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PRODUCTION OF PROTEASE AND UREASE BY KEROSENE UTILIZING FLUORESCENT PSEUDOMONADS ISOLATED FROM LOCAL RED LATIRITE SOIL

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ABSTRACT
The present work relates to a simple, safe, and efficient process for the complete utilization of kerosene using fluorescent pseudomonads. Fluorescent pseudomonads used in this study were isolated from local red soil collected at Acharya Nagarjuna University Campus, Guntur Dt., (AP) India. The isolates were identified as *Pseudomonas aeruginosa, Pseudomonas aureofaciens, Pseudomonas putida* and *Pseudomonas fluorescens* on the basis of biochemical characteristics. The isolates were screened for their ability to grow on nutrient broth medium in presence of petroleum hydrocarbon (kerosene) and also utilization of kerosene as their sole source of carbon and energy. All the isolates showed maximum growth in medium with 6% kerosene concentration when tested with different concentrations of kerosene viz., 0%, 2%, 4%, 6%, 8% and 10%. The isolates were able to grow well on kerosene and subsequently produced the extracellular enzymes protease and urease at significant level compared with control (without kerosene). Plasmid profile of the isolates revealed that, all the four *Pseudomonas* isolates harbored two low molecular weight plasmids one with 3Kb size and the other with 10Kb to 12Kb size. Thus, these four *Pseudomonas* isolates exhibited high potential in utilizing kerosene and therefore useful for bioremediation of sites that contaminated with kerosene.

INTRODUCTION

Environmental pollution with petroleum hydrocarbons has been recognised as one of the most serious current problems (Plohl *et al*., 2002). Studies on the microbial utilization of hydrocarbons have attracted increasing attention primarily from the point of view of converting hydrocarbon substrates to valuable compounds and pollution abetment (Fedorak and Westlake, 1983). Bioremediation has become an important way of remediation of oil polluted sites and it makes use of indigenous oil consuming miccroorganisms called petrophiles by enhancing and fertilizing them in their natural habitats. Petrophiles are very unique organisms that can naturally degrade large hydrocarbons and utilize them as a food source. This microbial cleanup method is perhaps the best, most environmentally safe process in use today. Interest in pseudomonads has increased because of their possible use in detoxifying chemical wastes through a wide range of enzymatic metabolic activities (Raaijmakers *et al*., 1995). By virtue of their versatility *Pseudomonas* species are known to be involved in the biodegadation of natural or man made toxic chemical compounds (Holloway *et al*., 1992). *P. aeruginosa* is considered to be a good candidate for bioaugmentation of petroleum products such as diesel oil, kerosene oil, and heavy oil in a liquid medium containing mineral salts (Wongsa *et al*., 2004). This potential of *Pseudomonas* species has led to several strains being genetically engineered to assist in cleaning up contaminated site.

The potential of microorganisms as biotechnological sources of industrially relevant enzymes has stimulated a renewed interest in the exploration of microorganisms for extracellular enzymatic activity. Protease refers to a group of enzymes whose catalytic function is to hydrolyze peptide bonds of proteins. Proteolytic enzymes are very important in digestion as they breakdown the protein foods to liberate the aminoacids needed by the body. Additionally, proteolytic enzymes have been used for a long time in various forms of therapy. Protease is one of the most important industrial enzymes occupying nearly 60% of the enzyme sales obtained from microbial, plant and animal sources (Godfrey and Reichelt, 1983). Extracellular protease finds various applications in industrial processes like in detergents, leather tanning, dairy, meat tenderization, baking, brewery, photographic industry etc (Moses and Cape, 1991).

Ureases (EC3.5.1.5) are nickel-dependant enzymes, widespread among plants, bacteria and fungi that hydrolyze urea into ammonia into carbon dioxide. Plant and fungal ureases are homotrimers or hexamers of a ~ 90kDa subunit, while bacterial ureases are multimers of two or three subunit complexes. Urease activity enables bacteria to use urea as the sole nitrogen source.

The aim of this study is to isolate and characterize the dominant fluorescent pseudomonas isolates capable of utilizing kerosene as a sole source of carbon and energy and ability of producing extracellular protease and urease. In addition, to demonstrate that with the kerosene utilizing capability of these

isolates the association of plasmids.

MATERIALS AND METHODS

Isoaltion and identification of microorganisms: The bacterial isolates used in this study were isolated from soil collected at Acharya Nagarjuna University Campus, Guntur District, Andhra Pradesh, India. For the isolation, fluorescent pseudomonad selective king's B medium 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 (King *et al*., 1954). 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 and used for further studies of utilization of petroleum hydrocarbon.

Kerosene (Petroleum Hydrocarbon)

Kerosene is defined as that group of hydrocarbons with a boiling temperature ranges approximately from 180ºC to 320ºC and contains hydrocarbons from C_{11} to C_{12}. Higher fractional kerosene can contains 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

Fluorescent pseudomonads were tested for the growth in the presence of kerosene. The cultures were inoculated into nutrient broth medium in tubes containing different concentrations viz., 0%, 2%, 4%, 6%, 8% and 10% of kerosene per 10mL of medium. After 24h 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 (0ºC, 10ºC, 20ºC, 30ºC, 35ºC and 40ºC) on growth of isolates was studied. For this, the broth medium with the most optimal concentration of kerosene was inoculated with cultures and incubated at above said temperatures. After incubation for 24 h, growth was measured in terms of OD values. In a similar way, the effect of different pH (pH = 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12) was also studied at the best level of kerosene in the medium.

Altogether four dominant strains were screened for extracellular enzymatic activity. For enzyme production, *Pseudomonas* isolates were cultivated at 37ºC for 24h at pH 8.0 in a 250mL erlenmeyer flask containing 50mL of nutrient broth medium with 6% kerosene and subjected to incubation for 24h. After incubation, culture supernatant fluid was obtained by centrifugation at 3000 rpm for 20min. The activity of protease and urease enzymes during kerosene utilization as well as absence of such oil in the medium was studied and expressed in terms of enzyme units by following the standard procedures. At the time of quantitative estimation of enzymes, the growth was also measured in terms of OD values.

Protease assay

Proteolytic activity was measured spectrophotometrically by using Folin-Ciocalteau reagent method (Nannipieri *et al*., 1980). The reaction mixture containing 1.0mL of casien solution and 1.0mL of enzyme sample was allowed to stand for 10min at 37ºC. To this mixture, 2.0mL of TCA was added and incubated for 20min. After incubation, the contents were filtered through Whatmann No 1 filter paper. To 1.0mL of this filtrate, 5.0mL of Na_2Co_3 solution and 1.0mL of the FC reagent were added and incubated the mixture at 37ºC for 30min. The OD was measured at 660 nm on spectrophotometer. The OD value can be taken as one enzyme unit.

Urease assay

Urease activity was measured spectrophotometrically by the method of Tabatabai and Bremner (1972). To the reaction mixture, 10mL of phosphate buffer, 10mL of urea solution, 1.0mL of $ZnSO_4.7H_2O$, 0.5mL of NaOH and 10mL of enzyme sample were added. The contents were allowed to stand for 15 min and filtered through Whatmann No. 42 filter paper. From the filtrate, 10mL aliquot was taken and diluted to 50mL by distilled water. To this, 10mL of EDTA and 3.0mL of Nessler's reagent were added and the developed yellowish-orange color was read at 440nm. Enzyme activity was measured by taking the OD values. The OD value 0.01 can be taken as 1 enzyme unit.

Isolation of plasmids

The plasmid DNAs of four *Pseudomonas* species were isolated and their molecular weight was determined by Agarose Gel Electrophoresis using gel Elute TM endotoxin-free plasmid midiprep kit method. To determine the molecular weight of the plasmid DNA Lambda DNA/*EcoR*I + *Hind* III marker standard was also loaded along with the samples for electrophoresis.

Statistical analysis

Two-way ANOVA analysis was done for Table 1 and student t-test for data of Tables 3 and 4 was carried out.

RESULTS AND DISCUSSION

Biodegradation by naturally occuring populations of microorganisms is the major mechanism for the removal of petroleum from the environment (Dua, 2002). Isolation and identification of hydrocarbonoclastic bacteria from phylloplane of ten tropical plants and their luxuriant growth on diesel and kerosene was reported earlier (Ilori *et al*., 2006). In the present study, potent hydrocarbon (kerosene) utilizing, extracellular protease and urease producing fluorescent pseudomonads were isolated from soil and identified as *P. aeruginosa, P. aureofaciens, P. putida* and *P. fluorescens* on the basis of morphological and bio-chemical characters with reference to Bergey's Manual of Systematic Bacteriology (Holt *et al*., 1994). The data on these biochemical characters of the isolates that are given in Table 1 revealed that all the four isolates were positive for catalase, tween 80 hydrolysis, arginine dihydrolase, litmus milk reaction and indole production, and negative for glucose fermentation, starch hydrolysis, while the other tests were variable.

The four *Pseudomonas* species were screened for their hydrocarbonoclastic ability to utilize various concentrations of kerosene as carbon source. During the study of optimization of kerosene level for better growth, all four isolates grow comparatively well in presence of kerosene at all concentrations relative to control. *P. putida* exhibited a greater

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

TEST	Ps1	Ps8	Ps11	Ps13
Morphological				
Size	0.6X1.7μm	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	+	-	+	+
H2S 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 (ODValues) of fluorescent *Pseudomonas* isolates

Pseudomonas isolates	Concentration of the Kerosene (in %)					
	0%	2%	4%	6%	8%	10%
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; Columns – F- cal value = 32.21; P-value = 0.00 **; Rows – F-cal value = 45. 76 ; P-value = 0.00 **; ** Values are < 0.01 (1%) , so results are highly significant

Table 3: Growth (OD values) and protease activity during Kerosene utilization

Pseudomonas isolates	A		B	
	Growth	protease	Growth	protease
P. aeruginosa	0.15 ±0.03	73 ± 2.28	0.22 ±0.04	138 ± 3.21
P. aureofaciens	0.12 ±0.02	106 ±3.07	0.19 ±0.03	120 ± 2.70
P. putida	0.08 ±0.01	80 ± 2.21	0.15 ±0.03	107 ± 2.67
P. fluorescens	0.07 ±0.01	98 ± 2.03	0.11 ±0.01	100 ± 2.02

A-Without oil; B-Kerosene; Values are average of triplicates with standard deviation; Growth – t-cal value = +3.48 ; p-value = 0.0022 **; Protease – t-cal value = +4.40; p-value = 0.0002 **; ** Values are < 0.01 (1%) , so results are highly significant

growth at all levels of kerosene tested, followed by *P. fluorescens,P. aureofaciens* and *P. aeruginosa* (Table 2), it can be seen that maximum kerosene utilization by the isolates took place at 6% kerosene concentration which indicated that 6% kerosene is the optimum concentration for growth. Although growth was observed at 2%, 4%, 8% and 10% concentrations of kerosene it was relatively less. *Pseudomonas* species isolated from petroleum contaminated soil was successfully utilized petroleum hydrocarbons including the kerosene (Emtiazi *et al*., 2005). Successful degradation of kerosene, diesel and waste oil by *Pseudomonas* and other bacterial genera was reported earlier (Livingston *et al*., 1976). Maximum utilization of diesel and kerosene by *P. aeruginosa* was observed (Modi and Patel, 1968). Also in the present study, all four *Pseudomonas* species showed better utilization of kerosene than glycerol and exhibited better growth when tested with different proportions of kerosene and glycerol. Optimization of temperature and pH conditions for better growth at 6% kerosene level was also studied and it was observed that 35°C temperature and pH 8.0 were optimum. So many factors affect degradation of oil and these include concentration of oil, temperature, salinity, pressure and water activity (Leahy and Colwell, 1990).

The results of the present work showed that all the four isolates exhibited greater protease and urease activity during the utilization of kerosene when compared to that of control (Tables 3 and 4).The order of efficiency of producing protease by the isolates during kerosene utilization was found as *P.*

Table 4: Growth (OD values) and urease activity during kerosene utilization

Pseudomonas isolates	A		B	
	Growth	Urease	Growth	Urease
P. aeruginosa	0.07 ±0.003	4±1.01	0.14±0.04	16±1.05
P. aureofaciens	0.10±0.008	8 ±1.28	0.11±0.02	11±1.04
P. putida	0.09±0.006	6±1.07	0.19±0.03	21±1.04
P. fluorescens	0.08±0.004	5 ±1.04	0.17±0.03	18±1.00

A-Without oil; B-Kerosene; Values are average of triplicates with standard deviation; Growth – t-cal value = + 4.95; p-value = 0.0001 **; Urease – t-cal value = +7.73; p-value = 0.0000 **; ** Values are < 0.01 (1%), so results are highly significant

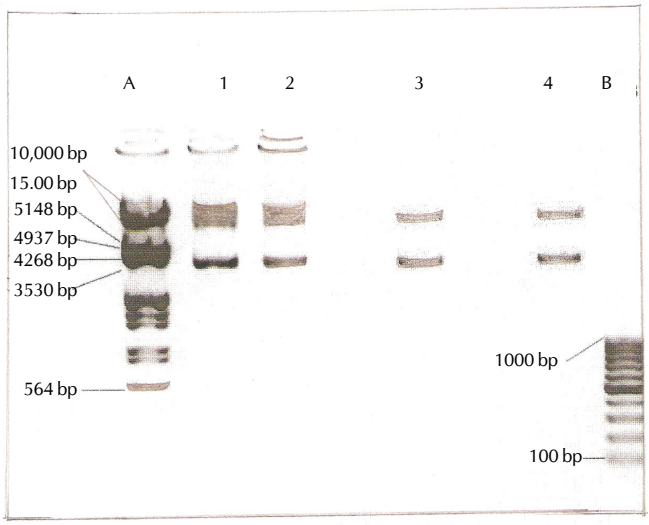

Figure 1: Plasmid profile of four hydrocarbonoclastic fluorescent pseudomonads on agarose Gel Electrophoresis
Plasmids isolated from four HCB; Lane-1: phage marker, Lane-2: *P. aeruginosa*, Lane-3: *P.aureofaciens*; Lane-4: *P.putida* and Lane-5: *P. fluorescens*.

aeruginosa > *P. aureofaciens* > *P. putida* > *P. fluorescens*. The order of efficiency of producing urease by the isolates during kerosene utilization was *P. putida* > *P. fluorescens* > *P. aeruginosa* > *P. aureofaciens*.

Enzymes that have been tested for their potential to monitor hydrocarbon removal include lipases, dehydrogenases, catalases and ureases. Dehydrogenases, catalases and ureases have been found to be useful for indicating the onset of bio degradation process as their activities decline rapidly after the rate of bio degradation has decreased (Margesin and Shinner, 1997). Although proteases are widespread in nature, microbes serve as a preferred source of these enzymes because of their rapid growth. As the genus *Pseudomonas* is a prolific producer of number of extracellular enzymes, there are several reports on the production of extracellular enzymes protease and urease by fluorescent pseudomonads. Studies on production of extracellular protease have been reported earlier by several researchers. The virulence of *P. aeruginosa* was associated with various extra cellular factors like elastase and alkaline protease and contribution of these enzymes in tissue destruction and bacterial invasion during infection (Yagci *et al*., 2002). An interesting application of alkaline protease was developed by Fujiwara and co workers. They reported the use of an alkaline protease to decompose the gelatinous coating of x-ray films, from which silver was recovered (Ishikawa *et al*., 1993). The effect of carbon and nitrogen sources on extracellular protease production by *Pseudomonas* species isolated from local soil (Dutta and Banerjee, 2006).

There are few reports on the production of urease by *Pseudomonas* species. The repressible mode of urease production was reported in *Pseudomonas aeruginosa* and *Pseudomonas* fluorescens (Moriya and Horikoshi, 1993). The production of urease by fluorescent pseudomonad species viz., *P. aeruginosa*, *P. fluorescens* and *P. putida* was also reported during utilization of various hydrocarbons (Margesin and Schinner, 1997).

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). 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 kb to 12 kb (Fig. 1). A similar observation harboring two low molecular weight plasmids with 4.2kb and 3.8 kb in *P. aeruginosa* were reported (Thavasi *et al*., 2007). Similar reports on the presence of plasmid DNAs in *Pseudomonas* species were documented well by several workers attributing to the biodegradation potential of the isolates (Volkova and Anokhina, 2005).

This study contributes to the isolation and characterization of a potent hydrocarbon (kerosene) utilizing and extracellular enzymes (protease and urease) producing fluorescent pseudomonads from local soil. The efficiency of kerosene utilization may be plasmid mediated by the presence of two low molecular weight plasmids in all the four isolates.

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