Naphthalene Biodegradation by Novel Soil Isolate of Vehicle Service Station Sites

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Abstract:- **New bacterial strains were isolated, identified and screened for their naphthalene degradation ability from the soil contaminated with oil (lubricating oil, petrol and diesel etc.) of 3 different vehicle service station sites of Chandigarh, India. Enriched media (0.5% peptone and 0.1% w/v naphthalene in basal salt mineral medium) was used to isolate the naphthalene degrading bacteria and the concentration of peptone was decreased to 0.25g, 0.1g and to 0.0g during successive enrichments. After one month of enrichment, out of the total 59 strains screened, only 3 strains were found to be potent naphthalene degrader. These 3 strains were further sub-cultured for 10 days and on the basis of naphthalene degradation (in percent), strain IR1 was found to degrade 74.8% naphthalene supplemented in BSM medium at 0.1% concentration (w/v) as sole source of carbon and energy and was identified as *Pseudomonas* sp. Antibiotic sensitivity test revealed that the strain IR1 - *Pseudomonas* sp. was resistant to cefadroxil and ampicillin among the seven antibiotics tested. Plasmid curing of the isolate lead to complete loss of plasmid and the naphthalene degradation activity suggesting that the plasmid could have a role in naphthalene degradation activity.**

Key words: **naphthalene, *Pseudomomas* sp., degradation, antibiotic, plasmid curing.**

I. 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. Polycyclic aromatic hydrocarbons (PAH) comprise a substantial fraction of petroleum mixtures, including crude oils, coal tar creosote, and refined fuel mixtures such as diesel. Soil is a valuable resource as it regulates biogeochemical cycles, filters and remediates pollutants and enables food production. As such, their presence in soil makes biodegradation of these compounds a prime concern (Miya and Firestone, 2000). PAHs are ubiquitous in the environment and cause great environmental concern because they are persistent, mutagenic and can be toxic and/or carcinogenic and as such PAH contamination is of ecological and human health concern (Juhasz and Naidu, 2000; Sverdrup *et al*., 2002).

Naphthalene is a bicyclic aromatic hydrocarbon with the chemical formula $C_{10}H_8$. In purified form, naphthalene is a white aromatic, crystalline solid hydrocarbon, best known as the traditional, primary ingredient of mothballs. It is volatile and is sparingly soluble in water (0.031 g/L). Its molecule consists of two fused benzene rings. Naphthalene is a natural constituent of coal tar and crude oil. It is obtained in purified form from these raw materials by fractional distillation. On the basis of the occurrence of adverse effects following exposure, naphthalene is expected to be absorbed via the gastrointestinal tract, the respiratory tract and the skin. Humans exposed to naphthalene via inhalation, combined inhalation and dermal exposure and oral exposure have developed haemolytic anaemia, jaundice, headache, confusion, nausea, vomiting and cataracts (U.S.E.P.A., 2003).

Possible fates for PAHs released into the environment include photolysis, chemical oxidation, photo-oxidation, bioaccumulation, adsorption on soil particle and volatilization. The major decomposition processes for their successful removal are currently believed to be microbial transformation and degradation. Biodegradation by natural populations of microorganisms represents one of the primary mechanisms by which petroleum and other hydrocarbon pollutants can be removed from the environment and is a cheaper process than other remediation technologies (Leahy and Colwell, 1990). Certain plasmids play an important role in adaptation of natural microbial populations to oil and other hydrocarbons. Some of the microbial catabolic pathways responsible for the degradation have been extensively characterized and are generally located on large catabolic plasmids (Gary *et al*., 1990). A large number of microorganisms are able to use polycyclic aromatic hydrocarbons (of two and three rings) as the sole source of carbon and energy (Bamforth and Singleton, 2005).

Microorganisms that biodegrade the components of petroleum hydrocarbons are isolated from various environments, particularly from petroleum-contaminated sites. Evaluations of indigenous microorganisms are needed so that bacterial community composition can be correlated with ability to degrade target pollutants (Alquaty *et al*., 2005). In this study, we investigated the potential of isolated bacteria and the role of plasmids for degradation of naphthalene.

II. MATERIALS AND METHODS

2.1 Collection of soil samples

For the isolation of naphthalene degrading bacteria, soil samples were collected from 3 different vehicle service station sites of Chandigarh, India. Samples were stored in sterilized polyethylene bags at 4°C for further use.

2.2 Enrichment and isolation of naphthalene 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 naphthalene.

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 naphthalene. Further sub-culturing was done with 0.25 g, 0.1 g and 0.0 g peptone with constant concentration of naphthalene. The naphthalene degrading microorganisms from the flask containing 0.1% w/v naphthalene and no peptone were isolated by spread and streak plate method and analyzed for their naphthalene degradation activity.

10 ppm solution of naphthalene in ethyl acetate was scanned from 190-600 nm on UV-VIS spectrophotometer. The λ_{max} was used to determine the concentration of naphthalene in ethyl acetate extracts. For preparation of standard curve of naphthalene, 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 naphthalene.

2.3 Screening of naphthalene degrading microorganisms

For screening of naphthalene degrading microorganism, 30 ml BSM broth containing 0.1% w/v naphthalene 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 naphthalene was monitored spectrophotometrically for 10 days by liquid-liquid extraction method as described by Manohar *et al.* (1999).

Further the naphthalene degrading capability of most efficient naphthalene degrading organism was monitored in 30 ml BSM broth containing 1.5% w/v naphthalene/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 naphthalene degrading microorganism was done on the basis of microscopic, morphological and biochemical characteristics.

2.4 Plasmid curing by acridine orange

BSM broth (18 ml) was taken in different flasks and was inoculated with 1 ml 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 photolysis of cells and 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 antibiotics were used to check the antibiotic sensitivity of most efficient naphthalene degrading microorganism by the 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 help of a cork borer in the agar at equal distances. Seventy µl of each antibiotic was poured in these wells.

2.5 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.

2.6 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.

III. RESULTS

3.1 Screening for most efficient naphthalene degrading microorganism

The λ max of naphthalene in ethyl acetate was determined to be 254 nm (Figure 1). It was used to determine the concentration of naphthalene in ethyl acetate extracts. 3 different soil samples were analyzed for their naphthalene degradation capability. After enrichment of 59 bacterial strains, 3 strains were found to be efficient naphthalene degrader. Further, the naphthalene degrading capability was monitored for 10 days in BSM medium supplemented with naphthalene as a sole source of carbon. On the basis of naphthalene degradation (%) ability, strain IR1 was found to be the most efficient naphthalene degrader with maximum degradation rate of 74.8% followed by strain IR2 and IR3 with values if 60.3% and 54.9% respectively (Figure 2). Strain IR1 was identified on the basis of cultural, microscopic, morphological and biochemical characteristics (Table 1). As determined by the Bergey's manual of systematic bacteriology the strain IR1 has been tentatively identified as *Pseudomonas* sp.

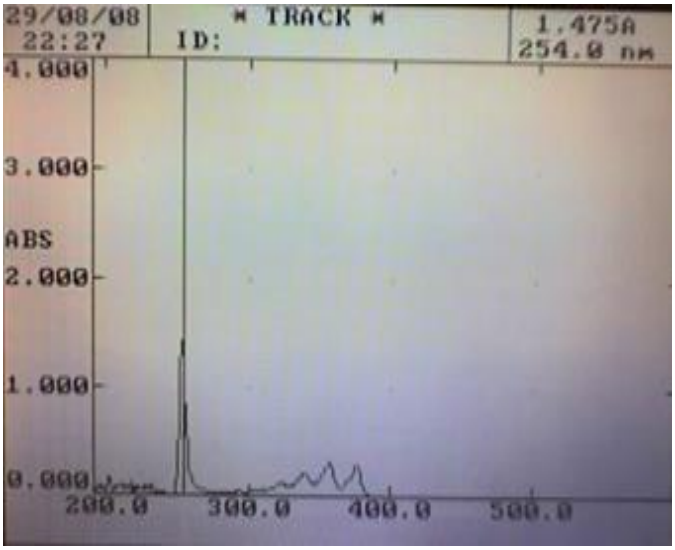

Figure 1. UV-Spectrum of Naphthalene in ethyl acetate. A single peak at 254 nm shows the max absorbance

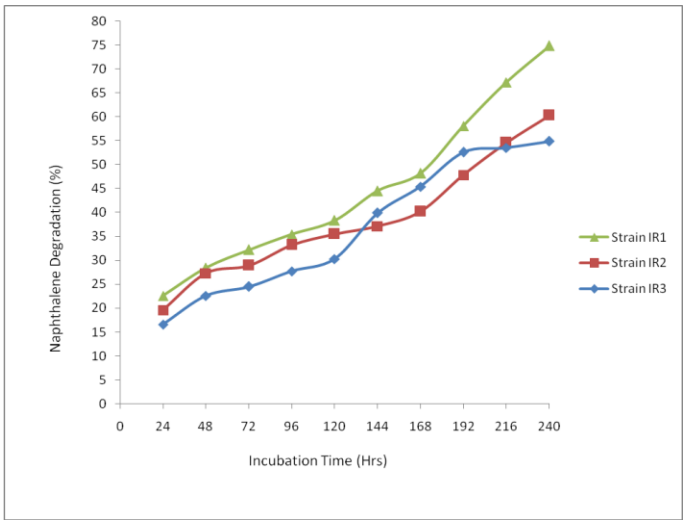

Figure 2. Naphthalene degradation by 3 soil isolates for 10 days of incubation period

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

S.N.	Morphological Characteristics	Results
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	**Results**
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	+

3.2 Naphthalene degradation study

The results of growth of the bacterium on naphthalene 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 naphthalene at 10th day of incubation.

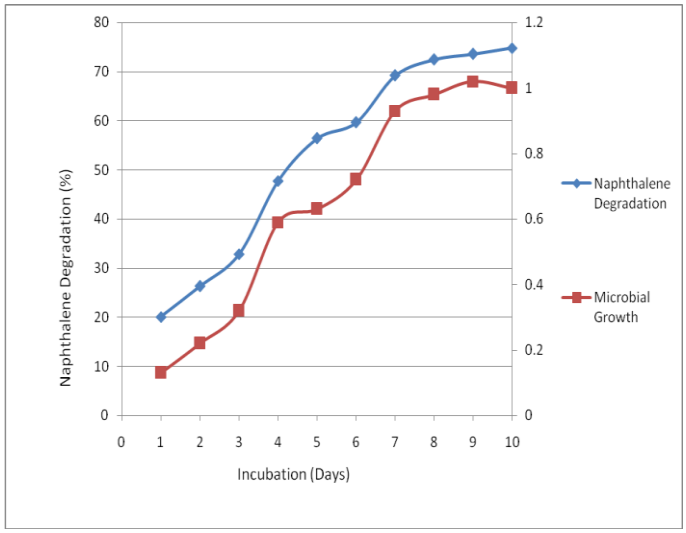

Figure 3. Growth (A_{600}) and Naphthalene degradation (%) by *Pseudomonas* sp.

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, cefixime 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).

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	Cefixime	CX	17
6	Ampicillin	AP	0
7	Amoxicilline	AM	6

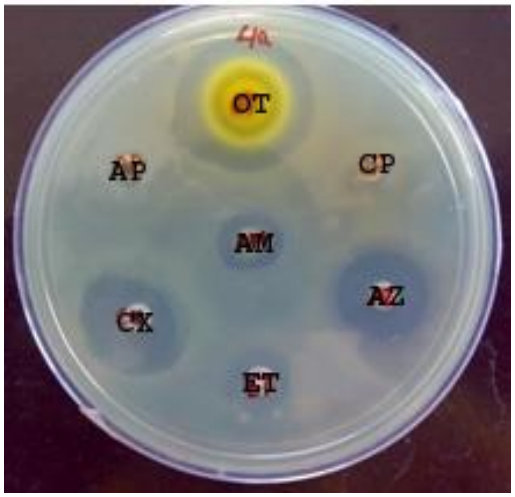

Figure 4. Sensitivity and resistance of *Pseudomonas* sp. against different antibiotics (OT- Oxytetracycline, CP- Cefadroxil, AZ- Azithromycin, ET- Erythromycin, CX- Cefixime, AP- Ampicillin, AM- Amoxicilline)

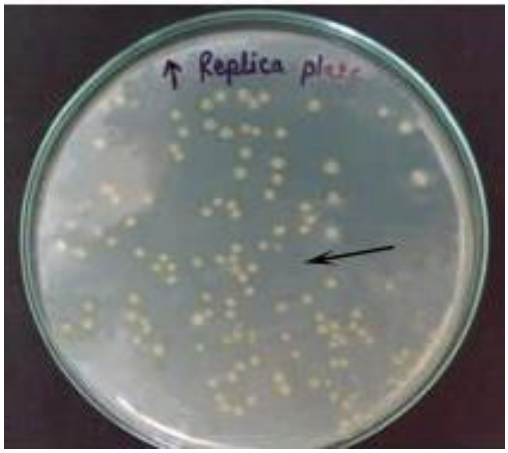

Figure 5. Replica plate showing the position of disappeared colony (arrow point) when incubated in presence of Cefadroxil, marker antibiotic.

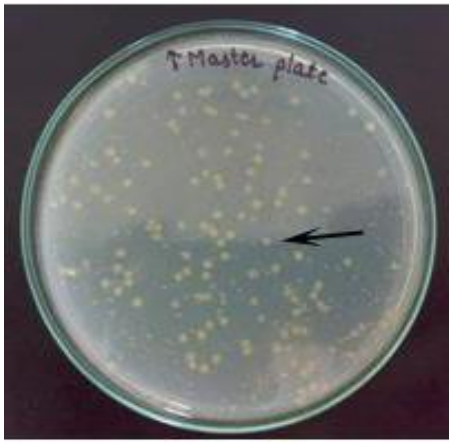

Figure 6. Master plate showing *Pseudomonas* sp. colonies on BSM agar plate when incubated in presence of Cefadroxil, marker antibiotic

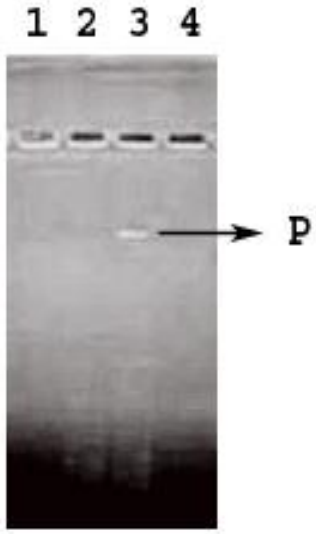

Figure 7. Gel Electrophoresis profile for DNA from *Pseudomonas* sp. Partially purified Plasmid was separated on 0.8% Agarose. P, Plasmid DNA. Lane - 2 and Lane - 3 represent DNA profile of cured and uncured plasmid, respectively.

IV. DISCUSSION

Pseudomonas sp. strain IR1 was isolated from vehicle service station site of Chandigarh, India and cultured in BSM broth supplemented with 0.1% w/v naphthalene as substrate. In contrast to other strains isolated in this study *Pseudomonas* sp. strain IR1 degraded 74.8% in 10 days of incubation. The addition of naphthalene to aqueous media was by dissolution in a carrier solvent, ethyl acetate (Moody *et al*, 2001; Nadalig *et al*, 2002). Safekordi and Yaghmaei (2001) isolated bacteria from soil contaminated with coal tar and studied biodegradation of PAH like naphthalene, phenanthrene and

anthracene and found that naphthalene degradation was 60% after 20 hrs and complete degradation was observed after 90 hrs. Mirdamadian *et al*. (2010) also reported similar results regarding biodegradation where 5 different strains were isolated and *Pseudomonas* strain L degraded 72% of crude oil and gave best degradation among all. Gomare and Lahane (2011) also observed similar results in their study where they found that out of 21 cultures isolated from contaminated soils of petrol pump stations *Pseudomonas* sp. degraded Naphthalene among the tested strains. After screening, *Pseudomonas* sp. strain IR1 was further cultured in BSM medium and during this period the maximum naphthalene degradation was found to be 74.8% on 10th day and maximum bacterial growth was measured on 9th day.

We have tested several antibiotics for their resistance against *Pseudomonas* sp. strain IR1 and observed that strain IR1 was resistant against cefadroxil and ampicillin antibiotics. This suggests that strain IR1 may possess 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 IR1 was not able to grow on BSM broth with naphthalene as sole carbon source. It is assumed that this may be because of the removal/inactivation of gene(s) responsible for naphthalene degradation from strain IR1. This indicates that gene(s) responsible for naphthalene degradation might be associated with plasmid DNA that has been cured, thus not allowing the colony to grow in BSM broth. Moreover, the colour 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 IR1. Coral and Karagoz (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 suggested that naphthalene degradation is plasmid associated. This is in accordance with the previous finding by Sanseverino *et al.* (1993) and Mirdamadian *et al*. (2010) 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 Naphthalene by *Pseudomonas* sp. was investigated. In conclusion *Pseudomonas* sp. is an efficient naphthalene degrading strain and could be use to develop an environmental friendly technology to overcome the problem of oil spills.

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