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Profiling marine bacterial dioxygenase gene involved in Polycyclic Aromatic Hydrocarbon degradation

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Abstract. *Mishra M, Das S. 2017. Profiling marine bacterial dioxygenase gene involved in Polycyclic Aromatic Hydrocarbon degradation. Ocean Life 1: 32-40.* The degradation of polycyclic aromatic hydrocarbons (PAHs) by bacteria has been widely reported. While many pure cultures have been isolated and characterized for their ability to grow on PAHs, little is known regarding the diversity of microorganism involved in PAH degradation in the environment. The aim of this study was characterization of the gene for enzymes involve in PAHs Degradation, and to make a bank collection of strains for further screening research. Pure bacterial cultures were sampled from a highly enriched consortium for biodegradation analysis. Bacterial strains capable of degrading pyrene and anthracene were isolated from Paradeep estuary (Odisha, India) water sample by selective enrichment. Five strains of pyrene and anthracene degrading pure cultures were collected, named as MP-1, MP-4, MP-9, MP-14, MP-18. Isolates were characterized by gram staining, utilization of citrate, sugar fermentation, swimming and swarming motility and antibiotic sensitivity test. In seven days isolates MP-1,MP-4,MP-9,MP-14,MP-18 shown to degrade 58.4%, 42.1%, 29.1%, 31.7%, 31.8% of pyrene at a concentration 100 mg/l and 56.3%, 46%, 44.6%, 30.4%, 48.2% of anthracene at a concentration100mg/l. Polymerase chain reaction (PCR) with PAH-specific primers successfully amplifies a dioxygenase gene in MP-4. The presence of dioxygenase gene may lead to unraveling the underlying mechanism on how bacteria develop the abilities to degrade high-molecular-weight PAH. The understanding to profile not only the bacterial community but also the dioxygenases which they encode provides a powerful way for both assessing bioremediation potential in the environment and the discovery of novel dioxygenase genes.

Keywords: Anthracene, biodegradation, dioxygenase gene, marine bacteria, pyrene

INTRODUCTION

Biodegradation is a viable bioremediation technology for organic contaminants. The purpose of bioremediation is to transform organic pollutants into harmless metabolites or mineralize the contaminants into carbon dioxide and water (Alexander 1985). A possible remedial technology requires microorganisms being capable of quick adaptation to and efficient uses of pollutants of interest in a particular case in a relevant period. Microorganisms can utilize pollutants as substrates or metabolize them under various factors. Hence, understanding the catabolic pathway, mechanisms, and responsible enzymes is an effective means to define essential factors for efficient cleanup of pollutants. Biodegradation involves uses of a wide range of microorganisms to break chemical bonds and has been well reviewed (Klein 2000). Nevertheless, biodegradation is a very active field, and new findings are rapidly contributed to the literature.

PAHs (Polycyclic aromatic hydrocarbon) are known as aromatic hydrocarbons with fused benzene rings, typically arranged of two or more rings. PAHs are generated during the thermal breakdown of organic molecules and their succeeding recombination, such as partial combustion at high temperature (500-800 °C) or subjection of organic material at low temperature (100-300 °C) for extended periods leads to PAH production. They appear as colorless, whitish, yellow solids with low solubility in water, high melting, high boiling points, and low vapor pressure. PAHs are the environmental pollutant that is found naturally or in several polluted soils as a result of industrial activities, as well those of creosote wood-treatment facilities (Mueller et al. 1989). PAHs have involved considerable attention due to their potential toxicity for higher organisms and resistance to microbial degradation (Kanaly and Harayama 2000). A wide range of microorganisms have been discovered that can degrade very stable, deadly organic compounds, e.g., polycyclic and aliphatic hydrocarbons (Kanaly and Harayama 2000; Habe and Omori 2003; Van Hamme et al. 2003). Among these microorganisms, numerous Arthrobacter species can degrade PAHs (Grifoll et al. 1992; Seo et al. 2006). The PAHs have gathered significant environmental concern due to their occurrence, recalcitrance, bioaccumulation potential, and carcinogenic activity.

Microbial degradation is the primary degradation process of PAHs (Bumpus 1989; Yuan et al. 2001). The process depends on the environmental conditions, number, and type of the microorganisms, nature and chemical structure of the chemical compound being degraded. PAHs are biodegraded into less complex metabolites, and through mineralization into inorganic minerals, H_2O, CO_2 (aerobic) or CH_4 (anaerobic). The rate of biodegradation depends on microbial population, oxygen, pH, temperature, accessibility of nutrients, the chemical structure of the

compound, chemical partition in growth medium, and cellular transportation properties (Singh and Ward 2004).

The majority of information on PAHs degradation pathways derived from studies on gram-negative bacteria especially the genus Pseudomonas (Simon et al. 1993; Bosch et al. 1999). Under aerobic conditions, bacterial degradation of PAHsbegins with the incorporation of both molecules of molecular oxygen to the aromatic ring by a dioxygenase. The case of naphthalene (smaller molecular weight PAHs) degradation by cultured microorganisms has been studied extensively (Cerniglia 1992). Microbial degradation of PAHs is an important decomposition process for these contaminants in nature, thereby, represents a potential solution to the environmental problems posed by these chemicals. In recent years, PAHs biodegradation studies have received much attention and many related reports have documented that PAH dioxygenase and catechol 2,3-oxygenase are identified as two key PAH-degrading-related enzymes (Resnick et al. 1996; Meyer et al. 1999).

Some recent studies on genetic diversity have been mainly conducted with microbial populations or many bacteria. It focuses on analyzing the variety of dioxygenase genes of a bacterium. *Pseudomonas rhodesiae* KK1 of utilize a wide range of monoaromatic compounds including polycyclic and heterocyclic aromatic hydrocarbons such as carbazole and naphthalene previously mentioned (Kahng 2002). Various types of heterocycles comprising oxygen, sulfur, and nitrogen are detected in the environment which derived from instinctive or anthropogenic sources. Dibenzofurans, dibenzodioxins, and dibenzothiophene are within the most critical environmental pollutants and well reviewed by Klein, 2000. Thereby, only aerobic bacterial degradation of non-halogenated heterocyclic aromatics is briefly discussed in this to make a general relation with PAH degradation.

However, recent studies on cloned dioxygenase from *Norcardioides aromaticivorans* IC177 demonstrated that some dioxygenase could catalyze both reactions. PAH dioxygenases can catalyze several responses, including reduction, mono-and de-oxygenation (Resnick et al. 1996). In addition to the multiple responses with specific dioxygenases, current genomic or proteomic search with several PAH-degrading bacteria (e.g., *Burkholderia* spp. and *Mycobacterium* spp.) exposed that multiple dioxygenases exist in a single bacterium, probably playing different roles in PAH degradation (Resnick et al. 1996; Liang 2006).

Generally, methyl-ethyl-naphthalenes and phenanthrenes are common contaminants in the environment and, however, a limited amount of studies have been done about bacterial degradation. Alkyl PAHs catabolism in aerobic bacteria suggests a wide diversity of enzymes involved. These include oxidation of methyl group to an alcohol, aldehyde, carboxylic acid, decarboxylation, demethylation, and deoxygenation. Even so, production of alkyl salicylate or alkyl phthalate suggests that the reaction may prefer non-substituted PAH systems. Many anaerobes can transform Nevertheless, PAHs and their alkyl derivatives through novel catabolic pathways. Proteomics has been recently employed in studies of environmental microbiology and has shown their vast impact on the field of biodegradation and bioremediation.

Majority of the studies on the microbial metabolism of PAHs have been carried out with strains that use the compound under investigation as a growth substrate (Cerniglia 1992; Kanaly and Harayama 2000). However, some degrading bacteria act on a variety of compounds that do not support their growth and secreted partially oxidized products (Grifoll et al. 1995). This versatility is partly due to the broad substrate specificity of the degradative enzymes, as has been widely established for naphthalene and toluene dioxygenases (Wackett et al. 1988; Gibson et al. 1995). The primary objective of present work is the localization of dioxygenase gene cluster, for the enzyme involved for degradation of PAHs by potential marine bacterial isolates.

The objective on this research was to (i) isolate and screen pyrene and anthracene degrading Bacteria, (ii) to study the degradation of pyrene and anthracene by isolates, (iii) amplify dioxygenase gene locus, (iv) characterize the strains.

MATERIALS AND METHODS

Isolation

The samples were obtained from Paradeep (N 20º 17.542' & E 86º 42.996'), Odisha. Water is kept in sterilized falcon tubes, stored in ice and transferred to the laboratory as soon as possible. One ml of sample was suspended into Basal Minimal media (BMM) broth (Dipotassium phosphate -7g/L, Monopotassium phosphate-2g/L, Magnesium sulphate-0.10g/L, Ammonium sulphate -1g/L, Sodium citrate -0.50g/L) (Sambrook et al. 1989) supplemented with 100 mg/L of pyrene and anthracene, as the sole carbon and energy source for growth and then it was kept at 37ºC/160 rpm for 15 days. After 15 days of incubation, the inoculum was diluted and inoculated on Sea Water Nutrient Agar plates. The isolated colonies from these plates were selected for further screening.

Screening

Each BMM agar plates was incorporated with pyrene and anthracene. The pyrene and anthracene stock solution (5mg/mL) was made in Hexane, and it was spread on prepared BMM agar plates. The plates were dried in laminar for few minutes so that hexane will evaporate. Next, the isolated colonies were streaked over the pyrene and anthracene. The same colonies were also inoculated in Minimal broth with 100mg/L and kept in the incubator shaker for seven days to monitor the growth and disappearance of PAH visually, which is further maintained for degradation studies.

Carbon source utilization

The strains were inoculated in freshly prepared LB broth and incubated at 37ºC for 24 hours. Following this step, the cell mass was collected and centrifuged at 4 ºC/7000 rpm for 10 minutes. Then, the supernatant was

discarded, and the pellet was resuspended in 1ml of BBM. 100 µL of the suspended cell was moved to 5ml BMM tubes with 100 mg/l of pyrene and anthracene separately as a sole carbon source. BMM supplemented with 1% glucose without any PAHs was kept as a control for growth. After that, these tubes were held at 37ºC/180 rpm for seven days. To monitor the growth, OD_{595} was taken at a regular time interval, i.e., 0 days, one day, three days, five days and seven days.

Standard curve of pyrene and anthracene

The stock solution of pyrene and anthracene was prepared in 5mg/mL of hexane. 5ml of pyrene and anthracene from the stock solution working solution in hexane of (0.5µg/mL, 1µg/mL, 10µg/mL, 50µg/mL and 100µg/mL) was prepared in triplicate for the standard curve. It was scanned to get the λ_{max} value and absorbance between 200-400nm using UV-Visible Spectrophotometer.

Degradation study

Degradation was performed by inoculating strains into test tubes containing 5 ml of Minimal Medium broth supplemented with 100mg/L of PAH (Pyrene and anthracene) (Kiyohara et al. 1982). PAH was delivered to media from the stock solution (5mg/mL) in hexane and kept for few hours to remove hexane from the media entirely. Thereafter, the BMM-PAH tubes were subsequently incubated at 37ºC/180rpm for seven days. After seven days, the cell was harvested by centrifugation, and it was again transferred to 200mg/L of BMM-PAH tubes (Pyrene and anthracene). The tubes were incubated at 37ºC/180 rpm for another seven days.

After seven days, BMM-PAH tubes were centrifuged min at 6000 rpm for 10 min. The supernatant was removed, while the pellet was rinsed with saline water two times. To above pellet, sterile basal minimal media was added without any carbon source. Then the OD was read at 595 nm and adjusted to 0.1. From 0.1 OD modified culture, 500 µL was transferred to BMM media with the addition of 100mg/l of PAH and incubated at 37ºC/160 rpm for seven days. After seven days the whole media was used for extraction and quantification of residual PAH (Pyrene and anthracene).

Extraction and quantification

Residual PAH was extracted from 5 ml of culture with an equal volume of Dichloromethane (DCM). For equal extraction volume of DCM was added to the degradation setup. The tubes were then vortex for 10 min and kept for another 10 min to separate the aqueous and organic phase. The upper organic layer was extracted and dried over sodium sulfate. Thereafter organic phase was pipette out and kept for drying overnight. The residual was resuspended in the same volume of n-hexane. The extracted pyrene and anthracene was diluted ten times in n-hexane. The residual pyrene and anthracene concentration was calculated from the standard curve of respective PAH. The absorbance of pyrene and anthracene was taken at 335nm and 254nm respectively.

Characterization of the isolates

Gram staining: gram reaction

The bacterial culture was smeared on a clean grease free slide with a sterile loop. The smear was air-dried and then fixed by heating method. Then, it was subjected to the following staining reagents: Flooded with Crystal violet (1 min), followed by washing with running distilled water. Next, flooded with Gram's Iodine (1 min), and washed with running distilled water. Then the slide was flooded with Gram's decolorizer (30 sec). After that, the slide was counterstain with Safranine (30 sec) and rinsed with running distilled water. The slide was air dried, and the cell morphology was checked under the microscope.

Biochemical tests: sugar fermentation tests

The sugar test was performed by using Bacillus Hi-media biochemical kit which contains different sugars. Kit A consists of Lactose, Maltose, Dextrose, Fructose, Raffinose, Xylose, Trehalose, Galactose, Melibiose, Sucrose, L-Arabinose, Mannose. Kit B includes Inulin, Sodium gluconate, Dulcitol, Glycerol, Inositol, Salicin, Sorbitol, Mannitol, Adonitol, Arabitol, α-methyl-D-glucoside, Erythritol. Kit C consists of Rhamnose, α-methyl-D-mannoside, Melezitose, Cellobiose, Xylitol, ONPG, Esculin hydrolysis, D-Arabinose, Citrate utilization, Malonate utilization, Sorbose. Sugar fermentation test was used to detect bacteria's ability to ferment sugar and produce gas and acid end product.

Swimming and Swarming motility tests

Swimming and swarming motilities are a rapid and a coordinated movement of a bacterial population across solid or semi-solid surfaces. This sort of motility is an example of bacterial multicellularity, an emerging concept in microbiology. Swarming motility was first described by (Henrichsen 1972) and has been mostly studied in genus *Serratia* (Alberti and Harshey 1990), *Salmonella* (Harshey 1994), *Escherichia* (Harshey and Matsuyama 1994), *Yersinia* (Young et al. 1999), *Aeromonas* (Kirov et al. 2002), *Bacillus* (Kearns and Losick 2004), *Pseudomonas* (Daziel et al. 2003*), Proteus* (Caiazza et al. 2005), *and Vibrio* (Rather 2005). This multicellular behavior is commonly observed in controlled laboratory conditions and depends on two critical elements: (i) the nutrient composition and (ii) viscosity of culture medium (i.e., the percentage of agar). One specific feature of this type of bacterial motility is the formation of dendritic fractal-like patterns generated by migrating swarms moving away from an initial position.

For swimming motility test, a lower agar nutrient concentration (0.2%) was prepared, and for swarming motility test, the higher content was used (0.5%). The agar was then poured plates in semi-solid condition, and ten µL of culture was inoculated at the center of the dishes, then the plates were kept in the incubator for 24 hrs at 37ºC.

Antibiogram

All the strains were examined for antimicrobial resistance by the method of Bauer et al. 1966 with Hi-Media, an antibiotic-impregnated media. All isolated

culture was swabbed in the MHA plates. Following this step, the antibiotics disc were kept over it. The following discs with concentration of the antibiotics as stated in the parenthesis were used, Gen: 10µg, AZM: 30µg, C: 50µg, AM: 30µg, E: 15 µg, NX: 10µg, S: 10µg, VA: 30 µg, MET: 5µg, T: 30µg, K: 30µg, AC: 10µg. Based on the diameter of the inhibition zones around the disc, the strains were characterized as susceptible or resistant based on diameter of the inhibition.

Preparation of lysates

Fresh cultures were transferred to 2 ml of LB medium and incubated at 37ºC for overnight. 1 ml of the suspension was centrifuged at 7500 rpm for 10 min at 4ºC. The supernatant was discarded, and the rest of the culture was transferred to the pellets again centrifuged at 7500 rpm for 10 min at 4ºC. The supernatant was discarded, and washed with 200 µL of sterile MQ water was added and mixed adequately by vortexing. This mixture was then centrifuged for 10 min at 4ºC at 7500 rpm, and the supernatant was discarded, followed by an additional washing step using sterile MQ as mentioned above. The tube was kept in a water bath at 100ºC for 10 min, and after that tube was kept on ice for 5 min, then centrifuged at 10,000 rpm for 5 min at 4ºC. The supernatant was transferred to a new fresh tand ube and used as the template for PCR or stored at -20ºC if not used immediately.

Amplification of pahs dioxygenase locus

The DNA sample was resuspended in sterilized distilled water to get the final concentration of 100mg/ µL. The primers for the amplification of the DNA were as follows: Forward primer-(GAG ATG CAT ACC ACG TKG GTT GGA); Reverse primer -(AGC TGT TGT TCG GGA AGA YWG TGC MGT T) (Cebron et al. 2008). 10X buffer contained 50Mm KC_2L, 1.5 Mm MgCl2, 20 Mm Tris-Cl (pH 8) and gelatine. The dNTP mixture was prepared with 2.5 Mm each of dATP, dTTP, dGTP, and dCTP to make a final concentration of 100 µL and kept at -20ºC. Taq DNA polymerase was stored buffer containing 50% glycerol. It was available at a concentration of 3 µL from the manufacturer.

Methodology

Genomic DNA was amplified in sterile PCR tubes with reaction volume of 25 µL containing the following components-10X assay buffer-2.5 µL, dNTP -0.5 µL, MgCl2 -1.5 µL, Forward primer -0.5 µL, Reverse primer-0.5 µL, Template -4 µL, Taq DNA polymerase-1 µL, autoclaved MQ water was added to make up the volume to 25 µL. The amplified PCR products were then stored at -20ºC for further use. Table 1 shows the PCR conditions.

Table 1. PCR conditions

		Temperature	Time
	Initial denaturation	95ºC	5 minutes
30 cycles	Denaturation	95ºC	30 seconds
	Annealing	57ºC	30 seconds
	Extension	72ºC	30 seconds
	Final extension	72ºC	7 minute

RESULTS AND DISCUSSION

Isolation and screening

The growth of isolates on Sea water nutrient is shown in Table 2. Twef inty-one isolated colonies were observed with distinct colony morphologies. These isolates were further screened for their potential to degrade PAHs. Preliminary screening was done merely by continuously monitoring the growth of strains on BMM-PAH agar plates (Table 3) and BMM-PAH broth for seven days (Figure 1). After seven days, among 21 isolates, continuous growth was observed in 5 strains (MP-1, MP-4, MP-9, MP-14, MP-18) and OD readings were found to be more than 0.5 at 595 nm.

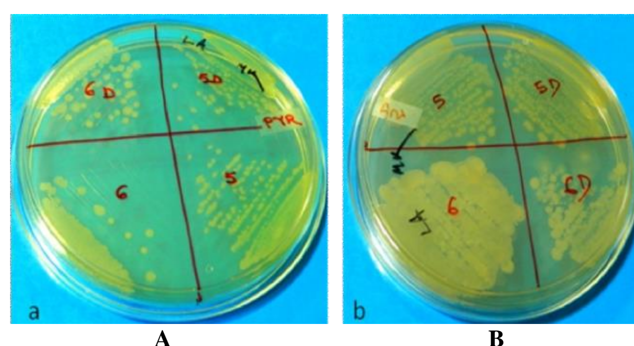
Carbon source utilization

To monitor the growth, OD_{595} was taken at a regular time interval, i.e., 0 days, one day, three days, five days and seven days by ELISA plate reader (Figure 2). The growth pattern of isolates in term of OD_{595} in different carbon source is presented in Table 4-5.

Table 2. Growth of isolates on Sea water nutrient agar plates.

Isolates	Growth on sea water nutrient agar plates
MP-1	+++
MP-2	+
MP-3	±
MP-4	+++
MP-5	–
MP-6	++
MP-7	–
MP-8	±
MP-9	+++
MP-10	++
MP-11	+
MP-12	–
MP-13	–
MP-14	+++
MP-15	++
MP-16	–
MP-17	+
MP-18	+++
MP-19	+
MP-20	++
MP-21	±

Note: +++ : excellent, ++ : good, + : satisfactory, ± : variable, -: negative



A B

Figure 1. Isolated colonies on basal minimal media. A. pyrene, B. anthracene

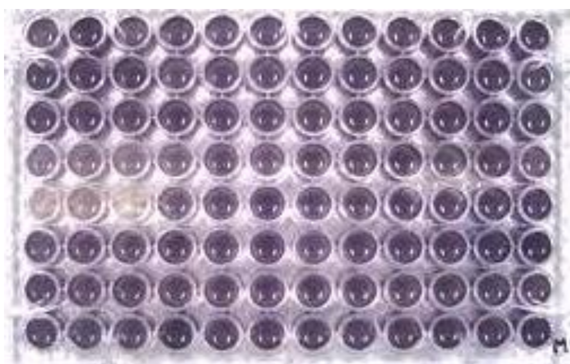


Figure 2. 96 well plate used to monitor the growth

Table 3. Growth of isolates on BMM-PAH agar plates

Isolates	Pyrene	Anthracene
MP-1	+	+
MP-4	+	+
MP-9	+	+
MP-14	+	+
MP-18	+	+

Table 4. OD_{595} for pyrene

Isolates	0 day	1 day	3 days	5 days	7 days
MP-1	0.011	0.104	0.229	0.339	0.5
MP-4	0.02	0.2	0.389	0.415	0.499
MP-9	0.022	0.197	0.303	0.415	0.495
MP-14	0.01	0.221	0.287	0.346	0.4
MP-18	0.012	0.2	0.299	0.395	0.487

Table 5 .OD_{595} for anthracene

Isolates	0 day	1 day	3 days	5 days	7 days
MP-1	0.03	0.24	0.47	0.64	0.82
MP-4	0.024	0.2	0.46	0.58	0.67
MP-9	0.021	0.34	0.5	0.66	0.84
MP-14	0.03	0.2	0.53	0.66	0.86
MP-18	0.03	0.29	0.46	0.65	0.79

Table 6. Standard curve of pyrene and anthracene at different concentration

Concentration	Pyrene (A_{335})	Anthracene (A_{254})
0.5 µg	0.0001	0.0001
1 µg	0.0004	0.0002
10 µg	0.064	0.0741
50 µg	0.306	0.323
100 µg	0.586	0.592

Standard curve of pyrene and anthracene

For anthracene and pyrene, λ was found to be 254nm and 335nm respectively, and the absorbance at this λ was considered to prepare the standard curve. λ_{max} and a standard curve of pyrene and anthracene are shown in Figure 3-4. Table 6 shows the absorbance value for different concentration.

Extraction and quantification

The results of degradation studies of these isolates were illustrated under quantification and extraction method. Before quantification and extraction, the prepared stock at different concentration was scanned within 200 to 400 nm in UV-Visible Spectrophotometer. pyrene shows its peak at 335 nm (Figure 3), and anthracene shows its peaks at 254 nm (Figure 4). Quantification and extraction were done using Dichloro-methane, then it was resuspended with 1mg/mL of hexane. After extraction OD was taken at 334nm for pyrene and 252 nm for anthracene for each sample (MP-1, MP-4, MP-9, MP-14, and MP-18). The results of degradation studies of these isolates in pyrene and anthracene were shown in (Table 7-8). The remaining concentration of pyrene and anthracene was calculated from the standard graph of the compound.

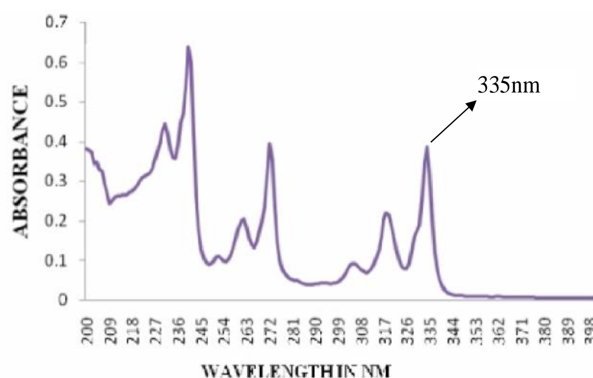


Figure 3. γ_{max} for pyrene (335nm)

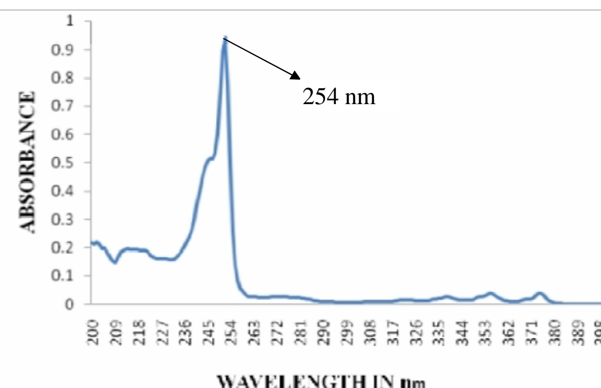


Figure 4. γ_{max} for anthracene (254nm)

Table 7. Percentage degradation of pyrene after 7 days.

Isolates	Initial Conc. (I) mg/L	Conc. (C) mg/L after 7 days	%Degradation =C/I*100
Control	100	0.997	0.997
MP-1	100	41.5366	58.4633
MP-4	100	57.8139	42.1860
MP-9	100	70.8106	29.1893
MP-14	100	68.2300	31.7691
MP-18	100	68.1499	31.8500

Characterization of isolates

All the five strains (MP-1, MP-4, MP-9, MP-14, MP-18) were found to be Gram-negative (Figure 5), motile and show positive Citrate Utilization test. The results of the characterization of these isolates were illustrated in Table 9-10.

Carbohydrate utilization

Sugar fermentation test was used to detect bacteria's ability to ferment sugar and produce gas and acid end product. The result of the carbohydrate utilization pattern was given in Table 11. Most of the strains varied in the pattern of utilization of 34 different sugars. Most of the isolates varied in the pattern of utilization of 34 different sugars.

Table 8. Percentage degradation of anthracene after 7 days

Isolates	Initial Conc. (I) mg/L	Conc. (C) mg/L after 7 days	%Degradation =C/I*100
Control	100	0.994	0.994
MP-1	100	43.6358	56.3641
MP-4	100	53.9531	46.0468
MP-9	100	55.3651	44.6348
MP-14	100	69.5418	30.4581
MP-18	100	51.7863	48.2136

Table 9. Characterization of isolates

Characteristic	MP-1	MP-4	MP-9	MP-14	MP-18
Gram stain	-	-	-	-	-
Colour of colonies	Cream	White	White	Cream	White
Motility	+	+	+	+	+
Citrate utilization test	+	+	+	+	+

Note: +: Positive, -: Negative

Table 10. Swimming and Swarming Motility Tests

Motility test	MP-1	MP-4	MP-9	MP-14	MP-18
Swimming(0.2%)	+	+	+	+	+
Swarming(0.5%)	+	+	+	+	+

Note: +: Positive

Table 11. Sugar Fermentation Tests

Sugars	MP-1	MP-4	MP-9	MP-14	MP-18
Lactose	-	-	-	-	-
Xylose	-	-	-	-	-
Maltose	-	-	-	-	-
Fructose	-	-	-	-	-
Dextrose	-	-	-	-	-
Galactose	-	-	-	-	-
Raffinose	-	-	-	-	-
Trehalose	-	-	-	-	-
Melibiose	-	-	-	-	-
Sucrose	-	-	-	-	-
L-Arabinose	-	-	-	-	-
Mannose	-	-	-	-	-
Inulin	-	-	-	-	-
Glycerol	-	-	-	-	-
Sodium gluconate	-	+	-	-	-
Salicin	-	-	-	-	-
Dulcitol	-	-	+	-	-
Inositol	-	-	-	-	-
Sorbitol	-	-	-	-	-
Mannitol	-	-	-	-	-
Adonitol	-	+	-	-	-
Arabitol	-	-	-	-	-
Erythritol	-	-	-	-	+
α-Methyl-D-glucoside	-	+	-	-	-
Rhamnose	-	-	-	+	-
Cellobiose	-	-	-	-	-
Melezitose	-	-	+	-	-
α-Methyl-D-Mannoside	-	-	-	-	-
Xylitol	-	-	-	-	-
ONPG	-	-	-	-	-
Esculin hydrolysis	+	+	+	+	+
D-arabinose	-	-	-	-	-
Malonate utilization	+	+	+	+	+
Sorbose	-	-	-	-	-

Note: +: Positive, -: Negative

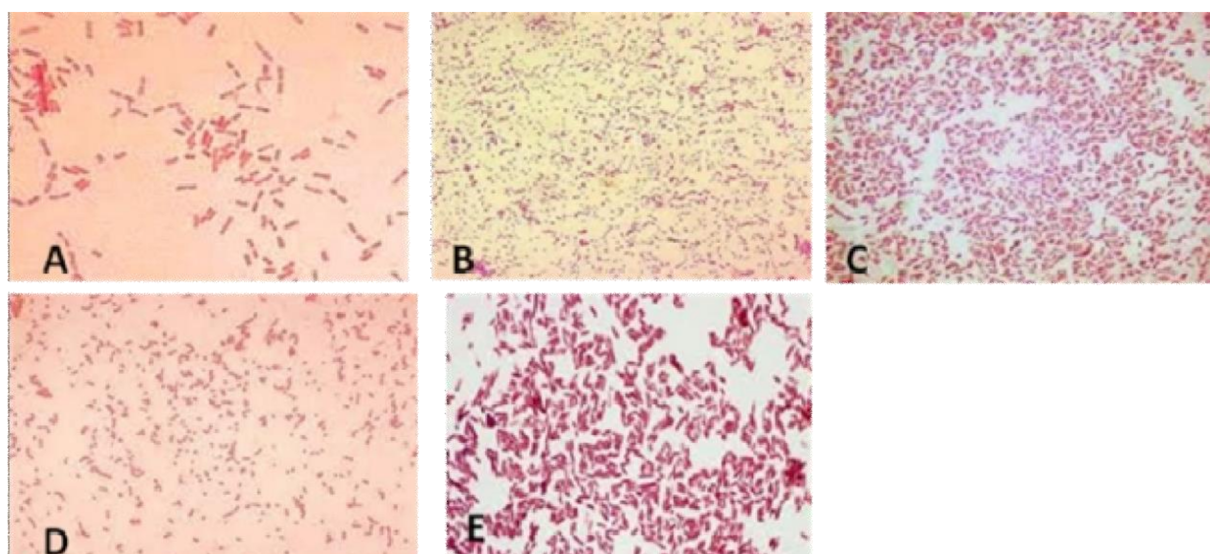


Figure 5. Gram Staining of five Isolates. A. MP-1, B. MP-4, C. MP-9, D. MP-14, E. MP-19

Antibiogram

All the five isolates were examined for antimicrobial resistance by the method of Bauer et al. 1966 with antibiotic-impregnated discs (Hi-Media). The strains were characterized as resistant or susceptible resistant based on the diameter of the inhibition zones around the disc (Fig .6). Gen10, C 50, E 15, S 10, AZM 30, T 30 and K 30 were sensitive to all five isolates but AM 30, and AC 10 is resistant in MP-1 and MP-14, VA 30 is resistant in MP-1, MP-4 and MP-9, NX 10 is resistant in MP-1 and all 4 isolates were sensitive, MET 5 are resistant in all the five isolates (Table 12 and 13).

Amplification of pahs dioxygenase locus

Polymerase chain reaction (PCR) amplification using PAH-specific primers revealed the presence of a dioxygenase gene in MP-1 (Figure 7). The size of the product was found to be 300 bp.

Discussion

Our study focused on the isolation and screening of potential pyrene and anthracene degrading bacteria from the marine source. Marine water and sediments receive waste from all terrestrial, atmospheric and freshwater source and being polluted by a variety of organic and inorganic pollutants. A total of 21 bacteria were isolated from the enriched contaminated water samples, mainly by the formation of inhibition zones on BMM with sprayed pyrene and anthracene as the sole carbon source. Five PAH-degrading bacteria from were further analyzed phenotypically for their ability to degrade PAH and whether they contain dioxygenase locus or gene which plays a vital role in the degradation of PAH. The analysis was designed to screen PAH-degrading isolates as potential sources for degradation of PAH, i.e., pyrene and anthracene.

After selective enrichment of PAHs degrading bacteria, five isolates named as MP-1, MP-4, MP-9, MP-14, MP-19 were studied for their degradation potential. In 7 days of observation, MP-1, MP-4, MP-9, MP-14 and MP-19, found to degrade 58.4%, 42.1%, 29.1%, 31.7%, and 31.8% of pyrene and 56.3%, 46%, 44.6%, 30.4% and 48.2% of anthracene respectively. The results obtained with the above test demonstrated that these particular isolates degrade more potentially pyrene than anthracene.

All isolates proceeded to PAH spray plate method, degradation, quantification. Physical and biochemical characterization was done through Gram staining, citrate utilization test, sugar utilization test, swimming and swarming motility test, antibiotic sensitivity test. All the isolates show different morphological aspects regarding Gram staining, the color of colonies, motility and citrate utilization. Among all the strains only MP-1 is Gram-positive and rest were Gram-negative. This particular isolate is highly motile. All the above strains are showing a positive result in Swimming and Swarming motility test.

Table 12. Zone (mm) of inhibition of antibiotics against five isolates.

Isolates	GEN	AM	AZM	C50	E 15	VA	NX	S	MET	T	K	AC
MP-1	16	0	18	17	19	0	0	12	0	28	19	0
MP-4	29	9	29	26	28	0	24	17	0	19	24	13
MP-9	28	9	3	19	24	0	25	16	0	19	24	13
MP-14	23	0	3	21	22	9	32	20	0	22	19	0
MP-18	21	19	24	19	15	16	16	19	0	24	14	22

Note: Gen 10-Gentamicin, Amp 30-Amphicillin, Azi 30-Azithromycin, Chl 50-Chloramphenicol, Ery 15-Erythromycin, Van 30-Vancomycin, Nor 10-Norfloxacin, Str 10-Streptomycin, Met 5-Methicillin, Tet 30-Tetracyclin, Kan 30-Kanamycin, Ac 10-Ac

Table 13. Antibiotic resistance pattern

Strain No.	Strain name	Antibiotic resistance pattern
1	MP-1	AC^R,MET^R,NX^R,VA^R,AM^R
2	MP-4	MET^R,VA^R
3	MP-9	MET^R,VA^R
4	MP-14	AM^R,MET^R,AC^R
5	MP-18	MET^R

Note: R: Resistant

Figure 6. Inhibition of bacterial growth on MHA by antibiotic discs in disc diffusion technique

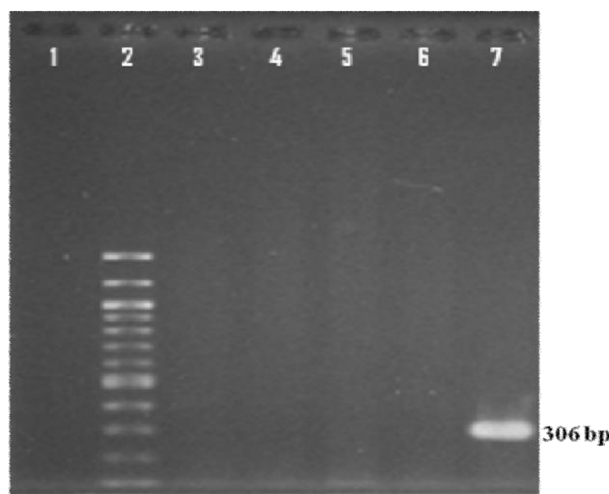


Figure 7. Gel Photograph showing amplification of *dioxygenase* gene. Lane 1: -ve Control, Lane 2: 100bp ladder, Lane 3-7: MP-4, MP-9, MP-14, MP-18 and MP-1

All the isolates show a negative result in the carbohydrate utilization test. They were subjected to the carbon source utilization process; degradation and quantification which displayed different types of effects, i.e., all strains were differentially potentially to degrade polycyclic Aromatic Hydrocarbons. The standard curve of the certain PAH, i.e., pyrene and anthracene show maximum absorbance at 335 and 254nm respectively. The results from degradation and quantification study suggested strong evidence about the percentage of degradation of isolates in PAHs. Among all the strains MP-1 is most potential, showing 58.4% degradation of pyrene and 56.3% degradation of anthracene. In the previous report, the degradation rate of anthracene was studied in four strains (*Escherichia coli,* a soil bacterium, *Alcaligenes* sp. and *Thiobacter subterraneus*). The mean degradation of anthracene demonstrated by these four bacterial strains was found to be 28.57%, 30.19%, 26.58% and 32.11% (Kampfer and Dott, 1988). Both *Pseudomonas* and *Alcaligenes sp.* are the most common bacteria occurring at polluted sites due to enhanced selection by a high concentration of organic xenobiotics (Abd-Elsalam et al. 2006).

The result of the present study confirmed that many of bacterial strains, especially Gram-negative bacteria were found capable of degrading PAH compound at various extents (Kiyohara et al. 1982), thus, indicates that most efficient of the PAH-degrading bacteria belong to the genus *Pseudomonas.* The previous report described that the pyrene induced pdoA1 and pdoB1 genes from *Mycobacterium sp*. is closely homolog to the nidA and nidB genes (Krivobok et al. 2003). These genes encode the terminal oxygenase component of the initial aromatic ring dioxygenase, nidA and nidB, whose products catalyze the conversion of pyrene to 4,5-dihydroxy-4,5-dihydropyrene, have been cloned and sequenced from *Mycobacterium vanbaalenii* (Khan et al. 2001). These results strongly suggest that not only the dioxygenase gene but also nidA and nidB genes are widely distributed among pyrene utilizing *Mycobacteria* which play an essential role in the degradation of PAH.

The result of the antibiotic sensitivity test revealed that all isolates were resistant towards methicillin. However, these strains exhibited different inhibition zone pattern towards different antibiotics. The PAH compound used for the studies were pyrene and anthracene, the metabolic pathway of which is essential for degradation study. These two compounds show different degradation pathways by forming different intermediates.

PCR amplification with PAH-specific primers identified the presence of a dioxygenase gene in MP-1 at 306bp of the product size306bp. There are many specific PCR primers designed directly on the nucleotide sequence, with either no or low degeneracy, with specific target for each type of PAH-dioxygenase genes (Laurie and Lloyd 1999; Lloyd Jones et al. 1999; Wilson et al. 1999; Ferrero et al. 2002;Widada et al. 2002; Baldwin et al. 2003; Brezna et al. 2003; Dionisi et al. 2004; Johnsen et al. 2006). Accordingly, there has been numerous PAH-degrading bacteria isolated from water sediments, to quantify the percent of degradation in the presence of the dioxygenase gene, followed by a trial before use.

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