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Determination of the Presence of Biosurfactant Produced by the Bacteria Present in the Soil Samples*

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Abstract: Polyaromatic hydrocarbons are the most obvious pollutant in both terrestrial and aquatic realm. Its damage in the agricultural fields and normal water system are well documented. PAHs pollution leads to drastic effected on the flora and fauna of the aquatic system and is directly affecting the human life, because of this carcinogenicity and mutagenicity. Biodegration appears to be the best method in controlling PAH pollution effectively. Biosurfactants are produced during hydrocarbon degradation by bacteria and they influence the degradation rate. As a result in the present study, an attempt on the significance role of bacteria in the production of biosurfactant was made.

Key words: Bacteria, biosurfactant, polyaromatic hydrocarbon

Introduction

The global network with the development of petroleum industries, there has been an increase in the amount of polycyclic aromatic hydrocarbons released into the environment, they are toxic, carcinogenic, or teratogenic, a variety of microorganisms can degrade certain PAHs completely to CO_2 and metabolic intermediates, enroute gaining energy and carbon for cell growth. These degradation are mainly influenced by production of biosurfactants produced by the degradative micro organisms.

During hydrocarbon degradation the degradative organisms produce an amphibolic compounds that influence the degradation rates. These compounds are known as Biosurfactants.

Literally, the term biosurfactants refers to any compound obtained from microorganisms, which has some influence on interfaces, is it brings drown the interfacial tension between the two liquids.

Biosurfactants produced as metabolic by products, are not only potentially as effective. But after some distinct advantages over the highly used synthetic surfactanrts. These increased bio degradability and reduced toxicity (Mulligan *et al*., 1989).

The biosurfactant molecule has both hydrophilic as well as hydrophobic moiety. They are generally classified on the basis of their biochemical nature. The classifications of microbial biosurfactants are Glycolipids, phospholipids and fatty acids, peptides and amino acid containing lipids, polymeric biosurfactants, emulsan, liposan. They are in the form of micelles or aggregate to form rod-shaped micelles, bilayers and vesicles (Shafi and Khanna, 1955).

A variety of microorganisms reported to produce biosurfactants, out of which some species require essentially hydrocarbons as substrate. Some microorganisms produce biosurfactants from both carbohydrates as well as hydrocarbons use, either separately or in combinations.

Biosurfactant production is essentially associated with hydrocarbon degradation in which facilitate its uptake, this may be the reason that most of the earlier reports on biosurfactant production are related to hydrocarbon degradation.

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The biosurfactants produced by *Pseudomonas* sp., from various carbon source such as glucose, glycerol and hydrocarbons have been characterized as glycolipids, they have been found increases dispersion of hydrophobic compounds in water and to enhance the recovery from soil of PAHs (Arino *et al*., 1996).

The production of biosurfactants depending on both nutritional and environmental factors includes, carbon source, nitrogen source, pH, cultivation temperature, scale of oxygen transfer and speed of agitation.

The interest in the potential applications of microbial biosurfactant is based on their broad range of function properties that includes emulsification, corrosion-inhibition and viscosity reduction.

Therefore, many areas of industrial applications where chemical surfactants could be substituted by biosurfactants in fields as diverse as agriculture food and beverage industries, industrial cleanings cosmetics, pharmaceutical industries and last but not least, petroleum and petrochemical industries (Shafi and Khanna, 1995).

The objectives of the present study are:

- Enumeration of microorganisms from fuel oil contaminated soil samples.
- Isolation, identification and characterization of bacterial isolates.
- Screening for biosurfactant producing bacteria.
- Chemical analysis of the isolated biosurfactant.

Materials and Methods

Sampling Area

For the study purposes, three sampling areas, (Thanjavur, Thiruvarur, Nagapattinam) were selected. From these three areas, different fuel oil contaminated soils were taken from random places. These fuel oil contaminated soil possesses a major impact on agricultural fields and fresh water systems that includes ground water and stored water.

Sampling Procedure (Kastner et al., 1994)

Soil samples were taken with sterilized shovels, after removing 20 cm of the surface layer. The samples were collected in sterilized containers. They were transferred and stored aerobically at 4°C.

Bacteriological Analysis
Extraction of the Sample

- The bacteria were isolated and enumerated from the different fuel oil contaminated soil samples by mixing 10 g of soil with 100 mL of sterile sodium and 3 g oil glass beads were added to the conical flasks.
- The flasks were closed and shaken for 2 h on rotary shaker (350 rpm).
- The solid particles were allowed to sediments for 30 min and aliquots of the supernatant phase were used as inoculum.
- Dilutions of the inoculum were prepared in a sterile sodium pyrophosphate solution up to 10^{-4}

Enumeration

Solid mineral medium was used for the enumeration of microorganism from fuel oil contaminated soil samples

- Solid mineral medium was prepared and sterilized at 121°C for 15 min.
- The sterilized solid medium was cooled up to 45°C and then poured into the sterilized petriplates.
- The plates were allowed to solidify aseptically.
- After solidification, 0.1 mL of serially diluted samples were spreaded on the solid mineral media plates, respectively.
- The plates were incubated for 14 days at 28°C.
- The colony forming units on the plates represents the number of microorganisms present per gram of fuel oil contaminated soil.
- Total viable counts of the samples were calculated.

Isolation

- Each single strain was isolated by repeated streaking on agar medium.
- The individual colonies were observed.
- The colonies were again taken for checking of their purity by microscopical methods.

Identification of Bacteria

The isolated colonies were identified by following microbiological and biochemical testes such as Gram's Reaction, spore Staining, motility, penicillin sensitivity test, oxidase test, catalase test, carbohydrate fermentation test, urease test, gelatinase test, fluorescent pigment production were performed to confirm the strain.

Biosurfactant Production

PAHs degrading bacteria were maintained on trypticase soy agar at 4°C for study their biosurfactant producing ability.

Inoculum Preparation

- Liquid minimal salt medium was prepared and sterilized at 121°C for 15 min.
- A glucose solution (20%) was prepared and filter sterilized.
- The filter sterilized glucose solution (20%) was added to the sterilized minimal salt media aseptically.
- Transfer a loopful of PAHs degrading bacteria from a trypticase soy agar plates to minimal salt medium.
- The flasks were incubated at 28°C for 2 days in rotary shaking at 200 rpm.

Production of Surfactant by Bacteria

- Five milliliter of each inoculum were added to the sterilized minimal salt medium flasks which containing a filter sterilized glucose solution (20%)
- The flasks were incubated at 28°C, 200 rpm for 7 days.

Extraction of the Biosurfactant

- Each cultures were centrifuged at 8000 rpm, 4°C for 10 min to harvest the cells.
- The culture supernatant was taken.
- pH of the culture supernatant was lowered to 2 with 5M HCl and incubating at 4°C for 24 h.

- The precipitate was separated by centrifugation at 8000 rpm for 20 min
- This white precipitate formed culture was selected

Chemical Analysis of Biosurfactants (Sawhnety and Sing, 2000)
Analysis of Amino Acids
Ninhydrin Test

It is a general test for all amino acids

- Add 2-5 drops of ninhydrin solution was added to a small amount of isolated Biosurfactants
- The tubes were mixed well and keep for 5 min in boiling water bath and observed the color formation.

Analysis of Carbohydrate

Anthrone Test

- A tiny amount of the isolated biosurfactant was added to the 2 mL of anthrone reagent and it was thoroughly mixed.
- Colour changes were observed.

Iodine Test

- 4 -5 drops of iodine solution was added to the a little amount of the isolated biosurfactant and it was mixed gently.
- The colour formation was observed.

Barfoed Test

- Two milliliter of Barfoed's reagent was added to the little amount of the biosurfactant.
- The tubes were kept in a boiling water bath.
- The formation of colour and also the time taken for its appearance was noted.

Bial's Test

- Two milliliter of Bial's reagent was added to 1-2 drops of biosurfactant.
- The tubes were heat in a boiling water bath.
- The color formation was observed.

Analysis of Lipids
Solubility Test

- Small amount of isolated biosurfactant was taken in three test tubes and water, alcohol, chloroform was added to each tube.
- Their solubility was tested.

Saponification Test

- Two milliliter of 2% NaOH solution was added to the small amount of biosurfactant and shaken well.
- The formation of soap was observed.

Acrolein Test for Glycerol

- 1.5 g potassium hydrogen sulphate was taken in a test tube and a little amount of isolated biosurfactant was added.
- The added biosurfactant was covered completely by adding more of solid potassium hydrogen sulphate on top of it.
- The test tube was slowly heated and noted the odor of the fumes evolved from the tube.

Results

Fuel oil contaminated soils were taken from three different areas (Thanjavur, Thiruvarur and Nagapattinam). Total viable count of the three area soil samples were calculated. When compared to Thiruvarur and Nagapattinam soil samples, the TVC of Thanjavur soil sample was higher (Table I).

The isolated bacteria from the three different areas confirmed by biochemical tests (Table 2). The identified bacteria were as follows, *Pseudomonas* sp., *Bacillus* sp., *Flavobactrium* sp., *Alcaligenes* sp., *Micrococcus* sp., *Aeromonas* sp., *Stretococcus* sp., *Rhizobium* sp. and *Vibrio* sp. Distribution of bacterial isolates in the three sampling areas were screened, (Table 3). *Pseudomonas* sp., *Bacillus* sp., *Flavobacterium* sp. *and Micrococcus* sp., were present in all the three area soil samples. Alcaligenes sp., was present in Thanjavur and Thiruvarur soil samples. But it was absent in Nagapattinam soil sample. *Aeromonas* sp., was present in Thanjavur sample *Streptococcus* sp., was present only in Nagapattinam sample where as *Rhizobium* sp., was present only in Thiruvarur soil sample, *Vibrio* sp., was present both Thiruvarur and Nagapattinam samples.

Biosurfactant producing ability of *the Pseudomonas* sp., *Bacillus* sp. *and Micrococcus* sp., were screened. The *Pseudomonas* sp., only produce biosurfactant (White precipitate) by using glucose as the substrate (Table 4).

The isolated biosurfactant chemically analysed, (Table 5) the presence of amino acids, carbohydrates and lipids in biosurfactant was carried out.

Ninhydrin test for amino acids, there was the absence of pink and purple of violet-blue complex formation was observed. It indicates the absence of amino acids.

Anthrone test for carbohydrates, there was a colour changes to bluish green, which indicates the presence of carbohydrates.

Iodine test for the identification of polysaccharides. There was the absence of blue or reddish brown complex. It indicates the absence of polysaccharides and the presence of mono or disaccharides.

Barfoeds tests for distinguish between mono and disaccharides the formation of red precipitate with 2-5 min was observed that indicates the presence of monosacharides.

Bial's test for pentose sugars, the formation of blue-green coloured complex was observed. It confirms the presence of pentose sugar in the isolated biosurfactant.

In the solubility test for lipids, the tested biosurfactant was insoluble in water, but soluble in alcohol and chloroform.

In saponification test for lipids, NaOH saponifies the lipid, which is present in the biosurfactant that indicates the presence of lipid in the isolated biosurfactant.

In achrolin test for glycerol the tested biosurfactant, does not produce pungent smell. It indicates the absence of glycerol.

Table 1: Enumeration of microorganisms from three fuel oil contaminated sites

Area of sample collection	Sample	TVC cfu g^{-1}
Thanjavur	Fuel oil	3.6×10^{-4}
Thiruvarur	Contaminated	3.3×10^{-4}
Nagapattinam	Soil	2.7×10^{-4}

Table 2: Identification of bacterial isolates isolated from three different fuel oil contaminated areas

Bacterial isolates	Gram's stain	Cell shape	Spore	Motility	Penicillin sensitivity	Oxidase	Catalase	O/F	Urease	Gelateinase	Fluorescent pigment
Pseudomonas sp.	-	Rods	-	+	-	+	+	+	-	+	+
Bacillus sp.	+	Rods	+	-	+	+	+	-	+	-	-
Flavobacterium sp.	-	Rods	-	+	-	+	+	-	-	-	-
Alcaligenes sp.	-	Rods	-	+	-	+	+	-	-	-	-
Micrococcus sp.	+	Cocci	-	-	+	+	+	-	+	-	-
Aeromonas sp.	-	Rods	-	+	-	+	+	+	-	+	-
Streptococcus sp.	+	Cocci	-	-	+	-	+	-	-	-	-
Rhizobium sp.	-	Rods	-	+	-	+	+	-	-	-	-
Vibrio sp.	-	Curved Rods	-	+	-	+	+	+	-	+	-

+ = positive ; - = Negative

Table 3: Distribution of the bacterial isolates in three different fuel contaminated soil samples

Bacterial isolates	Sample collected areas		
	Thanjavur	Thiruvarur	Nagapattinam
Pseudomonas sp.	+	+	+
Bacillus sp.	+.	+	+
Flavobacterium sp.	+	+	+
Alcaligenes sp.	+	+	-
Micrococcus sp.	+	+	+
Aeromonas sp.	+	-	-
Streptococcus sp.	-	-	+
Rhizobium sp.	+	-	-
Vibrio sp.	-	+	+

+ = Able to degrade; - = Unable to degrade

Table 4: Screening the surfactant producing bacteria

PAHs degrading bacteria	Biosurfactant production
Pseudomonas sp.	+
Bacillus sp.	-
Micrococcus sp.	-

+ = Presence of biosurfactant production; - = Absence of biosurfactant production

Table 5: Chemical analysis of biosurfactant produced by *Pseudomonas* sp.

Qualitative analysis	Result
Amino acids	-
Carbohydrates	+
Lipids	+

+ = Presence of the compound; - = Absence of the compound

Discussion

In present research, PAHs degrading *Pseudomonas* sp., produced biosurfactants in minimal salt medium supplemented with glucose. Biosurfactant are known to be produced by hydrocarbonoclistic microorganisms during their on growth on hydrocarbons and carbohydrates. The formation of biosurfactants by different species of *Pseudomonas* from various carbon sources such as glucose, glycerol and hydrocarbons has been reported (Jayani and Joshi, 1992).

Rhamnolipids are glycolipids, which contain one, or two molecules of Rhamnose linked to one or two molecules of β-hydroxy decanic acid. Several species of *Pseudomonas,* over the years, have

been shown to produce a variety of Rhamnolipids. In *P. aeruginosa* formation of rhamolipids of R1 was observed during hydrocarbon degradation (Shafi and Khanna, 1995).

In this study, isolated biosurfactant was analyzed chemically; the presence of carbohydrates, lipids was confirmed. That particular carbohydrate was found to be a pentose sugar and the glycerol was absent in the lipid, hence this indicates that the isolated biosurfactant was a glycolipid. Glycolipids containing sugar and lipid component and do not containing glycerol. The sugar constitutes most prevalent are glucose, galactose, mannose, glycocyamine have all been identified (Sawhney and Singh, 2000).

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