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Biodegradation of some aromatic hydrocarbons (Toluene and Xylene) by a bacterial strain isolated from petroleum contaminated site in Chennai

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Pseudomonas putida strain was isolated from petroleum polluted soil in Chennai by enrichment culture method to evaluate the biodegradation potential of toluene and xylene. The isolated bacterial strain in this study could grow in Bushnell Haas medium and mineral salt medium containing 1% each of toluene and xylene as the sole source of carbon and energy. The plasmid DNA was isolated by alkali lysis method and separated by agarose gel electrophoresis. The molecular weight of the plasmid was determined as 106Kb. Liquid culture studies were performed to determine the effect of pH and temperature on TX biodegradation. The isolated strain showed maximum degradation at 30^0 C and at pH 7.0. The kinetics of TX biodegradation was investigated at different time intervals (2.5 Hrs. - 12.5 Hrs.) and the biodegradation rate was maximum between 5 Hrs. and 12.5 Hrs. The conclusions that can be drawn from this study indicate that the microorganism present in this study could contribute significantly to bioremediation of TX pollution.

Key words : Biodegradation, Bioremediation, Toluene, Xylene, *Pseudomonas putida*

INTRODUCTION

Toluene is widely used as an organic solvent and starting material for the manufacture of plastics, pesticides and synthetic fibers. Its worldwide production was estimated to be more than 80,000 metric tons per year (Anonymons, 1985). Toluene and other organic solvents such as xylene, benzene and ethyl benzene are ubiquitous pollutants (Gibson and Subramanian, 1984) several environmental protection agencies have declared the removal of these pollutants to be high priority.

Compared to physicochemical methods, bioremediation offers an effective technology for the treatment of oil pollution, because majority of molecules in the crude oil and refined products are biodegradable and oil degrading microorganisms are ubiquitous (Chaineau *et al.*, 2000). Biological treatments for the removal of these organic compounds from contaminated water, soil and reactors are based on the action of degrading microbial communities, so detailed knowledge of the microbiology of petroleum hydrocarbons must be clear and understood to evaluate both the biodegradability of the most petroleum hydrocarbon compounds and the specific degradative activities of the different micro florae (Solano – Serena *et al*., 1999; Cavalca *et al*., 2000).

Contamination of ground water with toluene and xylene (TX) compounds is difficult to remedy because these compounds are relatively soluble in water and diffuse rapidly once introduced into an aquifer. Techniques for *in situ* bioremediation of the TX compounds are used to eliminate or reduce contamination levels in an aquifer. The biodegradability of the TX has been established using some pure bacterial strains or complex micro florae (Mallakin and Ward, 1996, Matteau and Ramsay, 1997); but little is known about the other microbial strains capacity, especially those which are wide-spreading in environment. This work describes the capability of a degrading microbial strain isolated from a polluted site in Chennai.

MATERIALS AND METHODS

Chemicals:

Toluene (99.5% purity), xylene (99% purity) were purchased from Merck Limited, Worli, Mumbai. All other chemicals used were of analytical grade.

Isolation of organism:

Soil samples were collected from petroleum polluted site at Manali, Chennai and were used to isolate

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Pseudomonas species by means of the enrichment culture procedure (Cappuccino and Sherman, 1999). Members of the genus *Pseudomonas* can utilize mandelic acid aerobically as their sole carbon and energy source. Therefore, this compound is the most important factor in the enrichment broth, which also contains a number of inorganic salts. The Erlenmeyer flask containing 20 ml of sterilized enrichment broth supplemented with 2 ml of 2.5% mandelic acid was inoculated with soil sample equivalent to the size of pea with a micro spatula. The culture broth was incubated at 30^0 C for 24 hours. The primary culture was monitored for growth by measuring the OD at 600 nm. One ml of primary culture was aseptically transferred into Erlenmeyer flask containing 20 ml of fresh enrichment medium. The secondary broth was incubated for 24 hours at 30^0 C. Sterile Petri plates containing enrichment media supplemented with 1.5% agar was prepared and inoculated with a loop full of secondary culture by four ways streaking. The plates were incubated for 24 hours at 30^0 C. The individual colonies were picked up and streaked on to secondary enrichment agar plates and nutrient agar plates, incubated for 24 hours at 30^0 C.

Identification of organism:

The isolated organism was characterized by standard biochemical tests and growth on selective medium (Cetrimide agar).

Isolation and determination of molecular weight of plasmid DNA:

The isolated organism was grown in 250 ml of Luria-Bertani (LB) broth and incubated at 30^0 C for 24 hours in a rotatory shaker. The plasmid was isolated by alkali lysis method. After 24 hours of incubation, the culture was checked for 0.6 O.D. at 600nm, blank was set by using the plain broth used for growing the culture. 10µl of the processed plasmid DNA sample was mixed with 10µl of gel loading buffer. The sample was then electrophoresed in 0.8% agarose gel for 1-2 hours. The gel was then stained with ethidium bromide and the plasmid DNA was observed under UV- Trans illuminator, photographed and analyzed with Total Lab System.

Effect of pH and temperature on TX degradation:

In to a series of 500 ml conical flasks marked (1 to 9), 250 ml of mineral salt medium was added. The culture flasks marked 1, 4, and 7 were adjusted to pH 5.0; culture flasks 2, 5, and 8 were adjusted to pH 7.0; and culture flasks 3,6 and 9 were adjusted to pH 9.0 using 1N HCl / 1N NaOH. Cells were initially grown in nutrient medium without TX compounds for about 10-12 hours, harvested by centrifugation at 6000 rpm for 10 minutes (4^0 C) and resuspended in mineral salt medium for biodegradation experiments. The initial cell concentration in the culture flasks was 0.4 mg per ml. 75 µl of toluene and 75 µl of xylene were injected to all the culture flasks as pure stock with a micropipette. The flasks were tightly sealed with a rubber stopper to prevent the loss of TX by evaporation. The culture flasks marked 1, 2, and 3 were incubated at 20^0 C; culture flasks 4, 5, and 6 were incubated at 30^0 C; culture flasks 7, 8, and 9 were incubated at 40^0 C for 8 hours.

Biodegradation kinetics of TX:

Biodegradation kinetics of TX compounds by the isolated bacterial strain was performed.

Into a series of 500 ml conical flasks marked S, and A to E, 250 ml of mineral salt medium was added. Cells were initially grown in nutrient medium without TX compounds for about 10-12 hours, harvested by centrifugation at 6000 rpm for 10 minutes (4^0 C) and resuspended in mineral salt medium for biodegradation experiments. The culture flask marked S (un inoculated) served as control, rest of the culture flasks marked A to E were inoculated with cell suspension. The initial cell concentration in the culture flasks was 0.4 mg per ml. 75 µl of toluene and 75 µl of xylene were injected to all the culture flasks as pure stock with a micropipette. The flasks were tightly sealed with a rubber stopper to prevent the loss of TX by evaporation. The culture flasks were incubated at 30^0 C. At different time intervals (A- 2.5 hrs; B- 5hrs; C-7.5 hrs; D-10 hrs; E-12.5 hrs), toluene and xylene concentrations remain in the culture flasks were determined by gas chromatography. Aqueous samples were extracted with 5.0 ml of chloroform. Samples were kept at 4^0 C in 10 ml screw cap vials with Teflon-lined rubber septa, until analysis. 2 µl of the extract was injected in to gas chromatography for analysis.

Determination of TX concentration by GLC:

Toluene and xylene concentrations in the culture flasks after incubation were determined by gas chromatography. Aqueous samples were extracted with 5.0 ml of chloroform. Samples were stored at 4^0 C in 10 ml screw cap vials with Teflon-lined rubber septa, until analysis. 2 µl of the extract was injected in to gas chromatography for analysis.

GC parameters:

Instrument: Netel gas Chromatograph,Model: Michro

9100, Column used: 10% SE 30 (methylsililone column), Column Length : 2 meter, Column Temperature: 60^0 C, Injection Temperature: 200^0 C, Detector:Flame ionization detector (FID), FID Temperature: 230^0 C, Carrier gas:Nitrogen, Flow rate: 30 ml per minute, Attenuation: 2.

The residual TX concentrations were expressed as mg/l. In the kinetics study the amount of toluene, xylene remains in the culture flasks at different time intervals were determined from Gas chromatogram and expressed in mg per litre of culture media.

RESULTS AND DISCUSSION

Bacterial isolation and identification was achieved at the end of the experimentation steps of enrichment culture technique and standard biochemical tests. The TX degrading isolate was identified to belong to *Pseudomonas* species (Table 1). The results of the isolated species provide useful information for comparison with other studies, and the isolated strain in this study have been reported to degrade a wide range of hydrocarbons (Cavalca *et al.,* 2000)

The plasmid DNA was isolated by alkali lysis method and separated by agarose gel electrophoresis. The molecular weight of plasmid was determined to be 106 kb in the present study. Similar work conducted by Keil *et al.* (1985) proved that the plasmid of *Pseudomonas putida* isolate showed molecular weight of 110 kb. The difference in result showed that *Pseudomonas putida* harbours different plasmids having different molecular weight.

Table 1: Shows the results of biochemical tests of the isolated organism

Sr. No.	Tests	Result
1.	Gram staining	Gram positive
2.	Motility test	Motile
3.	Catalase test	Positive
4.	Oxidase test	Positive
5.	Nitrate reduction test	Positive
6.	Indole production test	Negative
7.	Methyl red test	Negative
8.	Voges- Proskuer test	Negative
9.	Citrate utilisation test	Positive
10.	Carbohydrate fermentation test	Glucose positive
11.	Triple - Sugar - Iron test	No fermentation of lactose or sucrose
12.	Growth on Cetrimide agar plate	Large, irregular and moist colonies

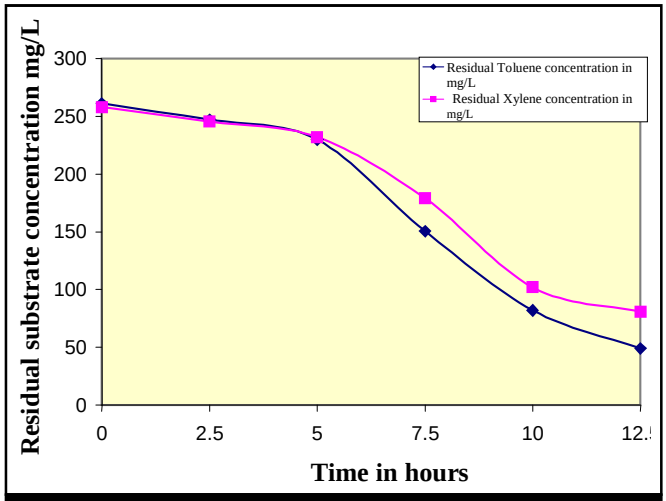

Fig. 1 : Experimental data and model output for batch biodegradation of toluene xlene mixture by *Pseudomonas putida*

In order to study the effect of different pH and temperature on TX biodegradation, 8 hours experiments in Erlenmeyer flasks were carried out as described in materials and methods. The isolated bacterial strain showed maximum TX degradation at 30^0 C. At low temperature, viscosity of the medium was increased, volatilization of alkanes reduced and the water solubility decreased, delaying the onset of biodegradation (Olivera *et al.,* 1997). The percentage of degradation decreased with decreased temperature, and higher temperature increased the rate of hydrocarbon metabolism to a maximum, typically in the range of 30-35^0 C, above that the membrane toxicity of hydrocarbons increased (Bossert and Bartha, 1984). A temperature of 30^0 C has been reported to be optimum for microbial growth and aromatic hydrocarbon degradation (Banat, 1995; Rahman *et al.,* 2002).

In an experiment to study the effect of pH on the degradation activity of bacterial strain under investigation, the culture pH was adjusted to 5, 7 and 9. It is clear from the data that the biodegradation activity of the bacterial strain was superior at pH 7 than pH 5 or pH 9. A neutral pH of 7.0 has been reported to optimal for biodegradation (Salmon *et al.*, 1998), and extremes in pH were shown to have a negative influence on the ability of microbial populations to degrade hydrocarbons (Rahman *et al.*, 1999; Meredith *et al.*, 2000).

Table 2 shows the residual TX concentrations in cultures after 8 hours of incubation at varying temperature (degrees) and pH.

Table 2: Shows the residual TX concentrations in cultures after 8 hours of incubation at varying temperature (degrees) and pH.

Sr. No.	Components	Residual TX remain in cultures after 8 days of incubation at different temperature degrees and pH (mg/l) #								
		20° C			30^0 C			40^0 C		
		pH 7.0	pH 8.0	pH 9.0	pH 7.0	pH 8.0	pH 9.0	pH 7.0	pH 8.0	pH 9.0
1.	Toluene	198.9	156.7	224	151	119	166	237	188	232
2.	Xylene	208.7	167.2	230.7	178	138	175	244	199	236

Mean value of three replicates
Initial concentration (mg/ l) of toluene: 261
Initial concentration (mg/ l) of xylene: 258

Table 3: Showing the amount and percentage of toluene and xylene (TX) degraded under varying conditions of temperature and pH after 8 hours of incubation

Components	Temperature in degree centigrade								
	20			30			40		
	pH 5.0	pH 7.0	pH 9.0	pH 5.0	pH 7.0	pH 9.0	pH 5.0	pH 7.0	pH 9.0
TX (Toluene + Xylene) degraded mg/L	111.31	194.99	64.21	189.22	260.18	176.84	36.93	130.86	50.35
Per cent TX (Toluene + Xylene) degraded mg/L*	21.44	37.5	12.37	36.45	50.13	34.07	7.11	25.21	9.70

Initial concentration of TX (Toluene + Xylene) 519 mg/L
*The percentage of degradation was determined as the amount of TX degraded to the initial amounts.

The kinetics of TX biodegradation were investigated by determining the residual components of TX through the experimental period, for every 2.5 hours up to 12.5 hours at 30^0 C with culture media pH 7. The obtained results showed that the degradation rate of TX was slow through the first 5 hours, followed by a fast degradation rate till end of the experiment of 12.5 hours. The kinetics study also indicated that the degradation rate of toluene was slightly higher than the degradation rate of xylene by the isolated strain (Fig. 1).

Table 3 Showing the amount and percentage of toluene and xylene (TX) degraded under varying conditions of temperature and pH after 8 hours of incubation.

Table 4: Showing the amount and percentage of toluene and xylene degraded at different time intervals incubated under optimum temperature 30^o C and pH 7

Components	Time in hours					
	0	2.5	5	7.5	10	12.5
Toluene degraded mg/L	0	14.05	31.10	110.56	178.85	211.70
Xylene degraded mg/L	0	12.65	26.12	78.91	156.11	177.23

Initial concentration: Toluene 261 mg/L, Xylene 258 mg/L

Table 4 shows the amount of toluene and xylene degraded at different time intervals. A steady decline in the residual TX compounds was observed between 5 hours and 12.5 hours. Similar observations have been reported by Tsao *et al.* (1998) and Arafa (2003) for aromatic hydrocarbon biodegradation.

Conclusion:

The general conclusion that can be drawn from this study is the necessity for site-specific evaluation when considering biodegradation as a remediation technique. Physical, chemical, and biological parameters of a contaminated environment should be investigated to optimize bioremediation efforts.

Results indicate that the microorganism investigated in this study could contribute significantly to bioremediation of hydrocarbons pollution.

REFERENCES

Anonymons, (1985). Toluene. Environmental health criteria, **52**. World Health Organization, Geneva, Switzerland.

Arafa, M.A. (2003). Biodegradation of some aromatic hydrocarbons (BTEXs) by a bacterial consortium isolated from polluted site in Saudi Arabia. *Pakistan J. Biol. Sci.*, **6**: 1482-1486.

Banat, I.M. (1995). Biosurfactants production and use of microbial enhanced oil recovery and pollution remediation: a review. *Biores. Technol.,* **51**: 1-12.

Bossert, I. and Bartha, R. (1984). The fate of petroleum in soil ecosystem. In: Atlas, R.M. (ed.), *Petroleum microbiology,* Mac Millan Publishing Co., New York, pp. 434-4756.

Cappuccino, J.G. and Sherman, N. (1999). Microbiology- A lLaboratory manual. (4th Ed.) Addison- Wesley, Longman Inc., California.

Cavalca, L., Confalonieri, A., Larcher, S. and Andreoni, V. (2000). Evolution of a degradative bacterial consortium during the enrichment of naphtha solvent. *J. Appl. Microbiol.,* **88** : 1009-1018.

Chaineau, C.H., Vidalie, J.F., Geneste, P., Ducreux, J. and Ballerini, D. (2000). Bioremediation of a crude oil polluted clay soil in a temperate zone. In Proceedings of the International Conference on Health, Safety, and the Environment. In: Oil and gas exploration and production. Society of Petroleum Engineers, Houston, Texas. pp. 1-12.

Gibson, D. T. and Subramanian, V. (1984). Microbial degradation of aromatic hydrocarbons. In: Gibson, D.T. (ed.) *Microbial degradation of organic compounds*. Marcel Dekker, Inc., New York, U.S.A. pp. 181-252.

Keil. H., Keil, S., Pickup, R.W. and Williams, P.A. (1985). Evolutionary conservation of genes coding for *meta*-pathway enzymes with in TOL plasmids pWWO and pWW53. *J.Bacteriol.*, **164:** 887-895.

Mallakin, A. and Ward, O.P. (1996). Degradation of BTEX compounds in liquid media and in peat biofilters. *J. Indust. Microbiol.,* **16**: 309-318.

Matteau, Y. and Ramsay, B. (1997). Active biofiltration of toluene. *Biodegradation,* **8**: 135-141.

Meredith, W., Kelland, S.J. and Jones, D.M. (2000). Influence of biodegradation on crude oil acidity and carboxylic composition. *Org. Geochem.,* **31**: 1059-1073.

Olivera, N.L. Esteves, J.L. and Commendatore, M.G. (1997). Alkane biodegradation by a microbial community from contaminated sediments in Patagonia, *Argentina. Int. Biodeter. Biodegrad.*, **40**: 75-79.

Rahman, K.S.M., Rahman, J.T., Lakshmanaperumalsamy, P. and Banat, I.M. (2002). Towards efficient crude oil degradation by a mixed bacterial consortium *Biores. Technol.,* **85**: 257-261.

Rahman, K.S.M., Vasudevan, N. and Lakshmanaperumalsamy, P. (1999). Enhancement of biosurfactant production to emulsify different hydrocarbons. *J. Environ. Pollut.,* **6**: 87-93.

Solano-serena, F., Marchal, R., Ropars, M., Lebeault, J.M. and Vandecasteele, J.P. (1999). Biodegradation of gasoline: Kinetics, mass balance and fate of individual hydrocarbons. *J. Appl. Microbiol.,* **86**: 1008-1016.

Tsao, C.W., Song, H.G. and Bartha, R. (1998). Metabolism of benzene, toluene, and xylene hydrocarbons in soil. *Appl. Environ. Microbiol.,* **64**: 4924-4929.