



**Isolation and identification of oil degrading *Pseudomonas aeruginosa* from marine sediment samples**

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**Abstract**

Oil contaminated marine sediment sample was collected from harbour, Chennai, Tamilnadu and transported to laboratory in sterile polythene bag. The diesel, petrol, kerosene and vegetable oils such as coconut oil, gingly oil, groundnut oil and sunflower oil used in this study were collected from local petrol bunk and oil shop and stored separately in bottles before being added aseptically to the growth medium. Bushnell Haas (BH) liquid medium was used as the enrichment medium with 1 % ( v/v) diesel as the sole carbon source to isolate diesel degrading bacteria. Serial dilutions (1/10) from the third enrichment process were plated out into BH agar plates, which were covered with 100 µl of diesel oil and incubated at 30°C for approximately one week. The single colonies were streaked into nutrient agar plates incubated at 30°C overnight and stored at 4°C until further use. The organism was identified morphologically by Gram's staining, motility test and biochemical tests such as catalase, oxidase, indole, methyl red, voges-proskauer, citrate utilization, Triple Sugar Iron fermentation and Urease test. Biosurfactant production in MS medium with diesel, kerosene, petrol and vegetable oil such as coconut oil, gingelly oil, groundnut oil and sunflower oil as carbon source. Extraction of biosurfactant by acid precipitation. Preliminary characterization by TLC and estimation of rhamnose. Screening of antimicrobial activity of biosurfactant against clinical isolates from diabetic foot ulcer was carried out.

**Keywords:** biosurfactant, Bushnell Haas (BH) liquid medium, antimicrobial activity.

**Introduction**

Surfactants are amphiphilic surface active agents possessing both hydrophilic and hydrophobic moieties that reduce surface and interfacial tensions by accumulating at the interface between two immiscible fluids like oil and water. They are of synthetic or biological origin. Due to their interesting properties such as lower toxicity, higher degree of biodegradability, higher foaming capacity and optimal activity at extreme conditions of temperatures, pH levels and salinity, these have been increasingly

attracting the attention of the scientific and industrial community (Kosaric, 1992).

Interest in microbial surfactants has been progressively escalating in recent years due to their diversity, ecofriendly nature, possibility of large-scale production, selectivity, performance under intense circumstances and their impending applications in environmental fortification (Ganesh *et al.*, 2009).

Biosurfactants are polymers, totally or partially extra cellular, amphipathic molecules containing polar and non polar moieties which allow them to form micelles that accumulate at interphase between liquids of different polarities such as water and oil thereby reducing surface tension and facilitating hydrocarbon uptake and emulsification.

Surfactants are key ingredients used in detergents, shampoos, toothpaste, oil additives and a number of other consumer and industrial products. They constitute an important class of industrial chemicals widely used in almost every sector of modern industry.

In order to reduce or eliminate the effect of oil spillage on the environment and living organisms, effort such as applications of chemical dispersant, skimming of the surface oils. Application of biological oil agents and inoculating the spilled area with relevant bacteria are the outcomes of intensive research. The most promising of many research carried out to deal with large scale oil spillage is the use of microorganisms to provide an effective alternative.

Surface-active compounds produced by microorganisms are of two main types, those that reduce surface tension at the air-water interface (biosurfactants) and those that reduce the interfacial tension between immiscible liquids, or at the solid-liquid interface (bioemulsifiers). Biosurfactants usually exhibit emulsifying capacity but bioemulsifiers do not necessarily reduce surface tension. Because of the presence of hydrophobic and hydrophilic groups, surfactants partition preferentially at the interface between fluid phase of different degrees of polarity and hydrogen bonding. These amphiphilic compounds have functional properties like surface and interface activity, emulsification, wetting, foaming, detergency, phase dispersing, solubilization and density reduction of heavy hydrophobic compounds and find wide applications in industries (Walter, 2010).

The total surfactant production has exceeded 2.5 million tons in 2010 for many purposes such as polymers, lubricants and solvents. From the total surfactants output, about 54% of them is consumed as household or laundry detergents, with only 32% destined for industrial use.

Almost all surfactants currently in use are chemically derived from petroleum. The choice of surfactant is based on product cost. Generally, surfactants has

been extensively used to save energy and consequently energy cost. For example, the new generation of detergents wash effectively at much lower temperatures, resulting in significant energy saving. Physicochemical behavior, charge-type, solubility and adsorption behavior are some of the most important selection criteria for surfactants. Synthetic surfactants exhibit a low rate of biodegradation and a high potential to aquatic toxicity. For these reasons, biosurfactants are seen to be the promising alternative for many purposes.

Biosurfactant is a structurally diverse group of surface-active molecule synthesized by microorganisms. Their capability of reducing surface and interfacial tension with low toxicity and high specificity and biodegradability, led to an increasing interest on these microbial products as alternatives to chemical surfactants. The interest in biosurfactant has been steadily increasing in recent years due to the possibility of their production through fermentation and their potential applications in such areas as the environmental protection (G.S. Kiran, 2010).

Majority of surfactants produced today is of petrochemical origin beside of the renewable resources like fats and oils. Petroleum-related industries have been identified as one of the major source of pollution in Kerala. At present, biosurfactants plays an important application in petroleum-related industries which is use in enhanced oil recovery, cleaning oil spills, oil-contaminated tanker cleanup, viscosity control, oil emulsification and removal of crude oil from sludge.

### **Classifications of biosurfactants**

Biosurfactants are classified mainly by their chemical composition and microbial origin. The major classes of biosurfactant include

- Glycolipids
- Lipopeptides and lipoproteins
- Phospholipids and fatty acids
- Polymeric surfactants and
- Particulate surfactants.

### **Materials and Methods**

#### **Collection of Soil Sample**

Oil contaminated marine sediment sample was collected from harbour, Chennai, Tamilnadu and

transported to laboratory in sterile polythene bag. The diesel, petrol, kerosene and vegetable oils such as coconut oil, gingelly oil, groundnut oil and sunflower oil used in this study were collected from local petrol bunk and oil shop and stored separately in bottles before being added aseptically to the growth medium.

### Isolation and identification of bacterial diesel degraders

Bushnell Haas (BH) liquid medium (Bushnell and Haas, 1941; Atlas and Bartha, 1992) was used as the enrichment medium with 1 % (v/v) diesel as the sole carbon source to isolate diesel degrading bacteria. 1 g of the marine sediment sample was added to 100 ml of the enrichment medium and incubated at 30°C in a rotary shaker at 160rpm. After two weeks, 1 ml of enriched medium was transferred into freshly prepared enrichment media and incubated at the same conditions as described earlier. Serial dilutions (1/10) from the third enrichment process were plated out into BH agar plates, which were covered with 100 µl of diesel oil and incubated at 30°C for approximately one week. The single colonies were streaked into nutrient agar plates incubated at 30°C overnight and stored at 4°C until further use.

### Characterization of biosurfactant-producing isolates

The selected biosurfactant-producing bacteria were characterized morphologically and biochemically.

### Morphological Analysis

#### Screening of biosurfactant producing microorganism

Biosurfactants production is detected by various techniques such as follows and performed in triplicates.

- \*Drop Collapsing technique
- \*Hemolytic activity
- \*Oil displacement test
- \*Emulsification stability test
- \*CTAB Agar Plate method
- \*Penetration assay
- \*Microtitre plate method

#### Drop collapsing technique

The isolates were grown in BH medium with diesel as carbon source, incubated with shaking for 48 hours at 37°C and 200 rpm. The glass slides used was rinsed

with hot water, ethanol and distilled water, and dried. The slides were coated with diesel and equilibrated for 24 hours to ensure a uniform oil coating. 1 µl of supernatant sample was then applied to the center of the oil drops using 10µl micropipette. The results were monitored visually after 1 hour. If the drop remained beaded, the result was scored as negative. If the drop collapsed, the result was scored as positive.

### Heamolysis test

Fresh colonies were prepared by streaking on nutrient agar and incubate at 37 °C for 24hrs. These fresh single colonies of culture are restreaked into blood agar plates and the plates were incubated at 37 °C 48-72hrs. The bacterial colonies were then observed for the presence of clear zones around the colonies. These clear zones were used as qualitative method for biosurfactant production.

### Oil displacement assay

The oil displacement or spreading assay was developed by Morikawa *et al.* 2000) For this assay, 2 µl of diesel is added to the surface of 15 ml of distilled water in a petridish to form a thin oil layer. Then, 10 µl of culture or culture supernatant are gently placed on the centre of the oil layer. If biosurfactant is present in the supernatant, the oil is displaced and a clear zone is formed. The diameter of this clearing zone on the oil surface correlates to surfactant activity, also called oil spreading activity.

### Emulsification ability assay

For measuring the emulsification index, diesel is added to an equal amount of culture. The mixture is vortexed at high speed for 2 minutes. After 24 hours, the height of the stable emulsion layer is measured. The emulsion index *E* is calculated as the ratio of the height of the emulsion layer and the total height of liquid. The same is done for petrol, kerosene and vegetable oil.

$$E = \frac{\text{Height of the emulsion}}{\text{Total height of the liquid}} \times 100$$

### CTAB Agar Plate method

The CTAB agar plate method is a semi-quantitative assay for the detection of extra cellular glycolipids or other anionic surfactants. It was developed by Siegmund and Wagner. The microbes of interest are

cultivated on a light blue mineral salts agar plate containing the cationic surfactant cetyltrimethylammonium bromide and the basic dye methylene blue. If anionic surfactants are secreted by the microbes growing on the plate, they form a dark blue, insoluble ion pair with cetyltrimethylammonium bromide and methylene blue. Thus, productive colonies are surrounded by dark blue halos.

### Penetration Assay

Maczek *et al* developed another assay suitable for high throughput screening, the penetration assay. This assay relies on the contacting of two insoluble phases which leads to a color change. For this assay, the cavities of a 96 well microplate are filled with 150 µl of a hydrophobic paste consisting of oil and silica gel. The paste is covered with 10 µl of oil. Then, the supernatant of the culture is colored by adding 10 µl of a red staining solution to 90 µl of the supernatant. The colored supernatant is placed on the surface of the paste. If biosurfactant is present, the hydrophilic liquid will break through the oil film barrier into the paste. The silica is entering the hydrophilic phase and the upper phase will change from clear red to cloudy white within 15 minutes. The described effect relies on the phenomenon that silica gel is entering the hydrophilic phase from the hydrophobic paste much more quickly if biosurfactants are present. Biosurfactant free supernatant will turn cloudy but stay red.

### Microplate Assay

The surface activity of individual strains can be determined qualitatively with the microplate assay developed and patented by Vaux and Cottingham. This assay is based on the change in optical distortion that is caused by surfactants in an aqueous solution. Pure water in a hydrophobic well has a flat surface. The presence of surfactants causes some wetting at the edge of the well and the fluid surface becomes concave and takes the shape of a diverging lens. For this assay, a 100 µl sample of the supernatant of each production medium is taken and put into a microwell of a 96-microwell plate. The plate is viewed using a baking sheet of paper with a grid. If biosurfactant is present, the concave surface distorts the image of the grid below. The optical distortion of the grid provides a qualitative assay for the presence of surfactants.

### Optimization of Growth

Bacterial growth was optimized using different parameters such as pH, temperature and nitrogen source.

### Effect of pH

The growth of biosurfactant producing *Pseudomonas aeruginosa* strain at different pH ranging from 6,6.5,7,7.5,8 and 8.5 was analysed.

### Effect of temperature

The growth of biosurfactant producing *Pseudomonas aeruginosa* strain at different temperature ranging from 35,36,37,38 and 39 was analyzed.

### Effect of nitrogen source

The growth of biosurfactant producing *Pseudomonas aeruginosa* strain on different nitrogen sources such as ammonium chloride, sodium nitrate, ammonium nitrate and ammonium sulphate was analyzed.

### Biosurfactant production on MSM supplemented with different carbon source in the form of oil

The *Pseudomonas aeruginosa* isolate was inoculated in Mineral salt liquid medium is used for production of biosurfactant. The pH was adjusted to 7 before autoclaving at 121°C and for 15 minutes. After sterilization, 2 % of the different carbon sources such as diesel, petrol, kerosene and vegetable oil such as coconut oil, gingly oil, groundnut oil and sunflower oil were added. Then bacteria was inoculated in to the mineral salt medium and it was placed on a reciprocal shaker at 100rpm at 37°C for 3 days.

### Biosurfactant recovery

The culture broth was centrifuged (10000 rpm, 15 min) to remove the cells and there after sterilized with membrane filter. The clear sterile supernatant served as the source of the crude biosurfactant. The biosurfactant was recovered from the cell free culture supernatant by acid precipitation method. The culture supernatant was acidified with 6N HCl to obtain pH of 2.0. The extraction is performed twice with an equal volume of ethyl acetate. Pooled solvent extract were concentrated using an evaporator under

reduced pressure. White precipitate was formed is used for TLC and AWD assay against selected human pathogens.

### 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 chloroform: methanol: water (70:10:0.5, v/v/v) developing solvent system. Anthrone reagent (1 g anthrone in 5 mL sulfuric acid mixed with 95mL ethanol) was used to detect the presence of rhamnolipid as yellow spot.

### Structural characterization

#### Rhamnose test

The presence of carbohydrate groups in the biosurfactant molecule was assayed by rhamnose

test. A volume of 0.5 ml of cell supernatant was mixed with 0.5 ml of 5%. Phenol solution and 2.5 ml of sulfuric acid, and incubated for 15 min before measuring absorbance at 490 nm.

Antimicrobial susceptibility test by AWD assay

The clinical isolate of pathogenic microorganisms obtained from diabetic foot ulcer were subjected for their susceptibility against the biosurfactant obtained by Agar Well Diffusion (AWD) assay on Muller-Hinton agar plates.

### Results

Characterization of biosurfactant-producing *Pseudomonas aeruginosa*

The results for the biochemical characterization of the isolate is given in Table – 1 and figure 2, 3 and 4.

**Table – 1 Biochemical characteristics**

S.NO	TEST	RESULT
1.	Gram Staining	Gram Negative rod shaped organism
2.	Motility test	Motile rods
3.	Catalase test	Positive
4.	Oxidase test	Positive
5.	Indole	Negative
6.	Methyl red	Negative
7.	Voges proskauer	Negative
8.	Citrate utilization	Positive
9.	Triple Sugar Iron	Acid butt, alkaline slant and H <sub>2</sub> S negative
10.	Urease	Negative

### Screening of Biosurfactant produced by *Pseudomonas aeruginosa*

The results for the screening of biosurfactant are given in the Table – 2, 3 and figure 6,7,8,9 and 10.

**Table – 2 Screening of Biosurfactant of *Pseudomonas aeruginosa***

S.No	Screening technique	Result
1.	Drop Collapsing test	+ (Droplet collapse with the hydrocarbon)
2.	Hemolysis activity	+ ( – hemolysis was observed)
3.	Oil Spreading technique	+
4.	Emulsification ability test	+
5.	CTAB Agar Plate method	+
6.	Penetration assay	+

### Emulsification index

The emulsification index  $E$  is calculated as the ratio of the height of the emulsion layer and the total height of liquid. The results for emulsification index of

Biosurfactant producing *Pseudomonas aeruginosa* for diesel, petrol, kerosene and vegetable oil such as coconut oil, gingly oil, groundnut was given in Table – 3. The maximum emulsification index is obtained for diesel which is shown in figure 6 and 7.

**Table – 3 Emulsification index**

S.No	Emulsification ability test (E24 index)	Height of emulsion in mm	% of emulsification index
1.	Diesel	<b>18 mm</b>	<b>52%</b>
2.	Petrol	15mm	44%
3.	Kerosene	17mm	<b>50%</b>
4.	Coconut oil	16mm	47%
5.	Giggly oil	15mm	44%
6.	Grounut oil	15mm	44%
7	Sunflower oil	17mm	<b>50%</b>

### Effect of pH

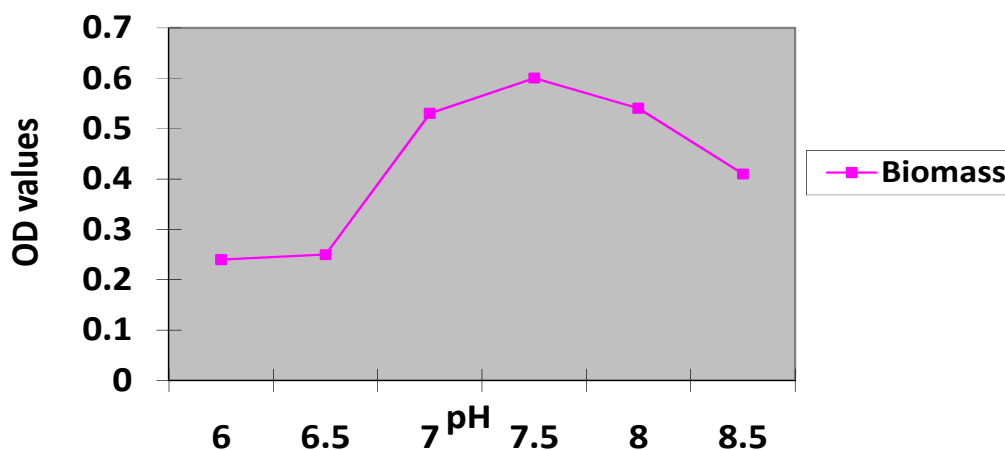
The growth of biosurfactant producing *Pseudomonas aeruginosa* strain at different pH ranging from

6,6.5,7,7.5,8 and 8.5 was given in Table – 4 and graph – 1 and figure – 11. Among the various pH, the slightly alkaline pH of 7.5 was found to yield maximum growth.

**Table – 4 Effect of Ph**

pH	Biomass
6	0.249
6.5	0.256
7	0.532
7.5	<b>0.601</b>
8	0.545
8.5	0.419

**Graph – 1 Effect of Ph**





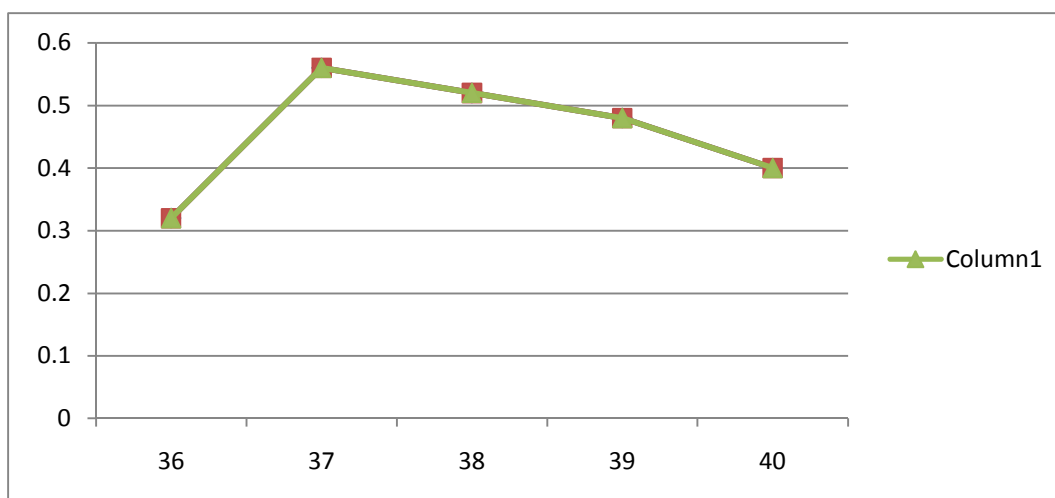
### Effect of Temperature

The results for the effect of temperature on optimization of growth of *Pseudomonas aeruginosa* was given in Table – 5, graph – 2.

**Table – 5 Effect of Temperature**

Temperature in °C	Biomass
36 °C	0.321
37 °C	<b>0.561</b>
38 °C	0.521
39 °C	0.482
40 °C	0.401

**Graph – 2 Effect of Temperature**



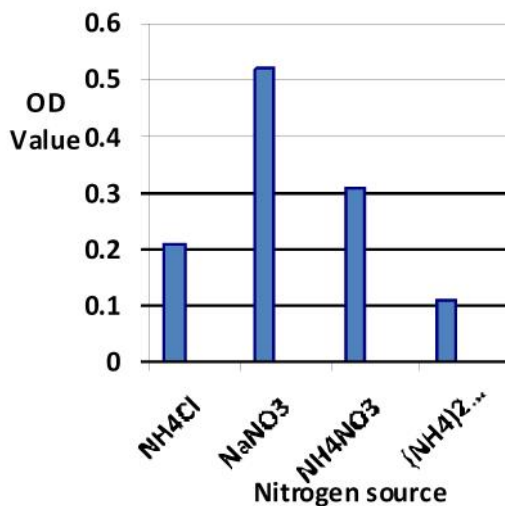
### Effect of Nitrogen source

The growth of biosurfactant producing *Pseudomonas aeruginosa* strain on different nitrogen sources such as ammonium chloride, sodium nitrate, ammonium nitrate and ammonium sulphate was given in Table – 6 and graph – 3.

**Table – 6 Effect of Nitrogen source**

Nitrogen source	Absorbance
NH <sub>4</sub> Cl	0.212
NaNO <sub>3</sub>	<b>0.524</b>
NH <sub>4</sub> NO <sub>3</sub>	0.312
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.118

### Graph – 2 Effect of Nitrogen source



#### Biosurfactant production on MSM

Biosurfactant production on MSM supplemented with various hydrocarbons such as diesel, petrol, kerosene and vegetable oil such as coconut oil, gingly oil, groundnut was given in figure 12, 13 and 14.

#### Biosurfactant recovery

The biosurfactant was recovered from the cell free culture supernatant by acid precipitation method was given in figure 15.

#### Thin Layer Chromatography

The results for the TLC biosurfactants obtained acid precipitation were given in the Table -6 and figure 16.

**Table -6 Thin Layer Chromatography**

Hydrocarbon	Rf Value
Diesel	0.72
Petrol	0.67
Kerosene	0.61
Sunflower oil	0.62

#### Rhamnose test

The results for the presence of carbohydrate groups in the biosurfactant molecule was assayed by rhamnose test was given in Table – 7.

**Table – 7 Optimization of Rhamnose**

Reagents	Blank	S1	S2	S3	S4	S5	Test
Rhamnose (Diesel)	–	0.2	0.4	0.6	0.8	1	0.5
Distilled Water	1	0.8	0.6	0.4	0.2	–	0.5
Phenol	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Sulfuric acid	2.5	2.5	2.5	2.5	2.5	2.5	2.5
OD at 490nm	0	0.76	1.02	1.10	1.38	1.70	0.80



**Antimicrobial susceptibility test by AWD assay**

The results for the antimicrobial activity of the biosurfactant by growing *Pseudomonas aeruginosa* in MSM was shown in Table – 8 and figure 17, 18, 19, 20 and 21.

**Table – 8 Antimicrobial susceptibility test by AWD assay**

S.No	Biosurfactant source	Zone of inhibition in mm				
		<i>Staphylococcus aureus</i>	<i>Escherischia coli</i>	<i>Klebsiella sp.</i>	<i>Salmonella typhi</i>	<i>Vibrio sp.</i>
1.	Diesel	7	12	18	12	20
2.	Petrol	15	15	15	10	20
3.	Kerosene	10	10	10	10	10
4.	Coconut oil	10	10	10	0	10
5.	Giggly oil	18	10	12	0	15
6	Grounut oil	2	10	8	0	10
7.	Sunflower oil	14	10	12	5	5
8.	Standard antibiotic	10	18	22	28	22

**Discussion**

Interest in microbial surfactants has been progressively escalating in recent years due to their diversity, ecofriendly nature, possibility of large- scale production, selectivity, performance under intense circumstances and their impending applications in environmental fortification (Ganesh *et al.*, 2009).

The total surfactant production has exceeded 2.5 million tons in 2010 for many purposes such as polymers, lubricants and solvents. From the total surfactants output, about 54% of them is consumed as

household or laundry detergents, with only 32% destined for industrial use.

*Pseudomonas* is a genus of Gram negative aerobic - proteobacteria, belonging to the family *Pseudomonadaceae* containing 191 validly described species. The members of the genus demonstrate a great deal of metabolic diversity, and consequently are able to colonize a wide range of niches. Their ease of culture in vitro and availability of an increasing number of *Pseudomonas* strain genome sequences has made the genus an

excellent focus for scientific research; the best studied species include *Pseudomonas aeruginosa*. Some members of the genus *Pseudomonas* are able to metabolise chemical pollutants in the environment, and as a result can be used for bioremediation.

The present study focused on studying the production of biosurfactant by bacteria isolated from marine sediment sample selectively *Pseudomonas aeruginosa*, which is assumed to be potent biosurfactant producer.

The screening of biosurfactant producing *Pseudomonas aeruginosa* by was investigated by hemolytic assay, drop collapse test, emulsification index, oil displacement test showed the results similar to the studies reported by Saravanan.V, 2012.

The optimization of growth that shows maximum yield of biomass obtained was pH 7.5 (OD - 0.601), temperature 37°C (OD-0.561) and the nitrogen source NaNO<sub>3</sub> (OD-0.524) which is nearly as similar as S.Dhail, 2013.

The results for TLC analysis of the biosurfactant was reported as done by Priya .T and G.Usharani, 2009 which shows the yellow colour development.

The results for antimicrobial activity of the clinical isolates from diabetic foot ulcer pathogens were performed and are reported by Andrea *et al.*, 2007.

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