ISSN : 0974 - 7451 Volume 6 Issue 2

Trade Science Inc.

Environmental Science
An Indian Journal
Current Research Papers

ESAIJ, 6(2), 2011 [80-86]

Degradation and quantification of polyaromatic hydrocarbon by *Pseudomonas* sp.

T.Kuberan[1]*, R.Vidhya Pallavi[1], J.Anburaj[1], C.Sundaravadivelan[1], P.Kumar[1], V.Prabakaran[2]
[1]Department of Microbiology, Ayya Nadar Janaki Ammal College, Sivakasi - 626 124, (INDIA)
[2]Research Department of Microbiology, Sengunthar Arts and Science College, Trichengode - 637 205, (INDIA)
E-mail: tku2007@yahoo.co.in
Received: 25th January, 2011 ; Accepted: 5th February, 2011

ABSTRACT

Polyaromatic hydrocarbon compounds are hazardous and found to be highly mutagenic and carcinogenic. Biodegradation is an eminent technique for reducing the toxic nature of polyaromatic hydrocarbon in petroleum amended soil by sustainable manner. *Pseudomonas* sp., having biodegradation property was isolated by selective media and characterized by various biochemical test. *Pseudomonas aeruginosa* and *Pseudomonas fluorescence* were isolated from the petroleum contaminated area and *Pseudomonas stutzeri* 2643 obtained from the MTCC which is used for the biodegration of PAH. These three cultures were screened for potential PAH degrading bacteria. The poly aromatic hydrocarbon degradation by *Pseudomonas aeruginosa*, *Pseudomonas fluorescence* and *Pseudomonas stutzeri* 2643 obtained were compared with the amount of PAH present in the control (Soil and Petrol) and was estimated by HPLC, which was found to be 55.6% (500µl petrol). The result indicates that the PAH quantity in *Pseudomonas aeruginosa* treated sample was found to be 23.40% (500µl petrol), in *Pseudomonas flourescens* treated sample the PAH Quantity was found to be 12.28% (500µl petrol) and in *Pseudomonas Stutzeri* 2643 treated sample which revealed 13.91% (500µl petrol). From this study it indicates that *Pseudomona*s sp., reduces the PAH pollutants from the environment

KEYWORDS

Polyaromatic hydrocarbon (PAH);
Biodegradation;
Carcinogenic;
Toxic;
Pollutants.

INTRODUCTION

"The Quality of human life depends upon the quality of environment", the environment are compact of various natural resources, and the soil is very important natural resource. The soil is economically important useful elements (minerals metals, rock and fossil fuels) for human beings are basically derived from the lithosphere. Now the soil are highly polluted because of the urbanization. Microbiology approaches is the best way for managing the environment[8]. Oil Sludge/Crude Oil contamination is a major environmental concern since many of the constituent hydrocarbons are toxic. Besides this incidence of crude oil spill is increasing despite the best effort of petro-chemical industry[7]. Large – scale production, transports, use and disposal of

petroleum have made it a leading contaminant in the environment[5]. In recent years the petroleum exploitation and production activities have increased which results in increased discharge of petroleum hydrocarbon into our environment. Petroleum is a complex mixture of solid, liquid and gaseous hydrocarbons. The poly aromatic hydrocarbons are classes of compounds consisting of fused aromatic rings in various structural configurations[10]. Poly aromatic hydrocarbon can be formed as products of the incomplete pyrolysis of organic materials and present in considerable quantification in fossil fuels (2-3%) and are released in the environmental directly. These organic chemicals will be able to enter the soil and cause serious pollution problems. The hydrocarbons are highly hydrophobic material, which are commonly found environmental contaminants. Though they are not usually classified as hazardous, these wastes can be hardly degraded or decomposed. In recent years the petroleum exploitation and production activities have increased which results in increased discharge of petroleum hydrocarbon into our environment. The development of transports, large-scale production of petrochemical based industries have led to the pollution of the aquatic and terrestrial ecosystem with oil spills. Hence oil spills is potentially a dangerous and a major environmental concern and highlights the need for cost effective and environmentally acceptable mitigation technologies[3]. Petroleum hydrocarbons are commonly found in the environmental contaminants, though they are not usually classified as a hazardous wastes. Many petroleum products are used in modern society, including those that are fundamental to our lives (i.e. transportation fuels, heating and power generating fuels). The volume of crude oil or petroleum products that is used today dwarfs all other chemicals of environmental and health concern.

Thousands of different species of bacteria exist everywhere in our world, and most of them carry bacterial digestion in some way. However some of them are found only in a specific environment, require specialized types of food or have unique niches. Considering this fact it would not be inappropriate to postulate that rather than pushing bacterial species to the verge of extinction, chemical contamination of soil actually may be contributing to a substantial increase in bacterial diversity. The microbial world is known to posses an incredible metabolic and physiological versatility that helps microorganisms to in habitat hostile ecological niches. Bacteria highly survive in contaminated niches, because they may be metabolically able to exploit it's resources. Contaminants always, potential energy sources for bacteria. The actual available concentration of most pollutants an environment is mostly in nM to μM.

Biodegradation is the transformation process in which microorganism are organic matter as their food and source of energy. Thus the organic compound is metabolized and is ultimately converted into CO_2, and biomass. Thus it has been proved that microbial degradation of poly aromatic hydrocarbon is thus predominant tool for natural clean-up of petroleum contaminated sites (Ahmed et al., 2006).

In order to evaluate the distribution of species diversity among the hydrocarbon degradation isolation and identification of the various strains recovered from petroleum spilled soil were carried out. Identification of 47 isolates revealed that all isolates belonged to genus Pseudomonas, which can be the predominant micro flora among the poly aromatic hydrocarbon degrading community, which is supported by other studies. Pseudomonas genus as a potential candidate that is recovered from oil polluted area. Which are clearly indicates that the crude –oil-degrading population has composed of several bacterial species but Pseudomonas is found to be the predominant micro flora. Biodegradation may refer to complete mineralization of the organic contaminants to carbon dioxide, water, inorganic and cell protein, or to transformation of organic contaminants to other organic compounds mediated by Micro organisms that satisfy energy requirements, detoxify the immediate environment, or occur fortuitously such that the organism receives no nutritional or energy benefit (Stoner, 1994). Over the past two decades there has been increasing interest in the bioremediation of environmental pollutants through manipulation and application of degradative Microorganisms. (Atlas, 1981; Leahy and Colwell, 1990)[2]. Microbial degradation of oil has been shown to occur by attack on the aliphatic or light aromatic fractions of the oil.

Microbial degradation is the major mechanism for the eliminating of spilled oil from the environment[1,4]. Besides, several genera of microorganisms have been found to be acute biodegradation of crude oil, but the

bacteria *Pseudomonas* stand out as most[6]. The poly aromatic hydrocarbon is degraded by many species of bacteria, fungi and yeast. In bacteria isolated and identify of various highly degradative strains recovered either from petrol spillage soil are carried by High Performance Liquid Chromatography (HPLC). Which play a key role in any preferential degradation of compounds occurring with the complex mixture. The present study is designed in such a way that, to determine the degrading ability of the bacteria collected from the oil contaminated site. The selected bacteria which exhibited higher degradation was tested over the contaminated site. Besides, a comparative study of degradation of three bacterial symbionts like *Pseudomonas aeruginosa, Pseudomonas fluorescens* and *Pseudomonas Stutzeri* 2643 were performed.

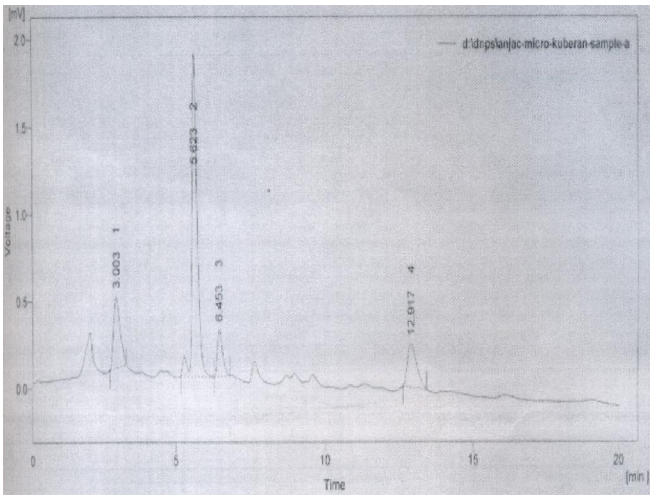

Figure 1 : Soil + 500 µl petrol (Control)

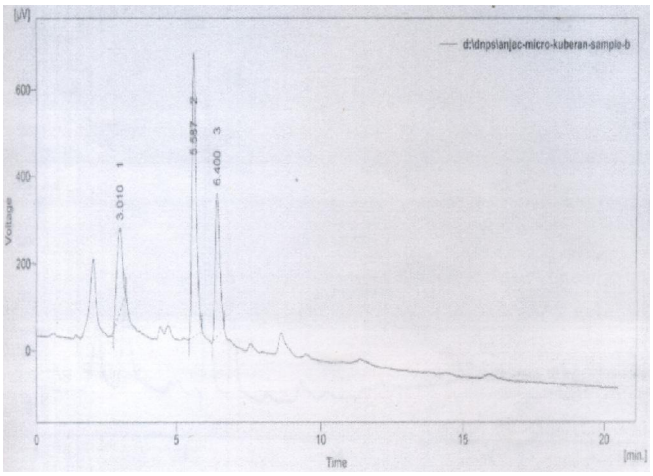

Figure 2 : Soil + 500 µl petrol + *Pseudomonos aeruginosa*

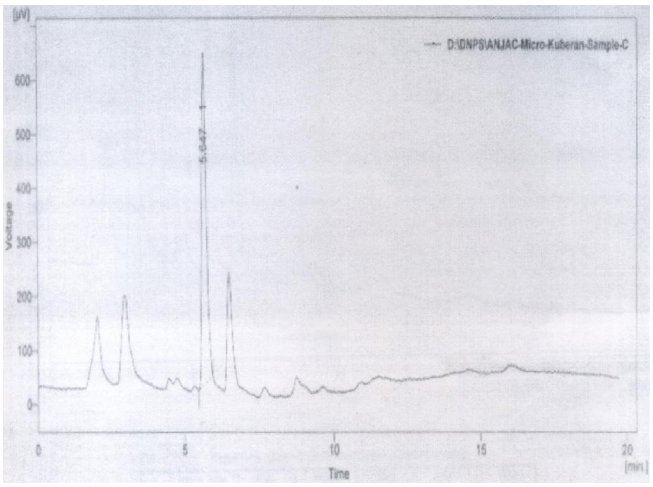

Figure 3 : Soil + 500 µl petrol + *Pseudomonos fluorescens*

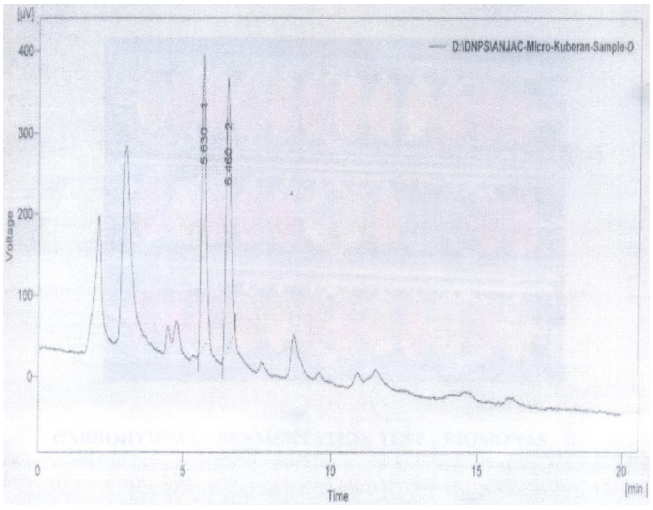

Figure 4 : Soil + 500 µl petrol + *Pseudomonos stutzeri 2643* (*MTCC*)

MATERIALS AND METHODS

Collection of soil samples

The soil Samples used in the present study were collected in a polythene bag from petroleum contaminant site of srivilliputtur, Tamil Nadu. Samples were collected with scalpel after removing 20 cm of the surface layer of the soil. Collected soil samples were stored aerobically at 4ºC.

Enrichment culture technique

1 gm of soil sample was added into different concentrations of petroleum as 200, 400, 600, 800, 1000ppm and these mixture was incubated for 15 days, after fifteen days, 1 g of mixture was enriched with 100ml of Bushnell Haas Broth (BHB), kept on a rotatory shaker approximately at 250 rpm. Bacterial growth was moni-

tored after 24 to 48 hrs by serial dilution technique.

Serial dilution

1 ml of culture was taken from the different concentration (200,400, 600,800,1000) of enriched soil mixture and mixed with 100ml of sterile distilled water. Then the samples were serially diluted from (10^{-1} to 10^{-9}). Spread plate technique was followed, were 0.1 ml of sample was spreaded on the Nutrient agar plates using sterile glass rod and incubated at 37ºC. After overnight incubation, the isolated colonies which showed distinct morphological variation was selected, brought to pure culture and stored in slants for further use.

Morphological and biochemical characterization

Colony characteristics, colour, motility, gram staining, fermentation of sugars, biochemical test were performed for identification of potential isolates of bacteria. Cultural characteristics of the colony such as margin, size, shape, type of colony, nature of colony (Mucoid, Rough, Smooth) transparently etc., were studied.

Differential media for confirmation of bacterial isolates

Acetamide agar

Acetamide centrimide glycerol mannitol is a selective medium for the growth of *Pseudomonas aeruginosa*. The positive result indicates deamidate acetamide turn the medium purplish red.

Isolation *of Pseudomonas* sp.

Isolation of fluorescents and non-fluorescent *Pseudomonas sp*.

Isolated single colony was streaked on certimide agar, which is a selective media for isolation of florescent *Pseudomonas* sp. A positive test indicates the growth of colonies in the plate.

Production of pyoveridin

Isolated single colony was streaked on King's B media, which is a selective media for pyoveridin production. A positive test indicates the production of yellow green coloured pigment with fluorescens on UV transilluminator.

Extraction of poly aromatic hydrocarbon from petroleum spilled soil

10mg of petroleum spilled soil was added with 20 ml of hexane in separating funnal and vortexed. (an organic solvent which dissolves hydrocarbon present in the soil sample) top layer of these extracts were used for quantification of PAH degradation analysis.

Biodegradation of PAH by isolated *Pseudomonas* sp in broth (BHB)

In a 100ml of conical flask, 25ml of BHB was added with 2ml of extract and 1 ml of microbial inoculum. The control flask was kept without microbial inoculum for comparison with treated sample. The culture was allowed to grow for 7 days and then it was centrifuged at a high speed for ten minutes. The resulting supernatant was discarded and final re-suspension was made in 25ml of BHB to yield a tenfold concentration of cells.

Extraction of PAH from broth

Extraction was carried out using liquid-liquid extraction procedure. 25ml of BHB which was added with 2ml of extract and 1 ml of microbial inoculum poured into the separating funnel and solvent hexane was added to dissolve the hydrocarbon present in the liquid system. Two distinct layers were found which make separation easier. Samples were separated, dried and dissolved in methanol then filtered and used for PAH analysis.

Quantification of PAH by high performance liquid chromatography

Chromatographic analysis was performed on a SHIMADZU LC AT Vp coupled with a SPD-10A Vp, ultraviolet (UV) visible detector, the absorbance of the UV at 254nm. The silicon C18 column was used. The flow rate of carrier for HPLC-UV was maintained at 0.8 ml/minute. The samples was injected by using 20µl micro syringe.

RESULTS

Hydrocarbon degrading bacteria were isolated from petro chemically polluted soil collected from in and around Srivilliputtur. The surface soil was removed and subsurface soil at a depth of 20 cm was collected and stored in 4^0C for further use. Physical parameters of oil spilled and normal Soil (control) was analysed and the result was noted on TABLE 1. The analysed soil samples were enriched and serially diluted by using serial dilution technique. The colonies grown on

higher petroleum concentration (1000ppm) was selected for further studies. The isolated petroleum degrading bacteria were identified based on morphological (TABLE 2) their biochemical characterization (TABLE 3) and carbohydrate fermentation test (TABLE 3A). The morphologically distinct organisms was further identified by using different selective media like King's B (*Pseudomonas fluorescence*), Certimide (*Pseudomonas* sp), Acetamide (*Pseudomonas aeruginosa*), Maleate Agar (*Pseudomonas fluorescence*) was observed (TABLE 4). The isolates (*Pseudomonas fluorescence*) and (*Pseudomonas aeruginosa*) were used for degradative studies which was named as Biomass I and Biomass II. The poly aromatic hydrocarbon degradation by *Pseudomonas aeruginosa, Pseudomonas flourescens* and *Pseudomonas stutzeri* 2643 obtained were compared with the amount of PAH present in the control (Soil and Petrol) and was estimated by HPLC, which was found to be 55.6% (500µl petrol). The result indicates that the PAH quantity in *Pseudomonas aeruginosa* treated sample was found to be 23.40% (500µl petrol), in *Pseudomonas flourescens* treated sample the PAH Quantity was found to be 12.28% (500µl petrol) and in *Pseudomonas Stutzeri* 2643 treated sample which revealed 13.91% (500µl petrol). From this study it indicates that *Pseudomona*s sp., which reduce the PAH pollutants from the environment.

TABLE 1 : Physical parameters of oil spilled and normal soil (Control)

Parameter	Control	Oil spilled
pH	8.0	6.8
Moisture content	0.896gms	0.346gms
E.C	0.42	1.30

TABLE 2 : Morphological & physiological characters

Appearance	Biomonas - I	Biomonas - II
Macroscopic appearance	Abundant, thin, white growth, with medium turning green	Large upaque irregular, iridescent patches, flurescent coloured colony
Microscopic appearance	Gram Negative	Gram Negative
Gram staining	Rod	Rod
Shape	Motile	Motile
Motility		

TABLE 3 : Identification of the isolates by various biochemical test

Biochemical test	Biomonas I	Biomonas II
Citrate utilization test	+	+
Indole production test	-	-
Methyl red test	-	-
VP test	-	-
Oxidase test	+	+
Catalase test	+	+
Triple sugar iron test	+	+
Nitrate reduction test	+	+
Gelatin liquification test	+	+
Starch Hydrolysis test	-	-
Growth at 41ºC	+	+
	Pseudomonas aeruginosa	*Pseudomonas fluorescens*

TABLE 3A : Carbohydrate fermentation test

Carbohydrate	Biomonas I	Biomonas II
Lactose	-	-
Xylose	+	+
Maltose	-	-
Fructose	-	-
Dextrose	+	+
Galactose	+	+
Ruffinosa	-	-
Trihalose	-	-
Melinbiose	-	-
Sucrose	-	-
L-arabinose	-	+
Mannose	+	+
Insulin	-	-
Sodium glveanate	-	-
Glycerol	-	+
Salicin	-	-
Gllucasamine	+	-
Dulcitol	-	-
Inositol	-	-
Sorbitol	-	-
Mannitol	-	-
Actonitol	-	-
Methyl – D- glucoside	-	-
Ribose	-	+
Rhamnose	-	-
Cellobiose	-	-
Melezitose	-	-

Carbohydrate	Biomonas I	Biomonas II
Methyl-D-mannoside	-	-
Xylitol	-	-
Onpg	-	-
Esuculin	-	
D-arabinose	-	-
Citrate	+	
Malonate	+	
Sorbose	-	-
	Pseudomonas aeruginosa	*Pseudomonas fluorescens*

DISCUSSION

Oil /petrol /crude oil contamination is a major environmental concern. since many of the constituents PAH toxic. Many petroleum products are used in modern society including those that are fundamental to our lives (transportation fuels, power generating fuels). Toluene, non polar aromatic compound cause pollution in ground water sediments and surface water (Chang et al.,1997). Among the various types of microorganisms used in biodegradation process,

TABLE 4 : Isolation of soil pseudomonas sp. by selective / differential media.

Selective / differential media	Cultural characteristic		Positive Organism	
	Selective	Differential	Selective	Differential
King's B medium	Yellow green	Defined growth	*P. aeruginosa*	Non fluorescent
	Pigment	but no yellow green	*P. fluorescens*	*Pseudomonas*
	Production	pigment production	*P. putida*	
Certimide Agar	Growth of defined colonies		Both fluorescent and non flurescent Pseudomonas	
Acetamide Agar	Production of purple	Defined growth	*P. aeruginosa*	Non fluorescent
	red colour pigment	but no pigment production		*Pseudomonas*
Maleate Agar	Defined growth	No growth	*P. fluorescens*	

Pseudomonas sp., in specific *Pseudomonas aeruginosa, Pseudomonas fluorescence* and *Pseudomonas stutzeri* 2643 play a vital role in degradation of petroleum hydrocarbons hence the present investigation, by using *Pseudomonas* sp., to degrade the petroleum hydrocarbon toluene. The result suggest that degradation of petroleum hydrocarbon treated with *Pseudomonas Aeruginosa*, revealed 23.40% when compare with control (soil and petrol) which was found to be 55.60%. among the *Pseudomonas sp., Pseudomonas fluorescence* exhibit 12.28%. which showed remarkable increased in degradation. Further this result emphasized the ability of best microorganism to utilize PAH (toluene as sole source of carbon and energy). The microbial utilization may be highly dependent on the chemical nature of phenolic compound such as toluene. Recent studies have been extended to make one of *the Pseudomonas aeruginosa, Pseudomonas fluorescence and Pseudomonas stutzeri* 2643 to degrade the aromatic hydrocarbon that might have been immobilized and applied to the fields to enhance the degradation efficiency (Battmann and Rehm,1984).

In accordance to the above send finding[9], reported that the microorganism break down (toluene) and use the energy to cellular components. An refined crude oil accounts for majority of oil spills where in gulf war made 1991 the worst year for catastrophic oil releases[11] *Pseudomonas* sp., is recognized has one of the most important ecofriendly microorganisms in the world. The recent may be due to the wide application of various strains of *Pseudomonas* sp., in biodegradation of hydrocarbons and oil pollutants in the environment. Further it has been proved by comparing the chromatograms of HPLC taken for control and experiments. The increase in peak height in different concentration proved that the metabolic enzyme have been synthesized and hence the concentration of PAH (toluene) was reduced in the medium by microorganisms. Thus the present work an HPLC analysis suggest that the biodegradation efficiency of microorganisms may be varied from species to species and even it may be differ strains of the same species, therefore in the field application of soil sample might be screened are chemical nature of contaminants using HPLC,GC-MS and IR-Spectroscopy.

CONCLUSION

The present investigation demonstrates the feasibility of adopting sustainable and ecofriendly approach to minimize the hydrocarbon pollutants.

REFERENCES

[1] R.M.Atals, R.Bartha; 'Biodegradation of Oil on Mater Surface: Polluting Petroleum Hydrocarbons', U.S. Patent, **3**, 127 **(1984)**.

[2] C.E.Cerniglia; Cur.Inbiotechnol., **4**, 331-338 **(1993)**.

[3] S.Chhatre, H.Purohit, R.Shanker, P.B.Khanna; Water Sci.Technol., **34**, 187-193 **(1996)**.

[4] R.R.Colwell, Walker; Crit.Rev.Microbiol., **5**, 423-445 **(1977)**.

[5] S.Jegadevan, S.Mukberji; Cen.Envion.Sci.and Eng.(CESE), **(2004)**.

[6] Miklosovicova, B.Trizilova; Biologia.(Bratislava), **46**, 219-228 **(1991)**.

[7] S.Mishra, J.Jyot, R.C.Kubad, B.Lal; Appl.Environ. Microbiol., **67**, 1675 **(2001)**.

[8] V.Prabakaran; Entitled as Biodegradation and Bioremediation of Pesticides in an Agro Ecosystem, Ph.D Thesis, **(2004)**.

[9] B.Shaw; Oils and Gas Jour, **4**, 85-88 **(2002)**.

[10] H.S.Stoker, S.L.Seager; Organic Chemical Pollution: Petroleum Pesticide and Detergents, Environ. Chem.Plenum Press, 401-415 **(1997)**.

[11] J.Welch, F.Yando; World Wide Oil Spill Incident Data Base: Recent Trends in Proc.Into Oil Spill Conf., American Petroleum Institute, Washington, D.C., 811 **(1993)**.