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RESEARCH ARTICLE

STUDIES ON THE BIOSURFACTANT PRODUCING *PSEUDOMONAS AERUGINOSA* AND ITS ANTIMICROBIAL ACTIVITY ON SELECTED HUMAN PATHOGENS

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Abstract

Oil contaminated soil sample was collected from a automobile workshop at banavaram, Kaveripakkam, Vellore District and transported to laboratory in sterile polythene bag. The diesel, petrol, kerosene and vegetable oil used in the studies were collected from local pump 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. 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. Screening of Biosurfactant by Drop Collapsing technique, Hemolytic activity, Oil displacement test, Emulsification stability test was carried. Optimization of Growth by using various parameters like pH, temperature, nitrogen source etc was done. Biosurfactant production was carried out in MS medium with diesel, kerosene, petrol and vegetable oil as carbon source. Biosurfactant was extracted by acid precipitation and characterized by TLC. Screening of antimicrobial activity of biosurfactant against selected human pathogens.
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Keywords: automobile workshop, diesel, petrol, kerosene and vegetable oil, Bushnell Haas (BH) liquid medium, Biosurfactant production, 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. 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. 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.

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)

Most biosurfactants are either neutral or negatively charged. The negatively charged is an anionic biosurfactants which is due to a carboxylate, phosphate or sulphate group. Least number of cationic biosurfactants contains amine functions (Karanth,1999 & Boboye *et al*., Shukor, 2010). A biosurfactant must have the ability to improve water loss, which can wet the solid surfaces. Some of the biosurfactants also has the ability to act as an emulsifier.

In the year 2000, the complete genome sequence of a *Pseudomonas* species was determined; more recently, the sequence of other strains has been determined,including *Pseudomas aeruginosa* strains PAO1 (2000), *Pseudomonas putida* KT2440 (2002), *Pseudomonas protegens* Pf-5 (2005), *Pseudomonas syringae* pathovar tomato DC3000 (2003), *Pseudomonas syringae* pathovar syringae B728a (2005), *Pseudomonas syringae* pathovar phaseolica 1448A (2005), *Pseudomonas fluorescens* PfO-1, and *Pseudomonas entomophila* L48

Aim and objectives

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

Isolation and identification of oil degrading *Pseudomonas aeruginosa* from oil contaminated soil using Bushnell Hass Medium.

Screening of Biosurfactant by Drop Collapsing technique, Hemolytic activity, Oil displacement test, Emulsification stability test, Optimization of Growth.

Biosurfactant production in MS medium with diesel, kerosene, petrol and vegetable oil as carbon source, Extraction of biosurfactant by acid precipitation, Preliminary characterization by TLC and estimation of rhamnose and Screening of antimicrobial activity of biosurfactant against selected human pathogens.

Materials and Methods

Collection of Soil Sample

Oil contaminated soil sample was collected from a automobile workshop at banavaram, Kaveripakkam, Vellore District and transported to laboratory in sterile polythene bag. The diesel, petrol, kerosene and vegetable oil used in the studies were collected from local pump 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 diesel contaminated soil sample was added to100 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. The organism was identified morphologically by Gram's staining, motility test and biochemically tests by catalase, oxidase, indole, methyl red,voges-proskauer,citrate utilization, Triple Sugar Iron fermentation and Urease test.

Growth and maintenance of bacterial isolates

Inoculum preparation

A fresh single pure colony of each bacterial isolates was transferred aseptically from agar plate into Bushnell Haas medium using a sterile loop. The inoculated medium was then incubated at 37ºC at 100rpm in orbital shaker.

Screening of biosurfactant producing microorganism

Biosurfactants production is detected by various techniques like

* Drop collapsing technique
* Heamolysis test
* Oil displacement test
* Emulsification index

Drop collapsing technique

The isolates were grown in BH medium with diesel as carbon source, incubated with shaking for 48 hours at 37ºC 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 $37^{o}C$ for 24hrs.These fresh single colonies of culture are restreaked into blood agar plates. And the plates were incubated at $37^{o}C$ 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 spreading assay

The oil 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 petri dish 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 displacement 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.

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

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 temparature

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 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 supernatent 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 selected organisms were tested for their susceptibility against the biosurfactant obtained by growing the organism in MSM broth is subjected to Agar Well Diffusion (AWD) assay on Muller-Hinton agar plates. A swab of the test culture was taken aseptically and inoculated to the surface of the Muller-Hinton agar plates so as to make a lawn. This was allowed atleast 5 minutes for the agar surface to dry before cutting the well by using gel punch. By using sterile tips, 20µl of the each biosurfactant obtained was added to the wells along with a standard antibiotic. The plates were incubated at 37ºC for 24 hours and observed for the formation of zone around the well.

Results

Characterization of biosurfactant-producing *Pseudomonas aeruginosa*

The results for the biochemical characterization of the isolate is given in Table – 1.

Screening of Biosurfactant produced by *Pseudomonas aeruginosa*

The results for the screening of biosurfactant is given in the Table – 2 and 3.

Emulsification index

The emulsion 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 was given in Table – 3.

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. Among the various pH, the slightly alkaline pH of 7.5 was found to yield maximum growth

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 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_2S negative
10.	Urease	Negative

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	+ (α – hemolysis was observed)
3.	Oil Spreading technique	+
4.	Emulsification ability test	+

Table 3. Emulsification index

S.No	Emulsification ability test (E24 index)	Height of emulsion in mm	% of emulsification index
1.	Diesel	**18 mm**	**76 %**
2.	Petrol	12 mm	64 %
3.	Kerosene	10 mm	56 %
4.	Vegetable oil	9mm	51 %

Table 4. Effect of pH

pH	Biomass
6	0.249
6.5	0.256
7	0.532
7.5	0.601
8	0.545
8.5	0.419

Graph.1 Effect of pH on biosurctant production

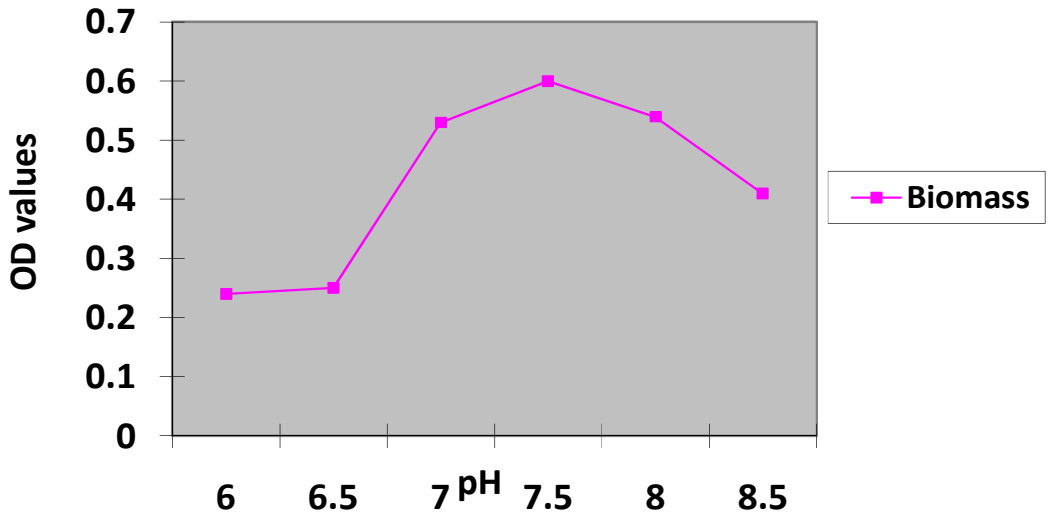

Table 5. Effect of Temperature on biosurctant production

Temparature in ºC	Biomass
36 ºC	0.321
37 ºC	0.561
38 ºC	0.521
39 ºC	0.482
40 ºC	0.401

Graph2. Effect of Temperature on biosurctant production

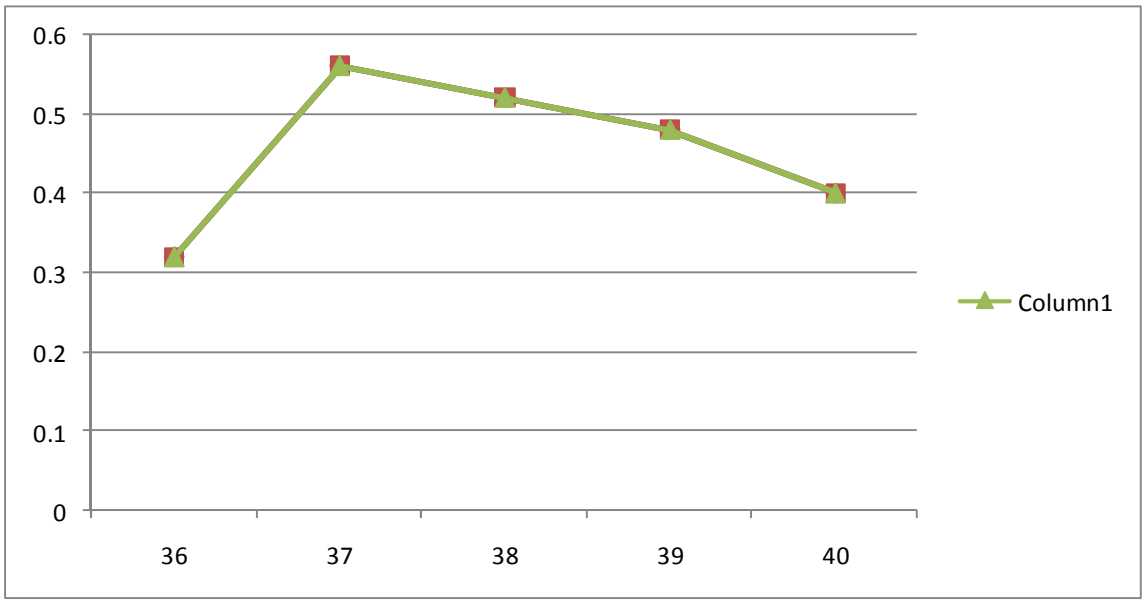

Table 6. Effect of Nitrogen source on biosurctant production

Nitrogen source	Absorbance
NH4Cl	0.212
NaNO3	0.524
NH4NO3	0.312
(NH4)2SO4	0.118

Graph 3. Effect of Nitrogen source on biosurctant production

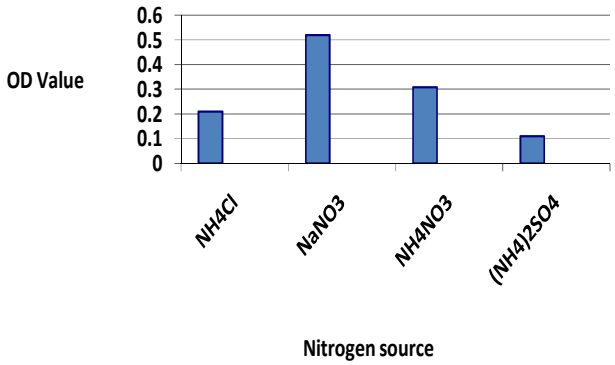

Table 6. Analysis of biosurfactant by Thin Layer Chromatography

Hydrocarbon	**Rf Value**
Diesel	0.72
Petrol	0.67
Kerosene	0.61
Vegetable Oil	0.62

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

Table 8. Antimicrobial susceptibility test by AWD assay

S.No	Biosurfactant source	Zone of inhibition in mm				
		Staphylococcus aureus	*Escherischia coli*	*Klebsiella sp.*	*Salmonella typhi*	*Vibrio sp.*
1.	Diesel	**12**	12	15	11	**11**
2.	Petrol	11	**15**	**16**	**12**	9
3.	Kerosene	10	11	13	10	8
4.	Vegetable oil	10	10	13	10	9
5.	Standard antibiotic	10	13	8	11	4

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.

Thin Layer Chromatography

The results for the TLC biosurfactants obtained acid precipitation was given in the Table -6.

Rhamnose test

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

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

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 γ-proteobacteria, belonging to the family Pseudomonadaceae containing 191 validly described species. The members of the genus demonstrate a great deal f metabolic diversity, and consequently are able to colonise 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 oil contaminated soil 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), temperature37°C (OD-0.561) and the nitrogen source $NaNO_3$ (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 and Usharani, (2009) which shows the yellow colour development. The results for antimicrobial activity of the selected human pathogens were performed and are reported by Andrea *et al*., (2007).

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