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Protease and urease production during utilization of diesel by fluorescent *Pseudomonas* species isolated from local soil

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ABSTRACT

Background and objectives: Bacteria, most prevalently the *Pseudomonas* species possess high capacity to utilize and degrade petroleum hydrocarbons and are classified as the hydrocarbonoclastic microorganisms. Many species of the genus *Pseudomonas* are notorious for their aerobic degradation capacity, extracellular enzyme production and are metabolically versatile organisms capable of utilizing a wide range of hydrocarbons and other compounds. In this study, the ability of diesel utilization by some locally isolated *Pseudomonas* species was tested.

Materials and Methods: From a local red laterite soil, four different *Pseudomonas* species were isolated on King's B medium, characterized, identified and tested their potential in utilizing diesel, a petroleum hydrocarbon. At the same time, production of protease and urease enzymes during the utilization of diesel was also assayed following the standard procedures.

Results: The isolates were grown well on diesel and subsequently produced the extracellular enzymes protease and urease at significant levels when compared to their production in the absence of diesel. Optimum temperature and pH for increased growth by four isolates was found to be 37°C and pH 8.0 indicating the maximum utilization of diesel. All the isolates showed maximum growth in medium with 100% diesel than 100% glycerol as carbon source, when tested with different proportions of diesel and glycerol as carbon sources. Plasmid profile of the isolates revealed that, all four *Pseudomonas* isolates harbored two low molecular weight plasmids; one with 3 Kb size and the other with 10 kb to 12 Kb size.

Conclusion: The four *Pseudomonas* isolates of the present study were found to have potential in diesel degradation and can be recommended for bioremediation of sites that are contaminated with diesel.

Key Words: Fluorescent *Pseudomonas*, diesel, plasmid, protease, urease.

INTRODUCTION

Public awareness of oil spills has increased over the years as more publicity has been focusing on this subject and the massive harm it does to our environment. Several causes of hydrocarbon spills occur including blowouts, leakage from tanks and dumping of waste petroleum products. Petroleum hydrocarbons are the major source of contaminants in soil and water environments. To control the environmental risks caused by petroleum products, various new regulations have been introduced and at the same time, research focusing on remediation of contaminated soils has increased. In contaminated ecosystems, petroleum hydrocarbons are mainly degraded by microorganisms (1). Among bacteria, the genus *Pseudomonas* is an effective hydrocarbon degrader and this potential has led to the discovery of several strains to assist in cleaning up contaminated site. By virtue of their versatility, *Pseudomonas* species are known to be involved in the biodegradation of natural or man-made toxic chemical compounds (2).Wongsa *et al*. (3) pointed out that *P. aeruginosa* is considered to be a good candidate for bioaugmentation of petroleum products such as diesel oil, heavy oil and kerosene in a liquid medium containing mineral salts. Petroleum hydrocarbon utilizing bacteria can tolerate oil-contaminated environments because they possess the capability to utilize oil as an energy source (4).

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. In-

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terest in *Pseudomonas* species has increased because of their possible use in detoxifying chemical wastes through a wide range of enzymatic metabolic activities (5). Protease is one of the most important industrial enzymes occupying nearly 60% of the enzyme sales obtained from microbial, plant and animal sources (6). Extracellular protease finds various applications in industrial processes like in detergents, leather tanning, dairy, meat tenderization, baking, brewery, photographic industry etc (7).

Ureases (EC 3.5.1.5) are nickel-dependent enzymes widespread among plants, bacteria and fungi that hydrolyze urea into ammonia and carbon dioxide. Plant and fungal ureases are homotrimers or hexamers of a ~90 kDa 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 adaptation of microbes to survive in the polluted environment is mainly based on their genetic make up. Plasmids that have been found to harbor genes encoding elements that transform environmental pollutants are known as catabolic plasmids. The purpose of this investigation was to determine the possibility of utilizing diesel as the sole carbon source and production of protease and urease by *Pseudomonas* isolates.

MATERIALS AND METHODS

Test organisms. Fluorescent *Pseudomonas* species used in this study were isolated from local red soil collected at Acharya Nagarjuna University Campus, Guntur Dt, Andhra Pradesh. The soil was subjected to ten fold serial dilutions. From this, 10^{-5}, 10^{-6} and 10^{-7} dilutions were spread on sterilized petriplates containing fluorescent *Pseudomonas* specific King's B medium (8). The plates were subjected to incubation for 24 hours at room temperature. From a total of fifteen isolates, four abundant isolates were characterized using standard morphological and biochemical characteristics and identified according to Bergey's manual of systematic bacteriology.

Diesel (petroleum hydrocarbon). Diesel is a petroleum derived hydrocarbon and its chemical composition is 75% saturated hydrocarbons including paraffins and 25% aromatic hydrocarbons including naphthalenes and alkyl benzenes. Its average chemical formula is $C_{12}H_{26}$ ranging from approximately $C_{10}H_{22}$ to $C_{15}H_{32}$.

Optimization of diesel level, temperature and pH. Growth in the presence of diesel was studied by growing the strains in flasks containing different concentrations of diesel (0%, 2%, 4%, 6%, 8% and 10%) and incubated for 24 hours. After completion of incubation, growth was measured in terms of OD values and the selected optimal level of diesel yielded maximum growth. Using the best level of diesel, the effect of different temperatures (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 the most optimal concentration of diesel 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 (pH=3, 4, 5, 6, 7, 8, 9, 10, 11 and 12) was also studied at the best level of diesel in the medium.

Utilization of diesel as sole source of carbon and energy. To study the possibility of utilizing diesel as carbon source, the isolates were allowed to grow in King's B broth medium containing different ratios of glycerol and diesel as carbon source (0:100, 25:75, 50:50, 75:25 and 100:0). After a period of incubation, growth was measured by taking the OD values.

Altogether, four dominant strains were screened for extracellular enzymatic activity. For enzyme production, *Pseudomonas* isolates were cultivated at 37°C for 24h at pH 8.0 in a 250 ml erlenmeyer flask containing 50 ml of nutrient broth medium with 6% diesel and subjected to incubation for 24 hrs. After incubation, culture supernatant fluid was obtained by centrifugation at 3000 rpm for 20 min. The activity of protease and urease enzymes during diesel utilization as well as in the 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 the enzymes, the growth was also measured in terms of OD values.

Protease assay. Protease activity was measured by using the standard procedure (9). The reaction mixture containing 1.0 ml of casein solution and 1.0 ml of enzyme sample was allowed to stand for 10 min at 37°C. To this mixture, 2.0 ml of Tri Chloro Acetic acid was added and incubated for 20 min. After incubation, the contents were filtered through Whatmann No. 1 filter paper. To 1.0 ml of this filtrate, 5.0 ml of Na_2CO_3 solution and 1.0 ml of the Folin-Ciocalteau reagent was added and incubated at 37°C for 30 min. The optical density was measured at 660 nm on spectrophotometer. The OD value of 10 was taken as one enzyme unit.

Urease assay. The urease activity was measured on spectrophotometer by employing the method of Tabatabai and Bremner (10). To 10ml of sample, 10ml of phosphate buffer, 10ml of urea solution, 1.0 ml of

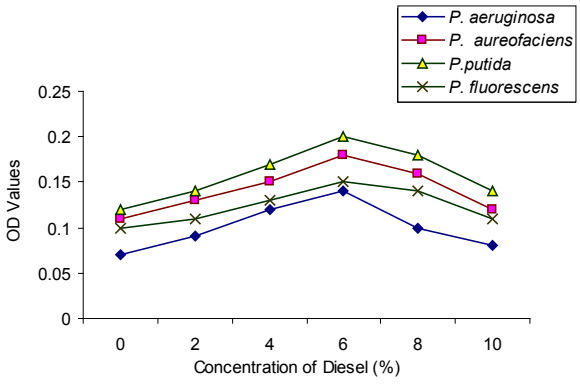

Fig. 1. Effect of different levels of Diesel on the growth of Fluorescent *Pseudomonas* spp.

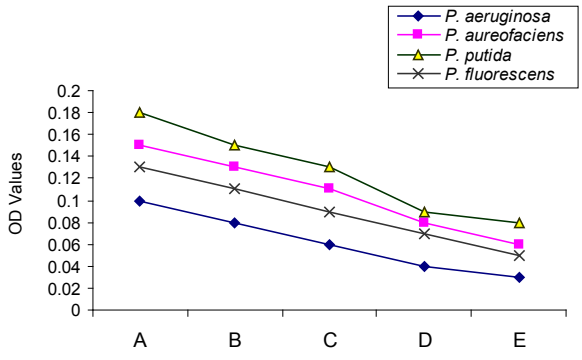

Fig. 2. Influence of diesel and glycerol in different proportions on growth of four *Pseudomonas* ssp.
A – 0% glycerol + 100% Diesel
B – 25% glycerol + 75% Diesel
C – 50% glycerol + 50 % Diesel
D – 75%glycerol + 25% Diesel
E – 100%glycerol + 0% Diesel

$ZnSO_4$. $7H_2O$ and 0.5 ml of NaOH were added in that order. The contents were allowed to stand for 15 min and filtered through Whatman No. 42 filter paper. From the filtrate, 10ml aliquot was taken and diluted to 50ml of distilled water. To this, 10 ml of EDTA and 3.0 ml of Nesslers reagent were added and the developed yellowish orange color was read at 440 nm. Enzyme activity was measured by taking the OD values. The OD value of 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 EluteTM endotoxin – free plasmid midiprep kit method. To determine the molecular weight of the plasmid DNA, Lamda DNA/ *Eco* RI+*Hind* III marker standard was also loaded along with the samples for electrophoresis.

RESULTS

In our present study, the most abundant four fluorescent *Pseudomonas* species were isolated from local red laterite soil and identified as *P. aeruginosa, P. aureofaciens, P. putida* and *P. fluorescens* based on morphological, staining, physiological and bio-chemical characterization, and with reference to Bergey's manual of systematic bacteriology (11). Data on these isolates are given in Table 1. All four isolates were positive to Tween 80 hydrolysis and Arginine dihydrolase activity.

All four isolates were tested for their ability to utilize various concentrations of diesel. Results showed that all four isolates were grown comparatively well in presence of diesel at all concentrations relative to control. Among the four isolates tested, *P. putida* showed highest growth rate in presence of diesel followed by *P. aureofaciens, P. fluorescens* and *P. aeruginosa*. From the data (Fig.1), it can be seen that maximum diesel utilization by the isolates took place at 6% diesel concentration which indicated that 6% diesel is the optimum concentration for growth. Although, growth was observed at 2%, 4%, 8% and 10% concentrations of diesel, it was relatively less. Also in the present study, all four *Pseudomonas* species showed better utilization of diesel than glycerol and exhibited better growth when tested with different proportions of diesel and glycerol (Fig. 2). All four species exhibited effective utilization of diesel when supplemented as sole carbon source than glycerol. Optimization of temperature and pH conditions for better growth at 6% diesel level was also studied, and it was observed that 35°C temperature and pH 8.0 are optimum (Figs 3 & 4).

Results of our study showed that all four isolates exhibited greater protease and urease activity during the utilization of diesel when compared to that of control (Tables 2 & 3). The order of efficiency of protease production by the isolates during diesel utilization was *P. aureofaciens* > *P. fluorescens* > *P. putida* > *P. aeruginosa.* The order of efficiency of producing urease by the isolates during diesel utilization was *P. aureofaciens* > *P. putida* >*P. fluorescens* > *P. aeruginosa.* Agarose gel electrophoresis, in the present study, of plasmid analysis revealed that all four isolates harbored two plasmids; one with the molecular weight of 3Kb and another with 10 to 12 Kb (Fig.5).

DISCUSSION

Hydrocarbon utilizing fluorescent *Pseudomonas* species are not only isolated from hydrocarbon contaminated sites, but also from normal soil environments. The existence of hydrocarbonoclastic bacteria in the soil environment has previously been documented by a researcher (12) which gives good support to our ba-

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

TEST	Ps1	Ps8	Ps11	Ps13
Morphological				
Size	0.6×1.7µm	0.8 × 2.0 µm	0.8 × 2.2 µm	0.8 × 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. Growth (OD Values) and protease activity during diesel utilization

Pseudomonas isolates	A		B	
	Growth	**protease**	**Growth**	**protease**
P. aeruginosa	0.15±0.03	73 ±2.28	0.25±0.04	125±3.03
P. aureofaciens	0.12±0.02	106±3.07	0.29±0.02	178±4.07
P. putida	0.08±0.01	80±2.21	0.17±0.03	130±3.64
P. fluorescens	0.07±0.01	98 ±2.03	0.13±0.01	162±3.21

Values are average of triplicates with standard deviation.
A-without oil, B-Diesel oil

Table 3. Growth (OD Values) and urease activity during diesel utilization.

Pseudomonas isolates	**A**		**B**	
	Growth	**urease**	**Growth**	**urease**
P. aeruginosa	0.07±0.003	4±1.01	0.17±0.01	19±1.02
P. aureofaciens	0.10±0.008	8 ±1.28	0.26±0.05	29±2.05
P. putida	0.09±0.006	6±1.07	0.23±0.04	25±1.07
P. fluorescens	0.08±0.004	5 ±1.04	0.19±0.02	22±1.03

Values are average of triplicates with standard deviation.
A-without oil, B-Diesel oil

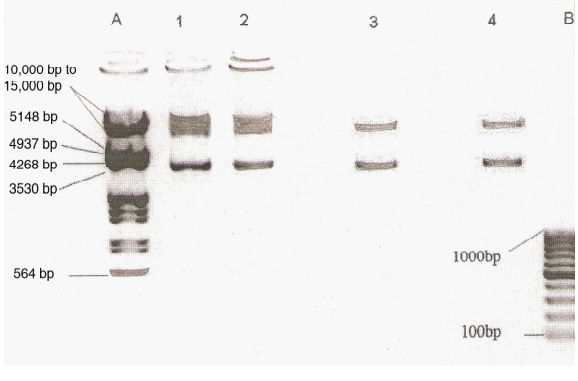

Fig. 5. Plasmid profile of four hydrocarbonoclastic fluorescent *Pseudomonas* spp. on agarose Gel Electrophoresis.
Lanes : A; Lamda DNA/*Eco* RI+*Hind* III Marker, B; DNA Ladder 100bp.
1; *P. aeruginosa,* 2; *P. aureofaciens*, 3; *P. putida*, 4; *P. fluorescens*.

sic attempt to isolate fluorescent *Pseudomonas* species having the capacity to utilize hydrocarbon from soil environments also. So, many factors affect the degradation of oil, including concentration of oil, temperature, salinity, pressure and water activity (1). According to Modi and Patel (13), hydrocarbons present in diesel and kerosene are readily utilized by *P.aeruginosa* as the sole carbon source at optimum conditions of 37° C and pH 7.0. *P. aeruginosa* was found as a powerful oil degrader and capable to survive for at least five years at oil contaminated sites (14). The optimum condition of 35°C and pH 8.0 noticed for the better diesel utilization by our four *Pseudomonas* species is also almost in match with the above report.

Our report of significant protease and urease production by the four *Pseudomonas* species of the present study during the utilization of diesel as a carbon source holds good with several earlier reports. As the genus *Pseudomonas* is a prolific producer of a number of extra cellular enzymes, there are several reports on the production of extracellular protease and urease by fluorescent pseudomonads. Studies on production of extracellular protease have been reported earlier by several researchers. Caballero *et al.* (2001) reported the production of multiple proteases by *P. aeruginosa* in association with the virulence property (15). Himelbloom and Hassan (16), observed the inhibition of growth and

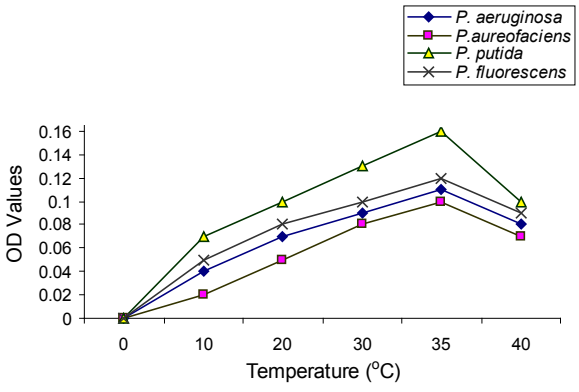

Fig. 3. Effect of temperature on growth of four *t*species during diesel utilization.

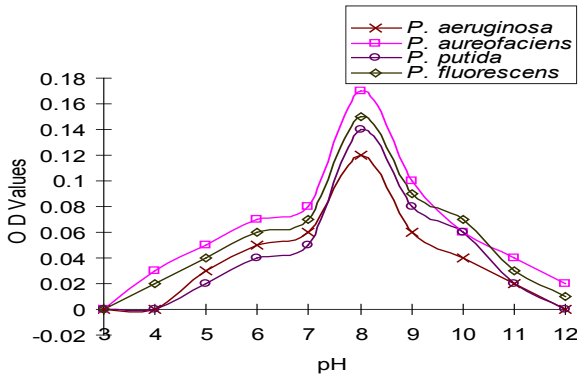

Fig. 4. Effect of pH on growth of four *Pseudomonas* isolates during diesel utilization.

extracellular protease production by *P. fluorescens* NS3 in presence of cysteine in the medium. An interesting application of alkaline protease was developed and reported the use of an alkaline protease to decompose the gelatinous coating of X-ray films, from which silver was recovered (17). Although proteases are widespread in nature, microbes serve as a preferred source of these enzymes because of their rapid growth.

Yaqci *et al*. (18) reported 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. It was also reported that extra cellular protease production by *P. aeruginosa* is under the control of nutritional factors like carbon sources, i.e. with increasing glucose concentration up to 0.07 M, there was a proportionate increase in growth and protease production and then these results declined at a higher concentration and in absence of glucose (both growth and protease production were poor) (19). A sensitive plate assay was developed for production of protease from *P. aeruginosa* isolated from patients with cystic fibrosis and these proteases are thought to play a major role in *P. aeruginosa* infections (20). Dutta and Banerjee (21) noted the effect of carbon and nitrogen sources on extracellular protease production by *Pseudomonas* species isolated from local soil. They reported that the optimum conditions for growth and enzyme production were pH 8.0 and 7.0, temperature 45°C and 60°C by wild type and mutant (UV radiation) strains, respectively.

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 the bio-degradation process as their activities decline rapidly after the rate of biodegradation has decreased (22). There are few reports on the production of urease by *Pseudomonas* species. Margesin *et al*. (23) reported the production of urease by fluorescent *Pseudomonas* species (*P. aeruginosa, P. fluorescens* and *P. putida*) during utilization of various hydrocarbons.

Catabolic plasmids effect physiological parameters, and efficiency of oil destruction by the *Pseudomonas* was reported earlier (24). Biodegradability of naphthalene and salicylate by *P. fluorescens* bearing seven plasmids was reported earlier (25). Presence of naphthalene degrading catabolic plasmids in *P. putida* was reported by Park *et al*. (26). The observation of presence of two plasmids of 3 Kb and 10-12 Kb molecular size in each of our four *Pseudomonas* species is in great concurrence with a very similar observation of two low molecular weight plasmids with 4.2 Kb and 3.8 Kb in *P. aeruginosa* reported earlier (27). Our attribution of diesel utilization capacity is due to the presence of plasmids in the presently studied *Pseudomonas* species. This is in agreement with similar reports on the presence of plasmid DNAs in *Pseudomonas* species documented well by several workers (28) attributing this to the biodegradation potential of the isolates. Deverenx and Sizemore (29) detected the 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.

The present work has focused on this approach, aiming to isolate novel *Pseudomonas* species, to study their diesel utilizing efficiency and extracellular enzyme production capacity during diesel utilization. From our study, we report that the isolates were able to grow at various concentrations of diesel and har-

bored two different sized plasmids suggesting plasmid mediated biodegradability of diesel and its possible exploitation in future bioremediation processes and with ability of producing significant levels of protease and urease in presence of diesel as a sole source of carbon in the production medium.

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