfactant by *Pseudomonas aeruginosa* strain was characterized by the appearance of carbonyl absorption arising from ester and carboxylic groups, (i.e.) component was identified as rhamnolipid. Similar results were reported by [27] and [28, 29].

## 5. CONCLUSION

In conclusion, the study represented surfactant activity of the bacterial strains isolated from oil contaminated soil samples wastes. This confirms that environment has an influence on the metabolism of the tested microbes. This study suggests that, *Pseudomonas aeruginosa* isolated from oil contaminated soil samples showed biosurfactant producing ability. Further study on the utilization of oil contaminated soil samples wastes as substrates for the large-scale production of biosurfactants is recommended.

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