Plasmid Associated Anthracene Degradation by *Pseudomonas* sp. Isolated from Filling Station Site

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Abstract: Bacterial strains were isolated from oil contaminated soil of 5 different filling stations of Himachal Pradesh, India and screened for their anthracene degradation ability. Enriched media was used to isolate the anthracene degrading bacteria with 0.5% peptone and 0.1% w/v anthracene in basal salt mineral medium and during successive enrichment the peptone concentration was decreased to 0.25 g, 0.1 g and to 0.0 g. After one month of enrichment 5 strains were found to be potent anthracene degrader out of total 76 strains screened. These 5 strains were further subcultured for 10 days and on the basis of percent anthracene degradation strain E was found to degrade 74.8% anthracene supplemented in BSM medium at 0.1% as sole source of carbon and energy and identified as *Pseudomonas* sp. As evident by antibiotic sensitivity test, *Pseudomonas* sp. showed resistance against Cefadroxil and Ampicillin among tested 7 antibiotics. Acridine orange induced plasmid curing of isolate lead to complete loss of plasmid and anthracene degradation activity. The study suggests that the plasmid could have a role in anthracene degradation activity. [Nature and Science 2010;8(4):89-94]. (ISSN: 1545-0740).

Key words: anthracene, *Pseudomomas* sp., plasmid curing, acridine orange, marker antibiotic

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of compounds containing carbon and hydrogen, composed of two or more fused aromatic rings in linear, angular and cluster arrangements. They are lipophilic in nature and relatively insoluble in water (Hafez *et al.*, 2008 and Johnsen *et al.*, 2005). PAHs are ubiquitous pollutants and are generated from anthropogenic activities such as the burning of fossil fuels, the use of wood preservatives such as creosote and the generation of wastes from coal gasification plants (Ni Chadhain *et al.*, 2006). PAHs have been identified as hazardous chemicals by different State and Central Pollution Control Boards because of their toxic, carcinogenic and tetragenic effects on living body (Ruma *et al.*, 2007). Anthracene, together with other PAHs, is a persistent and toxic soil contaminant (Hyotylainen and Oikari, 1999; Lotufo, 1997). Pollution by PAHs is usually found on the sites of gas factories and wood preservation plants. Bioremediation is an economically and environmentally attractive solution for cleaning those sites (Kastner and Maho, 1996). Environmental anthracene contamination originates from a number of anthropogenic sources/practices such as manufacturing of dyes, production of synthetic fibers, plastics and pesticides, petroleum spills as a result of pipeline rupture and tanker failure. Its natural sources are coal and tar, and can be released by incomplete combustion of fuels (such as coal, oil, gas). Therefore it is a constituent of exhaust from automobile and charcoal grills.

Overall bioremediation is an attractive process due to its cost effectiveness and the benefit of pollutant mineralization to carbon dioxide and water (Trindade *et al.*, 2005). Degradation of anthracene, that is its conversion to both carbon dioxide and water (mineralization) or other organic substances (degradation products) which is not toxic is one of the inexpensive way of removing large concentrations of anthracene from soil and water. Transfer of genes responsible for biodegradation of hydrocarbons plays an important role in bioremediation of pollutants (Wilson and Jones, 1993). In this study we described our experiment designed to determine whether the anthracene degradation was plasmid associated or associated with chromosomal DNA.

2. Materials and methods

2.1 Collection of soil samples

For the isolation of anthracene degrading bacteria, soil samples were collected from 5 different Filling station sites of Himachal Pradesh, India. Samples were stored in sterilized polyethylene bags at 4°C for further use.

2.2 Enrichment and isolation of anthracene degrading microorganism

One gram of each soil sample was suspended in 100 ml Basal Salt Mineral (BSM) medium (g/l: K_2HPO_4, 0.38; $MgSO_4.7H_2O$, 0.2; NH_4Cl, 1.0; $FeCl_3$, 0.05; Distilled Water, 1000 ml and pH, 7.0) broth containing 1.0 g peptone and 0.1% w/v anthracene.

Flasks were incubated at 30°C on a rotary incubator shaker at 150 rpm. After 1 week of incubation, 5 ml of inoculum was transferred from each flask to the fresh 100 ml BSM broth containing 0.5 g peptone and 0.1% w/v anthracene. Further subculturing was done with 0.25 g, 0.1 g and 0.0 g peptone with constant concentration of anthracene. The anthracene degrading microorganisms from the flask containing 0.1% w/v anthracene and no peptone were isolated by spread and streak plate method and analyzed for their anthracene degradation activity.

2.3 Analytical techniques

For the determination of λ max of anthracene, a 10 ppm solution of anthracene in ethyl acetate was scanned from 190-600 nm on UV-VIS spectrophotometer. The λ max was used to determine the concentration of anthracene in ethyl acetate extracts. For preparation of standard curve of anthracene, a stock solution of 10 ppm in ethyl acetate was prepared and aliquots in the range of 0.2 to 1 ppm were separately read at λ max of anthracene.

2.4 Screening of anthracene degrading microorganisms

For screening of anthracene degrading microorganism, 30 ml BSM broth containing 0.1% w/v anthracene as sole carbon source was taken in different flasks and inoculated with 5% inoculums ($A_{600,}$ 0.70) of different enriched soil isolates. All the flasks were incubated for 10 days at 30°C on rotary shaker incubator at 150 rpm. During incubation the residual concentration of anthracene was monitored spectrophotometrically for 10 days by liquid-liquid extraction method as described by Manohar *et al.* (1999).

Further the anthracene degrading capability of most efficient anthracene degrading organism was monitored in 30 ml BSM broth containing 1.5% w/v anthracene/ethyl acetate and 5% inoculum (A_{600} 0.70). The degradation was monitored by sampling 2 ml from each reaction set for 240 h at an interval of 24 h. Identification of most efficient anthracene degrading microorganism was done on the basis of microscopic, morphological and biochemical characteristics.

2.5 Plasmid curing by acridine orange

BSM broth (18 ml) was taken in different flasks and it was inoculated with 1 ml culture inoculum (A_{600} 0.70) with different concentrations of acridine orange ranging from 10-100 µg/ml (Fujii *et al.*, 1997). The flasks were wrapped in black paper to prevent the photolysis of cells then incubated at 40°C for 7 days with gentle shaking at 100 rpm. After incubation acridine orange treated cultures were serially diluted up to 10^{-11} times in sterilized saline.

Seven different antibiotics were used to check the antibiotic sensitivity of most efficient anthracene degrading microorganism by agar cup method. The culture was spread plate on BSM agar medium and incubated for 30 min. The wells of 4 mm diameter were made with the help of a cork borer in the agar at equal distance. Seventy µl of each antibiotic was poured in these wells.

2.6 Preparation of master and replica plate

Master plate was prepared on nutrient agar plate. Eighty µl of different dilution preparations were spread on the plates and were allowed to incubate at 37°C for 24 h. Replica plates were prepared by transferring the exact imprint of master plate. For replica plate (Nutrient Agar plate with marker antibiotic, Cefadroxil) every single colony from master plate was picked with sterilized tooth prick tip and placed on the corresponding site on replica plate. All the replica plates were incubated at 37°C for 24 h and master plates were preserved at 4°C.

2.7 Plasmid isolation and agarose gel electrophoresis

The plasmid was isolated by alkali treatment methods described by Kado and Liu (1981) and was electrophoresed on 0.8 % agarose gel in presence of ethidium bromide (1 µg/ml). DNA bands were visualized under UV light under UV transilluminator and photographed.

3. Results

3.1 Screening for most efficient anthracene degrading microorganism

The λ max of anthracene in ethyl acetate was determined to be 254 nm (Figure 1). It was used to determine the concentration of anthracene in ethyl acetate extracts. Different soil samples from 5 filling station sites were analyzed for their anthracene degradation capability. After enrichment of 76 bacterial strains, 5 strains were found to be efficient anthracene degrader. Further the anthracene degrading capability was monitored for 10 days in BSM medium supplemented with anthracene as sole source of carbon. On the basis of anthracene degradation (%) ability strain E was found to be the most efficient anthracene degrader with maximum degradation rate of 70.6%. Strain B had shown the least anthracene degradation, 15.5% (Figure 2). Strain E was identified on the basis of cultural, microscopic, morphological and biochemical characteristics (Table 1). As determined by Bergey's manual of systematic bacteriology the strain E has been tentatively identified as *Pseudomonas* sp.

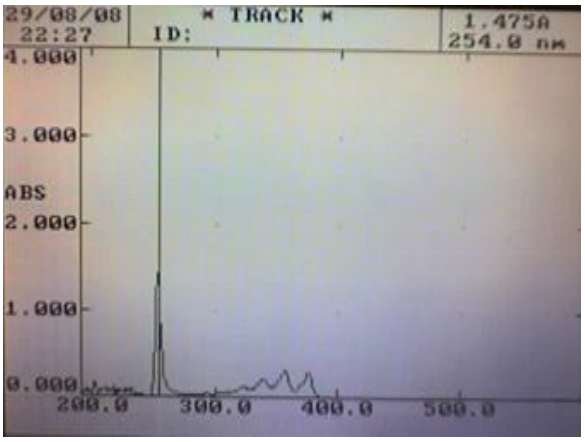

Figure 1. UV-Spectrum of anthracene in ethyl acetate. A single peak at 254 nm shows the max absorbance

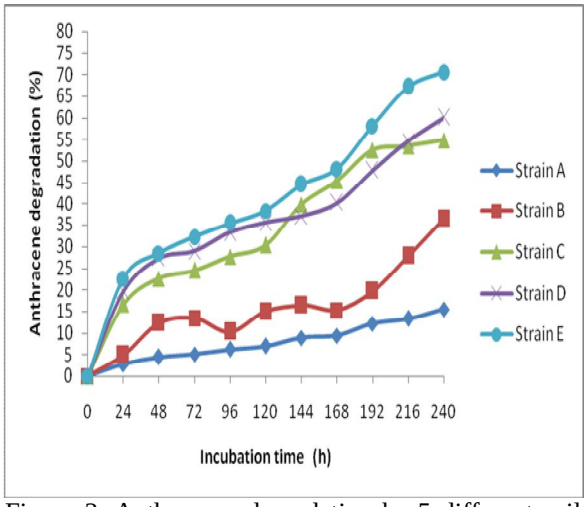

Figure 2. Anthracene degradation by 5 different soil isolates for 10 days of incubation period

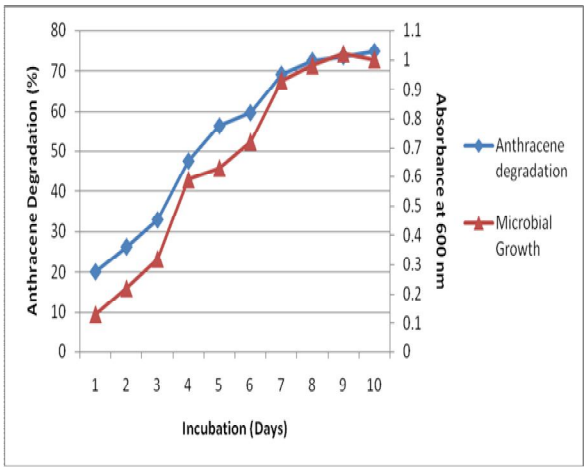

Figure 3. Growth (A_{600}) and anthracene degradation (%) by *Pseudomonas* sp.

3.2 Anthracene degradation study

The results of growth of the bacterium on anthracene and its utilization for different incubation periods were represented in Figure 3. Results revealed that there is an increase in cell growth with an increase in incubation period. The maximum growth of the bacterium was observed at 9th day of incubation (A_{600}, 1.02). The bacterium showed 74.8% utilization of anthracene at 10th day of incubation.

3.3 Plasmid curing by acridine orange

Pseudomonas sp. was tested for sensitivity and resistance against different antibiotics were used for sensitivity and resistance against *Pseudomonas* sp. Zone of inhibition was observed against oxytetracycline, azithromycin, erythromycin, cefixine and amoxyciline antibiotics while ampicillin and cefadroxil showed no zone of inhibition (Table 2, Figure 4). Replica plate showed the disappearance of colony when incubated in presence of acridine orange and marker antibiotic, cefadroxil (Figure 5). In contrast to this, the same colony was present on master plate which was untreated with acridine orange and incubated in presence of cefadroxil marker antibiotic (Figure 6). The curing of plasmid DNA was further supported by agarose gel electrophoresis of isolated plasmid DNA. A single band was observed for uncured culture while no band was visualized when the culture was treated with acridine orange at a concentration of 50 µg/ml (Figure 7).

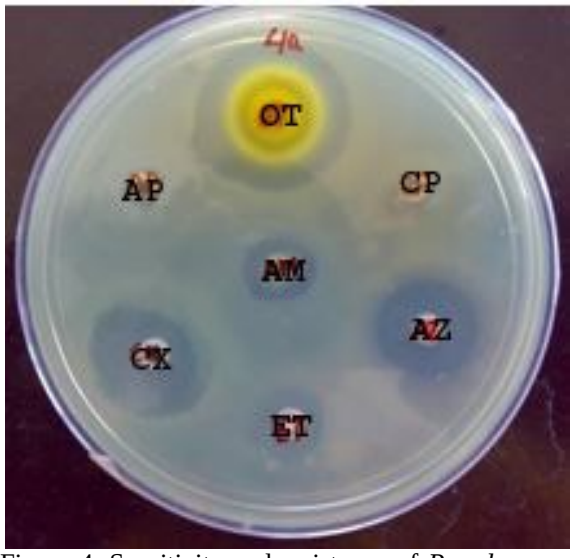

Figure 4. Sensitivity and resistance of *Pseudomonas* sp. against different antibiotics (OT- Oxytetracycline, CP- Cefadroxil, AZ- Azithromycin, ET- Erythromycin, CX- Cefixine, AP- Ampicillin, AM- Amoxicilline)

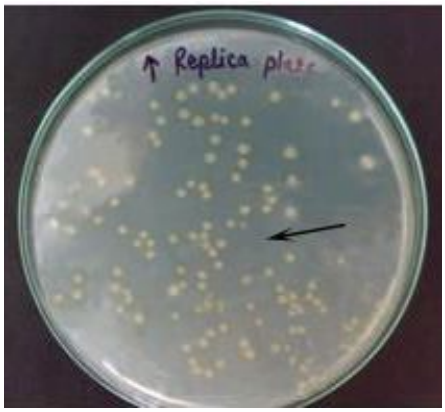

Figure 5. Replica plate showing the position of disappeared colony (arrow point) when incubated in presence of Cefadroxil, marker antibiotic.

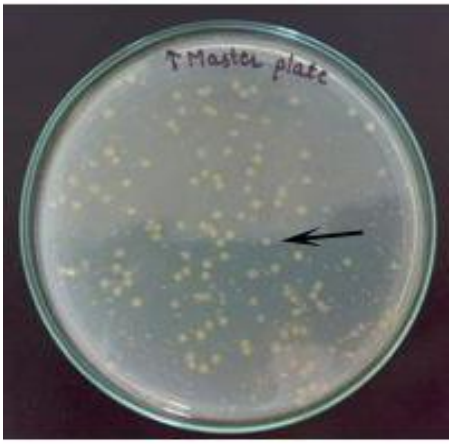

Figure 6. Master plate showing *Pseudomonas* sp. colonies on BSM agar plate when incubated in presence of Cefadroxil, marker antibiotic

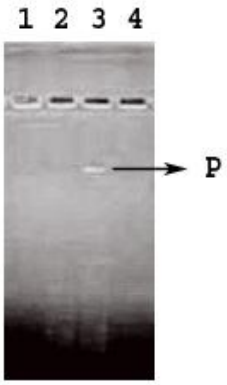

Figure 7. Gel Electrophoresis profile for DNA from *Pseudomonas* sp.. Partially purified Plasmid was separated on 0.8% Agarose. P, Plasmid DNA. Lane-2 and Lane 3 represent DNA profile of cured and uncured plasmid, respectively.

Table 1. Morphological and Biochemical characterization of *Pseudomonas* sp.

S.N.	Test	Results
	Morphological Characteristics	
1	Colony Character	Convex, Round, Creamish, Opaque, Greenish on white media
2	Simple Staining	+
3	Gram Staining	- , bacilli
4	Endospore	+, Green spores
5	Negative Staining	+
6	Motility	Motile
	Biochemical Characteristics	
7	Casein Hydrolysis	+
8	Urease	-
9	H_2S Production	-
10	Carbohydrate catabolism	-
11	Indole Test	-
12	Methyl-Red Test	-
13	Voges-Proskauer Test	-
14	Citrate Utilization	+
15	Catalase Test	+
16	Oxidase reaction	+
17	Nitrate reduction	+
18	Gelatin Liquefaction	+

Table 2. Zone of inhibition of different antibiotics against *Pseudomonas* sp.

S.N.	Antibiotics	Abbreviation	Zone of Inhibition (mm)
1	Oxytetracycline	OT	21
2	Cefadroxil	CP	0
3	Azithromycin	AZ	16
4	Erythomycin	ET	8
5	Cefixine	CX	17
6	Ampicillin	AP	0
7	Amoxicilline	AM	6

4. Discussion

Pseudomonas sp. strain E was isolated from filling station sites of Himachal Pradesh and cultured in BSM broth supplemented with 0.1% w/v anthracene as substrate. In contrast to other strains isolated in this study *Pseudomonas* sp. strain E degraded 74.8% in 10 days of incubation. The addition of anthracene to aqueous media was by dissolution in a carrier solvent, ethyl acetate (Moody

et al, 2001; Nadalig *et al*, 2002). Our results are in line with the results by Matthew *et al*. (2000), who have isolated *Pseudomonas aeruginosa, Alcaligenes eutrophus, Bacillus subtilis* and *Micrococcus luteus* from crude oil polluted soils using 0.1% w/v anthracene as the sole carbon and energy source resulted in a residual oil concentration of 22.2%, 33.3%, 39.3%, 44.0% and 91.7% respectively. Rodrigo *et al.* (2005) had also reported 71% of the anthracene degradation, added to the medium (250 mg L^{-1}) by *Pseudomonas sp.* isolated from a 14-year-old petrochemical sludge land farming site.

After screening, *Pseudomonas* sp. strain E was further cultured in BSM medium and during this period the maximum anthracene degradation was found to be 74.8% on 10^{th} day and maximum bacterial growth was measured on 9^{th} day. Similar studies have been reported for anthracene degradation & mineralization by *Pseudomonas, Sphingomonas, Nocardia, Beijerinckia, Rhodococcus* and *Mycobacterium* (Dean-Ross *et al*., 2000; Moody *et al.*, 2001). Another study has reported the complete degradation of added anthracene to autoclaved soil by *Burkholderia sp.* in 20 days (Somtrakoon *et al*., 2008). Manohar *et al*., (1999) had reported the complete anthracene degradation (2.8 mM) after 6 days of incubation and Eder *et al*., (2008) showed that after 48 days *Pseudomonas citronellolis* isolate 222A degraded 72% of anthracene.

We have tested several antibiotics for their resistance against *Pseudomonas* sp. strain E and observed that strain E was resistant against cefadroxil and ampicillin antibiotics. This suggests that strain E may posses the resistance gene for these two antibiotics and hence, these antibiotics were used as marker for the screening of plasmid cured bacterial colonies.

In the present study 50 µg/ml acridine orange concentration and 40°C temperatures was significantly effective for plasmid curing, suggesting that acridine orange can be used at sub-lethal temperature to cure the plasmid DNA. Curing agents such as acridine orange, if administered to bacterial populations in sub-lethal doses, can lead to the elimination of plasmid DNA without harming the bacterial chromosome and thus maintaining the ability to reproduce and generate offspring (Singleton and Sainsbury, 2001). Plasmid cured colony of strain E was not able to grow on BSM broth with anthracene as sole carbon source. It is assumed that this may be because of the removal/inactivation of gene(s) responsible for anthracene degradation from strain E. This indicates that gene(s) responsible for anthracene degradation might be associated with plasmid DNA that has been cured, thus not allowing the colony to grow in BSM broth. Moreover, the color of plasmid cured colony was changed to white from greenish (uncured). This study is in accordance with work carried out by Mesas *et al*. (2004) who had reported that the strains of *Oenococcus oeni*, RS2 (which carries the plasmids pRS2 and pRS3) were grown in the presence of different curing agents and at different temperatures. Sub lethal temperature together with acridine generated the cured strains, which lacking pRS3 plasmid and suggested that acridine orange is a better curing agent. Further the plasmid curing was confirmed by agarose gel electrophoresis of uncured and cured *Pseudomonas* sp. strain E. Gokhan and Serap (2005) also reported that catabolic pathways, which encode different aromatic hydrocarbon degradation routes, are frequently located on plasmids, although degradative genes can be located on either chromosome or plasmid.

The plasmid curing and agarose gel electrophoresis experiment suggest that anthracene degradation is plasmid associated. This is in accordance with the previous finding by Sanseverino (1993) who had proposed that NAH plasmid was involved in degradation of PAHs. Therefore, the possibility of the involvement of catabolic plasmid in the degradation of Anthracene by *Pseudomonas* sp. was investigated. In conclusion *Pseudomonas* sp. is an efficient anthracene degrading strain and could be use to develop an environmental friendly technology to overcome the problem of oil spills.

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