

BIOEMULSIFIER PRODUCTION AND MOLECULAR DETECTION OF *PSEUDOMONAS* SPP. ISOLATED FROM HYDROCARBON CONTAMINATED SOIL

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Abstract: The soil is the complex mixture and harbors different kinds of contaminants. Petroleum-derived hydrocarbons are among the most persistent soil contaminants which can be degraded by microorganisms by producing biosurfactant and increase bioavailability of these pollutants. Some microorganisms can degrade these hydrocarbons and produce surface active compounds. Present study was focused on bioemulsifier producing *Pseudomonas aeruginosa* isolated from hydrocarbon contaminated soil with ability to utilize hydrocarbon. From 30 hydrocarbon contaminated soil samples, six strains of *Pseudomonas* spp. were isolated and its ability to produce stable oil water emulsion with different vegetable and petroleum oil was determined. The isolated strains of *Pseudomonas* sp. were identified by 16S rRNA sequencing and these were screened for ability to produce high molecular weight bioemulsifier by conventional methods by measuring reduction in surface tension, oil spreading ability and emulsification index. The study reveals that the isolates have ability to utilize as well as produce low and high molecular weight bioemulsifier when cultivated on oil containing medium.

Keywords: Bioemulsifier, *Pseudomonas aeruginosa*, 16S rRNA sequencing.

INTRODUCTION

The accidental release petroleum and petroleum-derived products in soil causes pollution in soil and aquatic environment. These hydrocarbon molecules are very difficult to remove as it is persistent and immiscible in water (Rahman *et al.*, 2003). These contaminants can be removed by natural processes collectively called as weathering, but it takes several years. To accelerate the rate of degradation and mineralization of these molecules, such oil spills are often treated with synthetic surfactant. These synthetic surfactants increase the contaminants solubility but are often toxic in nature and may lead to the formation of toxic intermediates metabolites (Bognolo, 1999). The first investigation on microbial degradation of petroleum product as non expensive substrate for producing biomass was reported in 1960. Biosurfactant generally are of major two types, low-molecular weight biosurfactants and high

molecular weight biosurfactant. Low-molecular weight biosurfactants are structurally related with glycolipids and lipopeptides, while the high-molecular weight biosurfactants are structurally related with lipopolysaccharides, lipoproteins or a combination of both (Rosenberg and Ron, 1999; Christofi and Ivshina, 2002). These high-molecular weight compounds are associated with production of stable emulsions, but the lowering of the surface tension or interfacial tensions is not a usual trait of these and is frequently called as bioemulsifier (Bognolo, 1999).

Every microorganism present in soil and water are not capable to grow in contaminated oil and utilize the hydrocarbon due to flotation property and hydrophobicity of oil (Lin *et al.*, 2005). Noudeh *et al.*, (2007) studied the bioemulsifier production from bacteria *Bacillus licheniformis* isolated in nutrient broth. The microbial communities like *Acinetobacter*, *Arthrobacter*, *Pseudomonas*, *Halomonas*, *Bacillus*, *Rhodococcus*, *Enterobacter*, and yeast have been reported to produce bioemulsifier (Schulz *et al.*, 1991; Passeri *et al.*, 1992; Banat, 1993; Maneerat *et al.*, 2006). The bioemulsifier produced from the organisms has potential application in bioremediation of oil-polluted soil and water (Christofi and Ivshina, 2002), enhanced oil recovery, replacement of chlorinated solvents used in cleaning-up oil-contaminated pipes and the formation of stable oil-in-water emulsions for the food (Shepherd *et al.*, 1995). Despite the large application of bioemulsifier in various fields these were poorly studied. In this study the bioemulsifier producing bacteria *Pseudomonas aeruginosa* were isolated and studied for their potential to produce biosurfactant as well as bioemulsifier using diesel and coconut oil as source of hydrocarbon and fatty acid respectively.

MATERIALS AND METHODS

Collection site and sample collection: For isolation of bioemulsifier producing bacteria hydrocarbon contaminated soil samples were collected from different hydrocarbon contamination sites including automobile work shops and petrol pumps. The hydrocarbon contaminated soil samples were collected in zip lock plastic bag with the help of sterile scooper and were carried to the laboratory for further analysis.

Isolation procedure: A one gram of soil from each sample was transferred in sterile saline medium and transferred to a 250 mL capacity Erlenmeyer flask containing 100 mL mineral salt medium of composition: NaNO₃ 2.5 g/l, KH₂PO₄ 3.0 g/l, K₂HPO₄ 7.0 g/l, CaCl₂ 0.01 g/l, MgSO₄.7H₂O 0.5 g/l, and trace element solution containing FeSO₄.7H₂O 0.116 g/l, H₃BO₃ 0.232 g/l, CoCl₂.6H₂O 0.41 g/l, CuSO₄.5H₂O 0.008 g/l, MnSO₄.H₂O 0.008 g/l,

[NH₄]₆Mo₇O₂₄ 0.022 g/l, ZnSO₄ 0.174 g/l and 2% of respective oil as a carbon source Tambekar *et al.*, (2012). The continuous enrichment and subculturing of the culture was done by continuous shaking at 200 rpm for 72 h on orbital shaking incubator. The pure culture of the bacteria was isolated on solid nutrient agar plate by streak plate method. The isolated culture stocks were stocked and further identification was made.

Screening for bioemulsifier producing strain: The isolated bacterial strains were screened for bioemulsifier production. The culture broth was inoculated in the flask containing 200 mL mineral salt medium with 2% respective oil as carbon source. The broths were centrifuged after 5 days of incubation at 8000 rpm for 30 min and the cell free culture supernatant was collected. In order to distinguish between bioemulsifier and low molecular-weight surface active compounds, the surface tension of the cell free culture supernatant was measured (Morikava *et al.*, 1993). The bioemulsifier production was determined by the emulsification index determination method suggested by Cooper and Goldenberg, (1987). The bioemulsifier production ability of the isolates was determined by using coconut oil and diesel as source of carbon.

16S rRNA sequencing and phylogenic analysis: The bioemulsifier producing strains were identified by 16S rRNA sequencing and their phylogenetic analysis was done. The phylogenetic tree was constructed using software programme Mega version 4, by using neighbor joining method (Tamura *et al.*, 2007).

RESULTS AND DISCUSSION

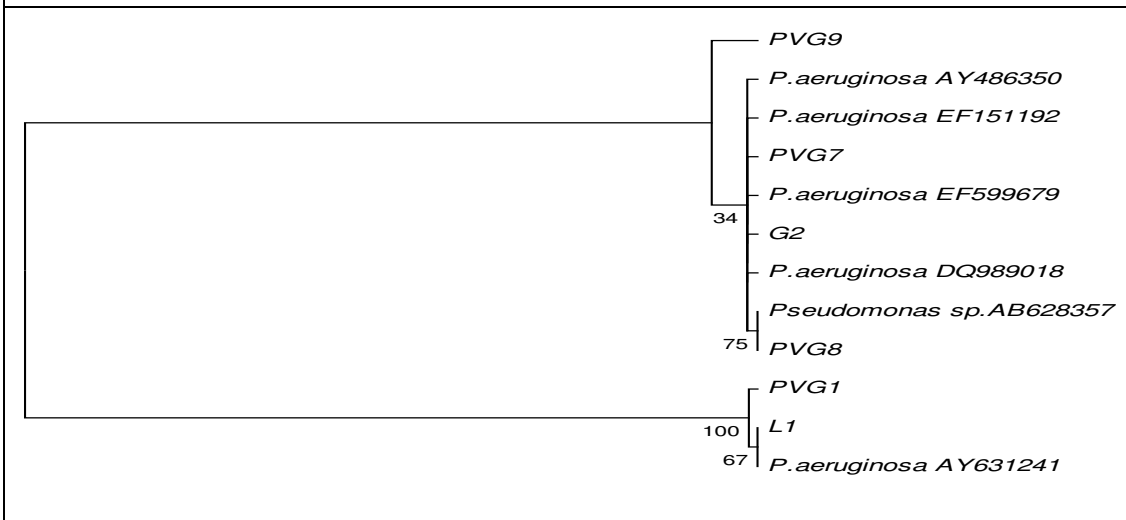
The bioemulsifier producing microorganism is generally screened by monitoring surface activity, emulsifying property and reduction in surface tension. The bioemulsifier producing bacteria were screened on diesel and coconut oil to avoid the over crowding of the results. From the 30 samples collected from the hydrocarbon contaminated soil 18 different bacterial strains were isolated. Out of these eighteen bacterial cultures, three were Gram positive and remaining fifteen was Gram negative. The Gram negative bacteria were predominating in hydrocarbon degradation and biosurfactant production. The results of the study were agreed with the results of the Bicca *et al.*, (1999). The isolated strains were further characterized by using standard biochemical characteristics and were identified by 16S rRNA sequencing from National Center for Cell Sciences (NCCS) Pune. Result of the sequencing showed that out of 18 bacterial cultures, six were belonging to phylum Proteobacteria and identified as *Pseudomonas aeruginosa* which were used in bioemulsifier production.

Table 1: Detection of reduction in surface tension and oil displacement ability of the culture supernatant

Isolation code	Species	Reduction in surface tension (mN/m)		Diameter of oil displacement (mm)	
		Coconut oil	Diesel	Coconut oil	Diesel
G2	<i>Pseudomonas aeruginosa</i>	21.45	35.97	75	70
L1	<i>Pseudomonas aeruginosa</i>	23.76	63.08	35	15
PVG1	<i>Pseudomonas aeruginosa</i>	28.17	43.12	25	20
PVG7	<i>Pseudomonas aeruginosa</i>	22.08	44.09	70	20
PVG8	<i>Pseudomonas species</i>	23.97	31.6	90	20
PVG9	<i>Pseudomonas aeruginosa</i>	23.14	33.54	80	55

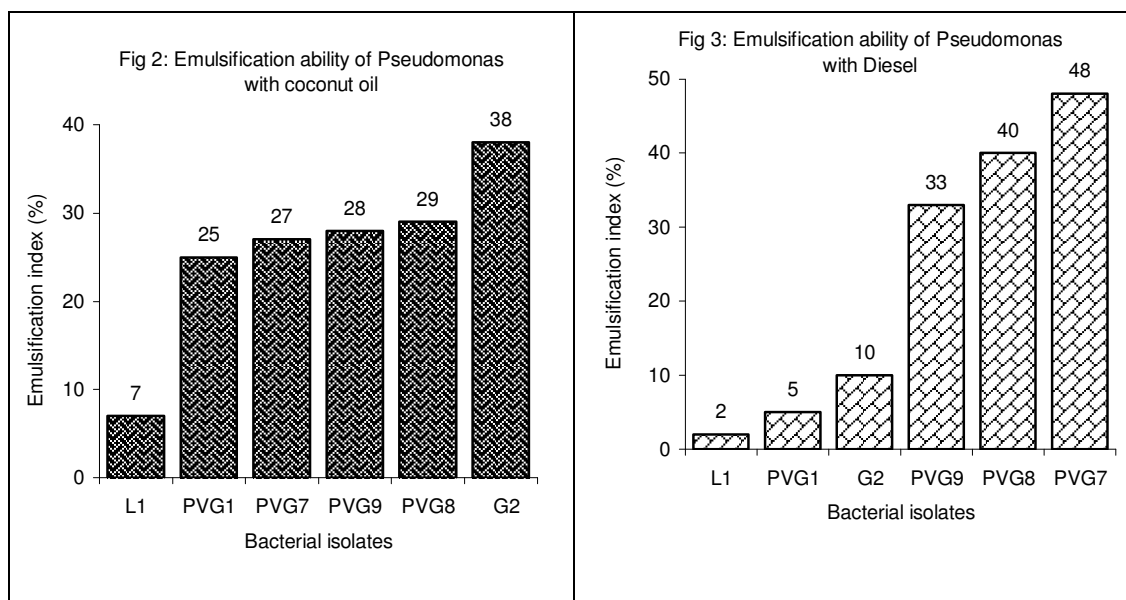
These biochemically characterized bacterial strains were screened for their ability to produce surface active agent using diesel and coconut oil which contain hydrocarbon and fatty acid source respectively. The production of low molecular weight surface active agent was firstly detected by measuring the reduction in the surface tension of broth by Stalagmometer (Morikava *et al.*, 1993) and the oil displacement test (Youssef *et al.*, 2004).

Fig 1: Phylogenetic Tree Based on a Comparison of the 16S Ribosomal DNA Sequences of the isolates, the Tree was Created by the Bootstrap Neighbor-Joining Method Using MEGA 4 Package



The isolates with ability to produce bioemulsifier were analyzed by Bootstrap neighbor joining phylogenetic analysis Bootstrap analysis was used to evaluate phylogenetic tree stability according to a consensus tree from the neighbor-joining based on 1,000 replicates for

each isolate. Phylogenetic analysis based on 16S rRNA gene sequences indicated that all the strain G2, L1, PVG1, PVG7, PVG8 and PVG9 were affiliated to phylum Proteobacteria with genera *Pseudomonas* (Fig 1). The highest similarity values with the sequences of G2 relate with *Pseudomonas aeruginosa* (EF599679) isolated from organophosphorus (OP) contaminated sites. The isolates L1 and PVG1 showed highest similarity value with *P. aeruginosa* (AY631241). While the isolates PVG7 and PVG9 matches with *P. aeruginosa* (AY486350) recovered from cystic fibrosis patients and PVG8 matches with *Pseudomonas* sp. (AB628357) isolated from Rhizosphere of potato plant.



The result of the primary detection showed that all these six isolates of *Pseudomonas* reduces the surface tension of broth below 30 mN/m in presence of coconut oil and also gives positive oil displacement test, while in presence of diesel the isolates gives positive oil displacement test but no significant decrease in the surface tension. The results were clearly indicating the ability of these strains to produce active low molecular weight surface active agent with easily digestible fatty acid source (Table 1). These results are contradict with studies of Sabina *et al.*, (2010), but similar results were obtained by Batista *et al.*, (2006). The ability of the isolates to produce bioemulsifier was determined by emulsification index using respective oil and cell free culture supernatant. The highest value for emulsification index of coconut oil was 38% showed by the isolates G2, while the lowest index 7% was observed for isolate L1. The other four isolates showed the emulsion range in between of 25% to 29% with coconut oil (Fig. 2). Jagtap *et al.*, (2010) isolated the bioemulsifier producing *Acinetobacter* species

from the human skin which showed 79.2% of emulsification index with coconut oil as well as also produce the emulsion with other carbon sources and hydrocarbon sources. Shubhrasekhar *et al.*, (2013) isolated the bioemulsifier producing bacteria by using mustard oil as carbon source and determined bioemulsification properties of isolates. The results of these studies were concordance with the present studies. The all *Pseudomonas* isolates were able to form emulsion with diesel. The highest emulsion was observed with isolates PVG7 (48%). Two isolates PVG9 and PVG8 forms emulsion between 33-40%, while the remaining three showed emulsion below 10% (Fig. 3). Ganesh and Lin, (2009) isolated the diesel degrading bacteria from hydrocarbon contaminated soil samples, all these isolates forms highest emulsion value 32.8% with diesel but in present study, the isolates PVG7 showed higher emulsion value 48%. Raza *et al.*, (2007) also studied the bioemulsifier production by *Pseudomonas aeruginosa* mutant using vegetable oil refinery wastes (paraffin, hexadecane and kerosene) but the values for emulsion formation was not exceeding more than 15%.

Conclusion: In present study all the *Pseudomonas* strains isolated from hydrocarbon contaminated soil showed the ability to degrade the fatty acid and hydrocarbon sources with simultaneous production of biosurfactant with high and low molecular weight compounds. The production was determined by using different carbon sources. Present study provides the supports previous findings on biosurfactant production and suggesting that the isolates with ability to produce biosurfactant or bioemulsifier could be suitable for effective in situ bioremediation of hydrocarbon contaminated sites.

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