RESEARCH PAPER Asian Journal of Bio Science, Vol. 4 Issue 1 : 24-28 (April to September, 2009)

Plasmid incidence and utilization of kerosene by hydrocarbonoclastic fluorescent pseudomonads isolated from local soil

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(Accepted : January, 2009)

Environmental concerns have led to the need of sustainable use of natural resources. It is important to use natural bacterial population for environmental cleaner techniques. One of those strategies is biodegradation of natural or man made xenobiotic compounds. The present investigation is on isolation of hydrocarbon utilizing pseudomonads from local red soil. The isolates were identified to species level by performing biochemical and physiological tests as *P. aeruginosa*, *P. aureofaciens*, *P. putida* and *P. fluorescens*. The isolates were screened for the utilization of petroleum hydrocarbon (kerosene) and the conditions (temp and pH) optimum for its utilization. The isolates were screened for their ability to utilize petroleum hydrocarbon (kerosene) as their sole source of carbon and energy. Biodegradation results revealed that, the highest growth was showed by *P. putida* followed by *P. fluorescens*, *P. aureofaciens* and *P. aeruginosa*. The results evidenced that, all the four isolates harbored two low molecular weight plasmids one with 3Kb and the other with 10kb to 12Kb.

Key words : Hydrocarbonoclastic bacteria, Fluorescent pseudomonads, Kerosene utilization, Plasmids.

INTRODUCTION

Environmental pollution is a cause of major concern affecting ecosystems globally. Fuels are significant pollutants of soils and ground water because of leaks of under ground storage tanks and defectiveness of transfer lines (Council on Environmental Quality, 1981). As industrialization expands, petroleum hydrocarbons became a greater potential source of contaminants in the water and soil environments (Margesin and Shinner, 2001). Bioremediation is the newest method of oil spill cleanup and far more effective than any of the mechanical methods used (Desai and Banat, 1997). Microbes are the main degraders of petroleum hydrocarbons in contaminated ecosystems. The most prevalent bacteria that degrade the hydrocarbons belong to the genus *Pseudomonas* (Atlas and Cerniglia, 1995) and it is classified as the most common hydrocarbonoclastic microorganisms in the list of Bossert and Bartha (1984). Interest in pseudomonads has increased because of their possible use in detoxifying chemical wastes through a wide range of enzymatic metabolic activities (Raijimarkers *et al.*, 1995). Ojo (2006) reported the use of native bacterial consortium with petroleum hydrocarbon utilizing capabilities in Southwest Nigeria. Bioremediation of long chain hydrocarbons such as kerosene, diesel and waste oil was reported by Livingston *et al.* (1976). A large kerosene spill in New Jersey was cleaned up by a combination of physical and biological techniques (Dibble and Bartha, 1979). Kerosene utilizing microorganisms belonging to the genus *Mycobacterium* and *Pseudomonas* were reported by Haas *et al.* (1941).The effective utilization of petroleum hydrocarbons *viz*., diesel, kerosene and petroleum waste by *P. aeruginosa* was reported earlier by Modi and Patel (1968).

Plasmids that have been found to harbor genes encoding the transformation of environmental pollutants are known as catabolic plasmids. The plasmid mediated bacterial utilization of various carbon compounds which could be found in the complex mixture of crude oil was also reported earlier (Chakraborthy, 1976). Presence of catabolic plasmids in *P. putida* was reported by Park *et al.* (2003). The present study was carried out to investigate the presence of plasmids in four isolates of hydrocarbonoclastic fluorescent pseudomonads and their biodegradation ability of kerosene oil.

MATERIALS AND METHODS

Microorganisms:

Fluorescent pseudomonads were isolated from soil collected at Acharya Nagarjuna Univesity Campus, Guntur district, Andhra Pradesh, India. For the isolation, fluorescent pseudomonad selective King's B medium (King *et al.*, 1954) with the composition of (g/L^{-1}): Protease peptone – 20.0, Purified Glycerol-15.0, K_2HPO_4-2.5, $MgSO_4.7H_2O$-6.0, Agar agar-20.0 and pH-7.2 was used. A total of fifteen isolates were isolated and among them four abundant isolates were identified to species

level by performing a series of biochemical tests as per Bergey's Manual of Systematic Bacteriology (Holt *et al.*, 1994) and used for further studies of utilization of petroleum hydrocarbon.

Petroleum hydrocarbon (kerosene):

Kerosene is defined as that group of hydrocarbons with a boiling temperature ranges approximately 180°C to 320°C and contains hydrocarbons from C_{11} to C_{12}. Higher fractional kerosene can contain alkanes up to C_{18} and aromatic compounds with higher molecular weight. The actual composition of kerosene is 80% of n-alkanes and branched alkanes, 13% alkyl mono and poly nuclear aromatics.

Optimization of kerosene level, temperature and pH:

Fluorescent pseudomonads were tested for the growth in the presence of kerosene. The cultures were inoculated into nutrient broth medium in tubes containing different levels *viz.*, 0.0ml, 0.2ml, 0.4ml, 0.6ml, 0.8ml and 1.0ml of kerosene per 10 ml of medium. After 24 hours of incubation, the growth was measured in terms of OD values and selected the best level of kerosene yielded maximum growth. Using the best level of kerosene, the effect of different temperatures *viz.*, 0°C, 10°C, 20°C, 30°C, 35°C and 40°C on growth of isolates was studied. For this, the broth medium with best level of kerosene was inoculated with cultures and incubated at above said temperatures. After incubation for 24 hours, growth was measured in terms of OD values. In a similar way, the effect of different pH *viz.*, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 was also studied at the best level of kerosene in the medium.

Utilization of kerosene as sole source of carbon and energy:

The influence of different ratios *viz*., 0:100, 25:75, 50:50, 75:25 and 100:0 of glycerol and kerosene on the growth of four isolates were also studied by using King's B medium.

Isolation of plasmids:

The plasmid DNAs of four *Pseudomonas* species were isolated and their molecular weight was determined by agarose Gel Electrophoresis using Gel EluteTM endotoxin – free plasmid midiprep kit.To determine the molecular weight of the plasmid DNA Lamda DNA/ EcoRI+Hind III marker standard was also loaded along with the samples for electrophoresis.

RESULTS AND DISCUSSION

Biodegradation by naturally occurring populations of microorganisms is the major mechanism for the removal of petroleum from the environment (Dua, 2002). The multiple antibiotic resistance exhibited by the isolates is a common phenomenon of fluorescent pseudomonads and it is important factor to consider the use of these organisms in biocontrol measures (Okoh *et al.*, 2001). Isolation and identification of hydrocarbonoclastic bacteria from phylloplane of ten tropical plants and their luxuriant growth on diesel and kerosene was reported earlier by Olusoji *et al.* (2006). In the present investigation, the four abundant fluorescent pseudomonads among the strains isolated from local red soil were identified as *P. aeruginosa*, *P. aureofaciens*, *P. putida* and *P. fluorescens* based on morphological, physiological and bio-chemical characters (Table 1) with reference to Bergey's Manual of systematic bacteriology (Holt *et al.*, 1994). The four *Pseudomonas* species were screened for their hydrocarbonoclastic nature using kerosene as carbon source. During the study of optimization of kerosene level for better growth, 6% kerosene level was found more optimum for all the four species. *Pseudomonas* putida exhibited a greater growth at all levels of kerosene tested, followed by *P. fluorescens*, *P. aureofaciens* and *P. aeruginosa* (Table 2). Optimization of temperature and pH conditions for better growth at 6% kerosene level was also studied and observed 35°C temperature and 8.0 pH as optimum (Fig. 1 and 2). However, Leahy and Colwell (1990) opined that so many factors affect the degradation of oil include concentration of oil, temperature, salinity, pressure and water activity. Successful degradation of kerosene, diesel and waste oil by *Pseudomonas* and other bacterial genera was reported earlier by Livingston *et al.* (1976). The successful utilization of petroleum hydrocarbons including the kerosene by *Pseudomonas* species isolated from petroleum contaminated soil was reported by Emtiazi *et al.* (2005). Dibble and Bartha (1979) reported the cleaning of a large kerosene spill in New Jersey by bioremediation and physical removal techniques. Also in the present study, all the four *Pseudomonas* species showed better utilization of kerosene than glycerol and exhibited better growth when tested with different proportions of kerosene and glycerol (Table 3). All the four species exhibited effective utilization of kerosene when supplemented as sole carbon source than glycerol. The order of potentiality of utilization of kerosene at 100% proportion by *Pseudomonas* species was found to be *P. putida* < *P. fluorescens* < *P. aureofaciens* < *P. aeruginosa*.

Table 1: Morphological, staining and bio-chemical characters of the four dominant fluorescent *Pseudomonas* isolates

Test	Ps1	Ps8	Ps11	Ps13
Morphological				
Size	0.6 x 1.7µm	0.8 x 2.0 µm	0.8 x 2.2 µm	0.8 x 2.5 µm
Shape	Rod	Rod	Rod	Rod
Staining				
Grams staining	Negative	Negative	Negative	Negative
Spore staining	Negative	Negative	Negative	Negative
Acid fast staining	Negative	Negative	Negative	Negative
Bio chemical				
Starch hydrolysis	-	-	+	+
Catalase	+	+	+	+
Nitrate reduction	+	-	+	+
H_2S production	+	+	+	-
Caseinase	-	+	+	-
Gelatin liquefaction	+	+	-	+
Indole production	+	+	+	+
Methyl red	+	-	-	+
Voges Proskaur	+	+	-	+
Citrate	+	+	+	+
Glucose fermentation	-	-	-	-
Litmus milk reaction	+	+	+	+
Tween 80 hydrolysis	+	+	+	+
Arginine dihydrolase	++	+	++	+
Growth				
Growth at 4°C	-	-	+	-
Growth at 40°C	+	+	+	+
Identification	*P. aeruginosa*	*P. aureofaciens*	*P. putida*	*P. fluorescens*

Table 2: Effect of different levels of kerosene on the growth (OD Values) of fluorescent *Pseudomonas* isolates

Pseudomonas isolates	Concentration of the kerosene (in ml)					
	0.0	0.2	0.4	0.6	0.8	1.0
P. aeruginosa	0.02±0.0	0.04±0.0	0.05±0.00	0.08±0.01	0.04±0.01	0.03±0.0
P . aureofaciens	0.04±0.0	0.06±0.01	0.07±0.01	0.09±0.02	0.06±0.00	0.05±0.0
P. putida	0.06±0.01	0.08±0.02	0.11±0.02	0.13±0.03	0.09±0.01	0.07±0.00
P. fluorescens	0.05±0.00	0.07±0.01	0.09±0.02	0.11±0.02	0.08±0.01	0.06±0.00

Values are average of triplicates with standard deviation

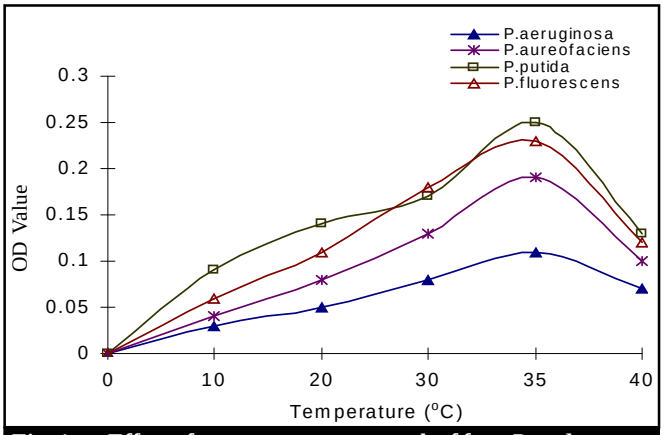


Fig. 1 : Effect of temperature on growth of four *Pseudomonas* species during kerosene utilization

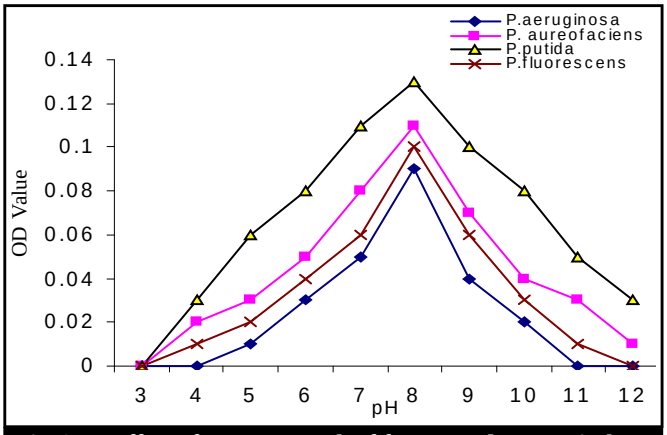


Fig. 2 : Effect of pH on growth of four *Pseudomonas* isolates during kerosene utilization

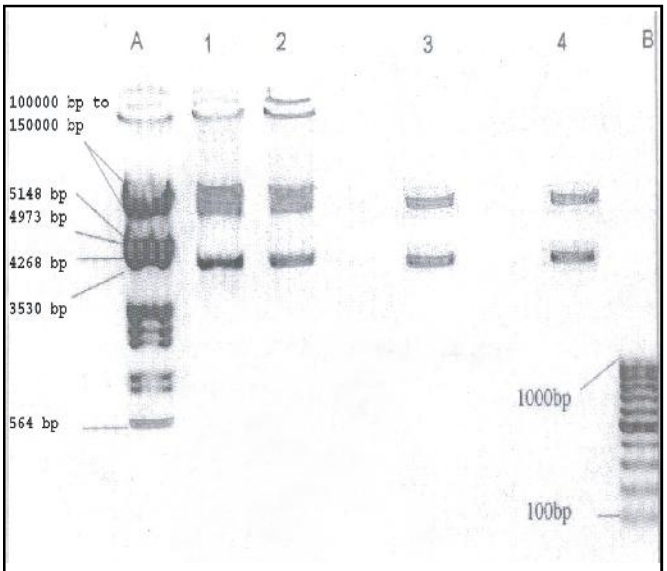
Table 3 : Influence of kerosene and glycerol proportions on the growth (OD values) of Four *Pseudomonas* isolates

Pseudomonas isolates	A	B	C	D	E
P. aeruginosa	0.09±0.003	0.07±0.001	0.05±0.002	0.04±0.002	0.02±0.001
P. aureofaciens	0.10±0.01	0.08±0.002	0.06±0.002	0.05±0.001	0.03±0.001
P. putida	0.14±0.03	0.12±0.005	0.10±0.03	0.08±0.002	0.06±0.001
P. fluorescens	0.11±0.02	0.09±0.003	0.07±0.003	0.06±0.001	0.04±0.001

Values are average o triplicates with standard deviation

A – 0% glycerol + 100% Kerosene B – 25%glycerol + 75% Kerosene C – 50%glycerol + 50% Kerosene

D – 75%glycerol + 25% Kerosene E – 100%glycerol + ±0% Kerosene



Plasmids isolated from four HCB, Lane-1 phage marker, Lane-2 *P. aerugionosa,* Lane-3 *P. aureofaciens*, Lane-4 *P. putida* and Lane-5 *P. fluorescens*

Fig. 3 : Plasmid profile of four hydrocarbonoclastic fluorescent pseudomonads on agarose Gel Electrophoresis

However, *P. aeruginosa* was reported as best kerosene degrader by Wongsa *et al.* (2004). Maximum utilization of diesel and kerosene by *Pseudomonas* aeruginosa was observed by Modi and Patel (1968). Similarly, effective utilization of kerosene and crude oil by *P. fluorescens* was reported by Barathi and Vasudevan (2001). According to Tang *et al.* (2006) from their studies, *P. aeruginosa* was found as the powerful oil degrader and able to survive for at least five years at oil contaminated site.

Catabolic plasmids effect on physiological parameters and efficiency of oil destruction by the *Pseudomonas* was reported earlier by Vetrova *et al.* (2007). Biodegradability of naphthalene and salicylate by *P. fluorescens* bearing seven plasmids was reported by Izmalkova *et al.* (2005). Carney and Leary (1989) reported the plasmid mediated hydrocarbon degradability in *P. putida* RS-3. Presence of naphthalene degrading catabolic plasmids in *P. putida* was reported by Park *et al.* (2003). Results in the present study of plasmid analysis revealed that all the four isolates harbored two plasmids one with the molecular weight of 3Kb and another with 10 to 12 Kb. The very similar observation harboring two low molecular weight plasmids with 4.2 Kb and 3.8 Kb in *P. aeruginosa* was reported earlier (Thavasi *et al.*, 2007). Similar reports on the presence of plasmid DNAs in *Pseudomonas* species were documented well by several workers (Deshpande *et al.*, 2001; Volkova *et al.*, 2005) attributing to the biodegradation potential of the isolates. Deverenx and Sizemore (1982) detected plasmids in 21 % of strains isolated from hydrocarbon contaminated sites and are similar to the plasmids observed in the four *Pseudomonas* isolates of our present study.

In conclusion, results obtained in the present study on utilization of kerosene inferred the possibility of applying these fluorescent pseudomonad bacteria for environmental cleaning of oil spill. Presence of plasmids in bacteria indicated that, the utilization of kerosene may be plasmid mediated.

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