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RESEARCH ARTICLE

PRODUCTION OF BIOSURFACTANT AND CHARACTERIZATION BY 16S rRNA SEQUENCING TECHNIQUE OF BACTERIA DEGRADING HYDROCARBON ISOLATEDFROM PETROLEUM CONTAMINATED SITES

Bhavani.T.M.B and Hemashenpagam.N

PG and Research Department of Microbiology, Hindusthan College of Arts and Science, Coimbatore-28

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Abstract

Biosurfactants are a structurally diverse group of surface activemolecules synthesized by microorganisms. These molecules reduce surface and interfacial tensions in both aqueous solutions and hydrocarbon mixtures, which makes them potential candidates for biodegradation.The diversity at the genomic level will help to understand the evolutionary and phylogenetic perspectives of hydrocarbon degraders.In this study 6 strains of optimized culture were processed for the production of biosurfactant and the molecular level characterization was done by performing 16S rRNA sequencing technique of bacteria degrading hydrocarbon.Among the six strains Pseudomonas sp produced biosurfactant on performing16S rRNAsequencingtechniqueit was clearly found as *Pseudomonas aeruginosa.*

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Introduction

Chemical and biosurfactants are amphiphilic compounds which can reduce surface and interfacial tensions by accumulating at the interface of immiscible fluids and increase the solubility and mobility of hydrophobic or insoluble organic compounds [19, 23, 14]. During hydrocarbon degradation the degradative organisms produce an amphibolic compounds that influence the degradation rates. These compounds are known as Biosurfactants. Biosurfactants produced as metabolic by products, are not only potentially as effective. But after some distinct advantages over the highy used synthetic surfactants[15], Such as lower toxicity; higher biodegradability [34]; better environmental compatibility [5]; higher foaming [21]; high selectivity and specific activity at extreme temperatures, pH, and salinity [13, 33]; and the ability to be synthesized from renewable feedstocks.
Due to the wide variety of complex individual substrates constituting total petroleum hydrocarbons and different metabolite interactions, there are certain inherent difficulties in characterizing microbial communities impacted by total petroleum hydrocarbons. Despite these problems, there has been extensive research to analyze the microbial abundance in such contaminated sites and to associate the microbial communities with the ecosystem function. For the proper exploitation of the bioremediation potential of indigenous microflora, the study of microbial diversity of site contaminated with total petroleum hydrocarbons with a polyphasic approach becomes imperative. The elucidation of diversity at the genomic level will help to understand the evolutionary and phylogenetic perspectives and will lead to conservation ofindigenousmicroflora, which are of economic and environmental importance. Whereas detection of the functional variability of the bacterial strains from the contaminated sites by investigating the degradative phenotype will allow the selection of specific bacterial strains for designing bioremediation strategies [22, 2]. Hence the objective of the research is to examine the biosurfactant production potential of optimized bacterial isolates from petrol contaminated sites and the biosurfactant producing strains were further characterized by genetic methods using the 16S rRNA sequencing technique to find out by species level.

Material and Methods

2.1 Production of Biosurfactant

The optimizedhydrocarbon degrading bacteria were maintained on trypticase soy agar at 4ºC for studying their biosurfactant producing ability.

2.1.1 Inoculum preparation

Liquid minimal salt medium was prepared and sterilized at 121ºC 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 trypticase soy agar plates to minimal salt medium. The flasks were incubated at
28ºC for 2 days in rotary shaking at 200 rpm.

2.1.2 Production of Surfactant by Bacteria

5ml 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 28ºC, 200rpm for 7 days.

2.1.3 Extraction of the Biosurfactant

Each cultures were centrifuged at 8000 rpm, 4ºC for 10 min to harvest the cells. The culture supernatant was taken. p^H of the culture supernatant was lowered to 2 with 5M HCl and incubating at 4ºC for 24 hrs. The precipitate was separated by centrifugation at 8000 rpm for 20 mins. This white precipitate formed culture was selected.

2.1.4 Chemical analysis of Biosurfactants [27]

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 2ml of anthrone reagent and it was thoroughly mixed. Colour changes were observed.

Iodine Test

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

Barfoed Test

2 ml of Barfoed’s reagent was added to the little amount of the biosurfactat. The tubes were heated in a boiling water bath. The formation of colour and also the time taken for its appearance was noted.

Bial’s Test

2ml of Bial’s reagent was added to 1-2 drops of biosurfactant. The tubes were heated 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 and chloroform was added to each tube. Their solubility was tested.

Saponification Test

2ml 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 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.

2.2 Identification of the Potential Hydrocarbon degrading strain.

2.2.1 Identification and phylogenetic analysis

The identification and phylogenetic relatedness of the isolates was assessed based on the partial 16S rRNA gene sequences using universal primer. To identify unknown bacterial isolates, the 16S rDNA sequences obtained were subjected to basic local alignment search tool (BLAST) search. This search was performed with Microseq identification and analysis software “Microseq™ Analysis software v. 1.40, Microseq™ 16S rDNA Sequence Databases v. 1.01” (PE Applied Biosystems, USA). The sequences were also analyzed with the BLAST (N) search against the non-reduntantGenbank+EMBL+DDBJ+PDB databases using NCBI web service: www.ncbi.nlm.nih.gov/blast. A phylogenetic tree was constructed using the neighbor-joining method (Saitou andNei, 1987). Tree topologies were evaluated through bootstrap analysis by MEGA 4.0 package [31].

Result

Production of Biosurfactant

The selected 6 strains of *Bacillus sp. Pseudomonas sp.* and *Micrococcus sp.*(B4, B5, P1, P2, P3 and M1) by optimization were subjected to check the ability of biosurfactant production.

Among 6 strains *Pseudomonas sp.*P1 only producedbiosurfactant (white precipitate) by using glucose as the substrate (Table 1).

The isolated biosurfactant was chemically analysed, (Table1) the presence of amino acids, carbohydrates and lipids in biosurfactant was carried out. (Table 2)

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 change 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 are performed to distinguish between mono and disaccharides the formation of red precipitate with 2-5 min was observed that indicates the presence of monosaccharides. 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, NaOHsaponifies 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.

16SrRNA Sequencing

In our study we have isolated and sequenced our tentative organism's 16SrRNA sequence and performed the nucleotide comparison analysis with the help of NCBI BLAST to identify the organism at species level. As showed in the table the top hit showed clearly that the organism must be belongs to Pseudomonas genera and the species might be *P.aeruginosa* and it has showed more than 99% identity and confirmed that the isolated organism belongs to the species *P.aeruginosa*and also the Phylogenetic tree was constructed (Figure 1).

Sequence Allignment of *Pseudomonas aeruginosa*

>120814-38_O07_M2_800R.ab1 781

**CCGGTCTTTTCGCACTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTTCGC
CACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATT
CCACCACCCTCTACCGTACTCTAGCTCAGTAGTTTTGGATGCAGTTCCCA
GGTTGAGCCCGGGGATTTCACATCCAACTTGCTGAACCACCTACGCGCGC
TTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTTCGTATTACCGCGG
CTGCTGGCACGAAGTTAGCCGGTGCTTATTCTGTTGGTAACGTCAAAACA
GCAAGGTATTAACTTACTGCCCTTCCTCCCAACTTAAAGTGCTTTACAAT
CCGAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCA
TTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCT
CAGTTCCAGTGTGACTGATCATCCTCTCAGACCAGTTACGGATCGTCGCC
TTGGTAGGCCTTTACCCCACCAACTAGCTAATCCGACCTAGGCTCATCTG
ATAGCGTGAGGTCCGAAGATCCCCCACTTTCTCCCTCAGGACGTATGCGG
TATTAGCGCCCGTTTCCGGACGTTATCCCCCACTACCAGGCAGATTCCTA
GGCATTACTCACCCGTCCGCCGCTGAATCCAGGAGCAAGCTCCCTTCATC
CGCTCGACTTGCATGTGTTAGGCCTGCCGCCAGCGTTCAATCTGACGGAA
AAAAAAAACCAAAAAAAAAAGGGGAGCTGCA**

Table 1: Chemical Analysis of biosurfactant produced by the isolates

Sl.No	Qualitative Analysis	Production of Biosurfactants by the strains					
		B1	B2	P1	P2	P3	M1
1	Amino acids	-	-	-	-	-	-
2	Carbohydrates	-	-	+	-	-	-
3	Lipids	-	-	+	-	-	-

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

Table 2: Strains showing Results of Chemical Analysis Test for Biosurfactant Production

S..No	Strains	Chemical Analysis test							Solubility test		
		Ninhydrin test	Anthrone test	Iodine test	Barfoeds test	Bial's test	Saponification test	Achrolin test	Water	Alcohol	Chloroform
1	B4	-	-	-	-	-	-	Pungent smell is present	Soluble	Insoluble	Insoluble
2	B5	-	-	-	-	-	-	Pungent smell is present	Soluble	Insoluble	Insoluble
3	P1	-	+	-	-	+	+	No pungent smell	Insoluble	Soluble	Soluble
4	P2	-	-	-	-	-	-	Pungent smell is present	Soluble	Insoluble	Insoluble
5	P3	-	-	-	-	-	-	Pungent smell is present	Soluble	Insoluble	Insoluble
6	M1	-	-	-	-	-	-	Pungent smell is present	Soluble	Insoluble	Insoluble

+ = Positive; - = Negative

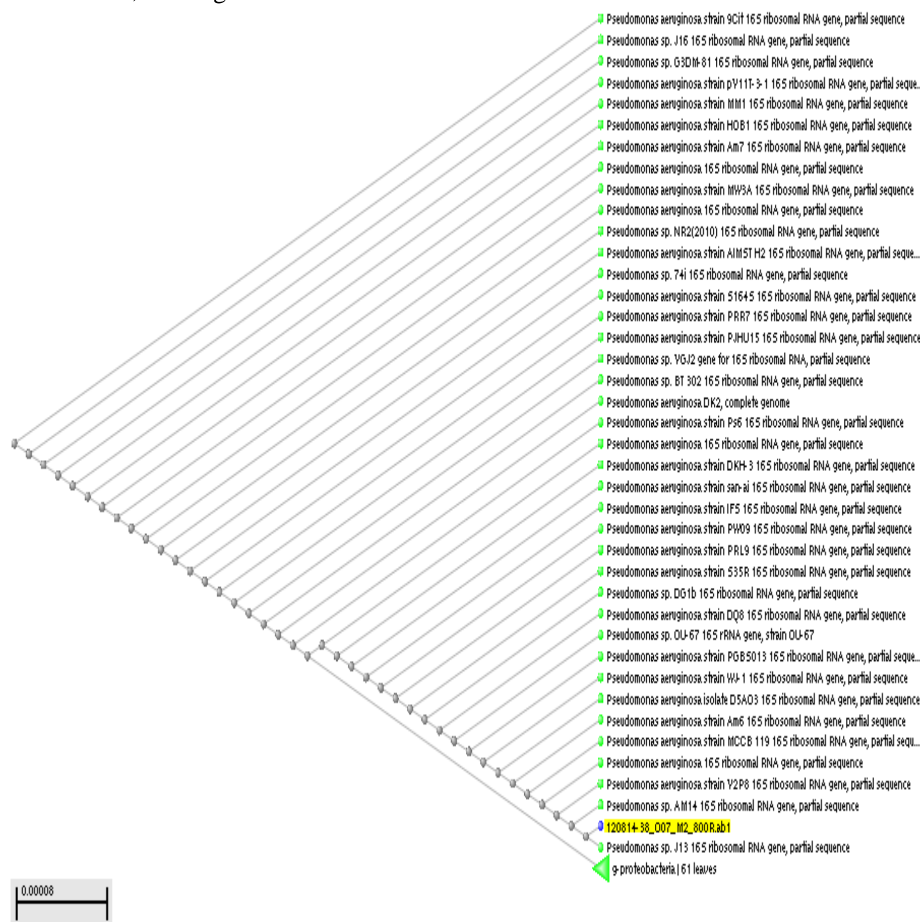

Figure 1: Phylogenetic Tree of *P. aeruginosa*showing 99% sequence similarity.

Discussion

Pseudomonas sp. is the most common bacterial hydrocarbon degrader reported in the literature, which is widespread in nature and can degrade a wide range of Xenobiotics (Rusansky*et al*.,1987; Kiyohara*et al*., 1992; Jonhson*et al.*, 1996; Barathi and Vasudevan, 2001; Pokethitiyook*et al*., 2003; Van Hamme*et al.,*2003). The present study proved it and *Pseudomonas sp*., was isolated as one of the isolates.
In present research, hydrocarbon 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 *Pseudomonassp* 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-hydroxydecanic acid. Several species of *Pseudomonassp*, 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, the optimized *Pseudomonassp* was analyzed for biosurfactant production, chemically; the presence of carbohydrates, lipids was confirmed. That particular carbohydrates, 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).
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.

Conclusion

Cleaning up of petroleum hydrocarbons in the substance environment is a real world problem. A better understanding of the mechanism of biodegradation has a high ecological significance that depends on the indigenous microorganisms to transform or mineralize the organic contaminants. Microbial degradation process aids the elimination of spilled oil from the environment after critical removal of large amounts of the oil by various physical and chemical methods. This is possible because microorganisms have enzyme systems to degrade and utilize different hydrocarbons as a source of carbon and energy.
Species level identification, 16S rRNA sequence analysis was performend to identify the potent degrading strain as *P. aeruginosa.* Finally, the strain P1 (*P. aeruginosa*) has the ability of producing biosurfactant. It can be concluded that in terms of biodegradability and environmental security, these compounds are more suitable for environmental applications.

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