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Isolation and characterization of polyaromatic hydrocarbons-degrading bacteria from different Qatari soils

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Polycyclic aromatic hydrocarbon (PAH)-degrading bacteria were isolated from contaminated soil samples collected from the industrial zone at Umm-Saied city, state of Qatar. Isolation was occurred by enrichment using naphthalene, phenanthrene or anthracene as the sole source of carbon and energy. The isolates were characterized using a variety of phenotypic, morphologic and molecular properties. Three isolates showed the highest growths during screening as demonstrated by the increase in their optical densities (OD600) were selected for further studies. The isolates showed a concentration-dependent growth in all examined PAH-compounds they grew in. There were visible changes in the color of the growth medium of the isolates during their incubation, suggesting the production of different metabolites. Each isolate of them was able to grow on the three tested PAH compounds. Molecular identification of the isolates based on partial 16S rDNA gene sequences assigned them to *Pseudomonas geniculata* and *Achromobacter xylosoxidans*, respectively. This study indicates that the contaminated soil samples contain a diverse population of PAH -degrading bacteria and the use of soil-associated microorganisms has the potential for bioremediation of PAH contaminated sites.

Key words: Biodegradation, polyaromatic hydrocarbons, Qatari soils, *Pseudomonas*, *Achromobacter.*

INTRODUCTION

Polycyclic Aromatic Hydrocarbons (PAHs) are pollutants produced via natural and anthropogenic sources, generated during the incomplete combustion of solid and liquid fuels or derived from industrial activities (Gemma, 2006). They are ubiquitous contaminants of aquatic and terrestrial ecosystems whose presence is attributable to a number of petrogenic and pyrogenic sources, which had increased since the end of the Second World War (Jonsen et al., 2005). All PAHs have in common a singular feature that is based on two or more fused benzene rings (Chaudry, 1994). Their biochemical persistence in the environment arises from dense clouds of -electrons on both sides of the ring structures, making them resistant making them resistant to nucleophilic attack (Jonsen et al., 2005).

Previously, studies using animals have shown the specific carcinogenic, mutagenic and teratogenic effects of some PAHs (Autrup, 1990). Even though higher molecular weight PAHs such as those containing four or more benzene rings are considered to be responsible for the majority of the potential hazards of these compounds to the environment and human health (EPA, 1984), lower molecular weight types such as naphthalene (the simplest containing two benzene rings), anthracene and phenanthrene (both of which contain three benzene rings) are known to have health effects that though are comparatively mild could be potentially hazardous (Klaasen, 2001). As a result of these hazardous effects of PAHs, there is much interest in their environmental effects.

Despite some physical processes such as volatilization, leaching, chemical and photo oxidation are often effective

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in reducing the environmental level of PAHs (Heitkamp et al., 1988), biodegradation using microorganisms is usually the preferred and major route of PAH removal from contaminated environments because of its cost effectiveness and complete cleanup (Pothuluri and Cerniglia, 1994). Besides, the physical processes are often limited to aquatic environments only. The microorganisms should possess all the necessary enzymes needed to degrade PAHs.

It is established that selection of PAH-degrading microorganisms as with other chemicals occurs as a result of their previous exposure to this substances in the environment (Lewis et al., 1984). However, these adaptations occur slowly and usually depend on the recalcitrance or biodegradability of the particular substance involved (Spain et al., 1980). This is especially so considering that PAHs usually have low aqueous solubility and thus, are poorly available (low bioavailability) for microbial utilization (Jonsen et al., 2005).

Several isolated microorganisms have been successfully utilized in major hazardous waste clean-up processes, as for example, in industrial process streams and effluents (Levinson et al., 1994). Unfortunately, most of these studies were carried out in Western countries and to a limited extent in South America and Asia (Prantera et al., 2002). In Qatar, there is limited information on microbial degradation of polycyclic aromatic hydrocarbons. In this work, we report the isolation and characterization of some PAHs (naphthalene, phenanthrene and anthracene)-degrading bacteria from soils in Qatari environment.

MATERIALS AND METHODS

Sampling

About 10 g soil samples were aseptically collected with a sterile spatula from soils at the industrial zone (Qatar). All samples were placed into sterile polythene bags and stored at 4°C immediately they were brought to the laboratory.

Isolation of bacteria

Isolation was performed using an enrichment medium containing separately naphthalene, phenanthrene or anthracene. The medium consisted of (g/L) $NH_4 SO_3$, 2.5; $Na_2 HPO_4$, 1.0; $MgSO_4$, 0.5; $Fe_2(SO_4)_3$, 0.01; $CoCl_2$, 0.005; $CaCl_2$, 0.001; KH_2PO_4, 0.0005; $MnSO_4$, 0.0001; $(NH_4)_6Mo_7O_2.4H_2O$, 0.0001. The naphthalene, phenanthrene or anthracene was added after autoclaving the medium. First the medium was dispensed in 30 ml volumes into 150 ml Erlenmeyer flasks and autoclaved.

Subsequently, 1.0 g of each soil sample was added into each flask of the medium and incubated at 30^{o}C in a shaker (120 rpm) for 7 days. After that, 1.0 ml sample was taken from each culture and transferred into fresh enrichment medium, followed by incubation as described above for one week. The enrichment procedure was repeated for the third time, before their bacterial contents was isolated using a solid medium containing the enrichment medium and 15.0 g/l of pure agar. Inoculated plates were purified by repeatedly sub-culturing. Pure cultures obtained by this procedure were stored in slants of enrichment medium with 15.0 g/ l pure agar and also in nutrient agar and stored at 4^{o}C.

Screening for PAHs degraders

Each isolate was inoculated into large test tubes containing 25 ml of screening medium. The screening medium was the same as the enrichment medium, except that 15 mg of naphthalene, phenanthrene and anthracene dissolved in DMSO was added to each tube after autoclaving, as sole source of carbon. Thereafter, the test tubes were statistically incubated by keeping on the laboratory bench at room temperature (23 – 25^{o}C) for three days. The ability of each isolate to utilize naphthalene, phenanthrene and anthracene was indicated by an increase in turbidity of the medium measured at 600 nm using a UV spectrophotometer.

Effect of PAH concentrations on the growth of the isolates

Three liters of the enrichment medium were prepared in a four liters flask and dispensed in 75 ml volumes into thirty-two 250 ml flasks before autoclaving. The flasks were then divided into six sets of six flasks each. Thereafter, the following levels of naphthalene, phenanthrene and anthracene (dissolved in DMSO as before) were added to each isolates: 50, 100, 150, 200, 25 and 300 ppm. Inoculated flasks were then incubated as previously described for three days. Five milliliter sample was aseptically collected from each flask and assayed for the level of microbial growth by measuring the OD as described above. In addition, the ability of the isolates to grow on various amounts of naphthalene, phenanthrene and anthracene was studied.

Biochemical analysis of the isolates

The activity of catalase was determined by the appearance of air bubbles after addition of a drop of 30% hydrogen peroxide solution to an overnight grown single bacterial colony. To determine the ability of isolates to hydrolyze starch, 50 µL of liquid cultures of each isolate were dropped on starch-based solid medium containing per liter, 3 g meat extract, 10 g starch and 15 g agar. After one day, the inhibition zones were determined. For casine hydrolysis, 50 µl of liquid cultures of each isolate were dropped on casein-based solid medium containing per liter, 10 g casein and 15 g agar.

After one day incubation, the inhibition zones were determined. Carbohydrates fermentation was determine by the production of gasses during incubation. The test was performed for fructose, sucrose, glucose, xylose and lactose. Producing of Tryptophan deaminase was also detected by the color change of the media after adding 10% ferric chloride. Glycerides hydrolysis and nitrate production were determined. In addition, isolates were subjected to gram staining procedure.

Comparative sequence analysis of 16S r DNA

Molecular identification of the selected isolates was performed by the amplification of 16S rDNA with eubacterial universal primers27F and 1492R (Lane, 1991). Sequencing was performed using ABI PRISM dye terminator cycle sequencing kit with AmpliTaq DNA polymerase and an Applied Biosystems 373 DNA sequencer (Perkin-Elmer, Foster City, Calif.). The sequences were analyzed using the CHECK CHIMERA and the SIMILARITY RANK programs of the Ribosomal Database Project (Altschul et al., 1990) also analyzed using the BLAST program (National Centre for Biotechnology Information) to determine the closest available database sequences. Selected rDNA sequences were aligned using the Clustal W program (Shingler, 1996). Published sequences were

obtained from GenBank. A phylogenetic tree was constructed using Clustal W by distance matrix analysis and the neighbour-joining method (Saitou and Nei, 1987). Phylogenetic trees were displayed using TREEVIEW (Page, 1996).

RESULTS

Isolation of PAH-degrading bacterial strains

The PAH-degrading strains isolated in this study are listed in Table 1. A total of 51 isolates were obtained from the collected soil sample, using naphthalene (20 isolate), phenanthrene (25 isolates), or anthracene (6 isolates) as the sole source of carbon. Three isolates, N6, A3 and P3, which were found to utilize naphthalene, phenanthrene and anthracene better than the rest of the isolates, were subjected for further analysis and characterization. The biochemical characteristics of the three isolates are shown in Table 2.

Effect of PAH concentrations on the growth of the isolates

Isolates N6, A3 and P3 were tested for the ability to degrade PAHs in cell suspensions. Each isolate was tested for the ability to utilize the three examined PAHs individually. Figure 1 (A-C) shows the optical density ($OD_{600\ nm}$) of PAH degraders under different PAH concentrations (15 - 100 ppm) . In general, results demonstrated that strains isolated on phenanthrene (P3) and anthracene (A3) were able to grow better on the three tested PAHs than the strain isolated on naphthalene (Figure 1) . The growth of the isolates on PAH was concentration dependent. In isolate P3, the lowest optimum growth of 0.01 (OD_{600}) was observed when the level of phenanthrene was higher than 100 ppm, while the highest optimum growth of 0.09 was recorded when the level of phenanthrene was 15 ppm. Similarly, the lowest optimum growth in isolate A3 was 0.05 at anthracene level of 30 ppm, while the highest was0.45 when the anthracene level was 15 ppm (OD_{600}). However, no significant growth of isolates P3 and A3 was observed under different naphthalene concentrations ranged from 15 - 100 ppm. The maximum growth rate of 0.25 (OD 600) was recorded only with isolate N6 at naphthalene concentration 15 ppm.

Biochemical analysis of the isolates

As shown in Table 2, Gram-staining revealed that all selected isolates are Gram-positive bacteria. No dioxygenase activity (indigo assay) was recorded for all tested isolates (data not shown) . Catalase and nitrate reduction showed positive results with all tested isolates. Only isolates N6 and P3 gave positive results in starch hydrolysis assay.

Table 1. Bacteria isolates recovered during screening.

Isolate NO.	Optical Density (OD 600 nm)		
	Naphthalene	Anthracene	Phenanthrene
1	0.194	0.450	0.082
2	0.14	0.365	0.059
3	0.117	0.452	0.091
4	0.069	0.288	0.031
5	0.088	0.21	0.042
6	0.253	0.432	0.012
7	0.094		0.056
8	0.078		0.034
9	0.01		0.033
10	0.089		0.021
11	0.098		0.008
12	0.147		0.025
13	0.077		0.016
14	0.109		0.024
15	0.098		0.045
16	0.138		0.021
17	0.135		0.034
18	0.191		0.017
19	0.132		0.05
20	0.082		0.044
21			0.023
22			0.016
23			0.045
24			0.024
25			0.028

Table 2. Biochemical characterization of PAH-utilizing isolates.

Biochemical test	Appearance and reaction		
	N6	A3	P3
Carbohydrates fermentation			
Fructose	-	-	-
Sucrose	-	-	-
Glucose	-	-	-
Xylose	-	-	-
Lactose	-	-	-
Catalase	+	+	+
Indol test	-	-	-
Starch hydrolysis	+	-	+
Glycerides hydrolysis	-	-	-
Nitrate reduction	+	+	+
Casein hydrolysis	+	-	-
Gram staining	+	+	+

16S rRNA gene analysis

Comparative sequence analysis of the 16S ribosomal

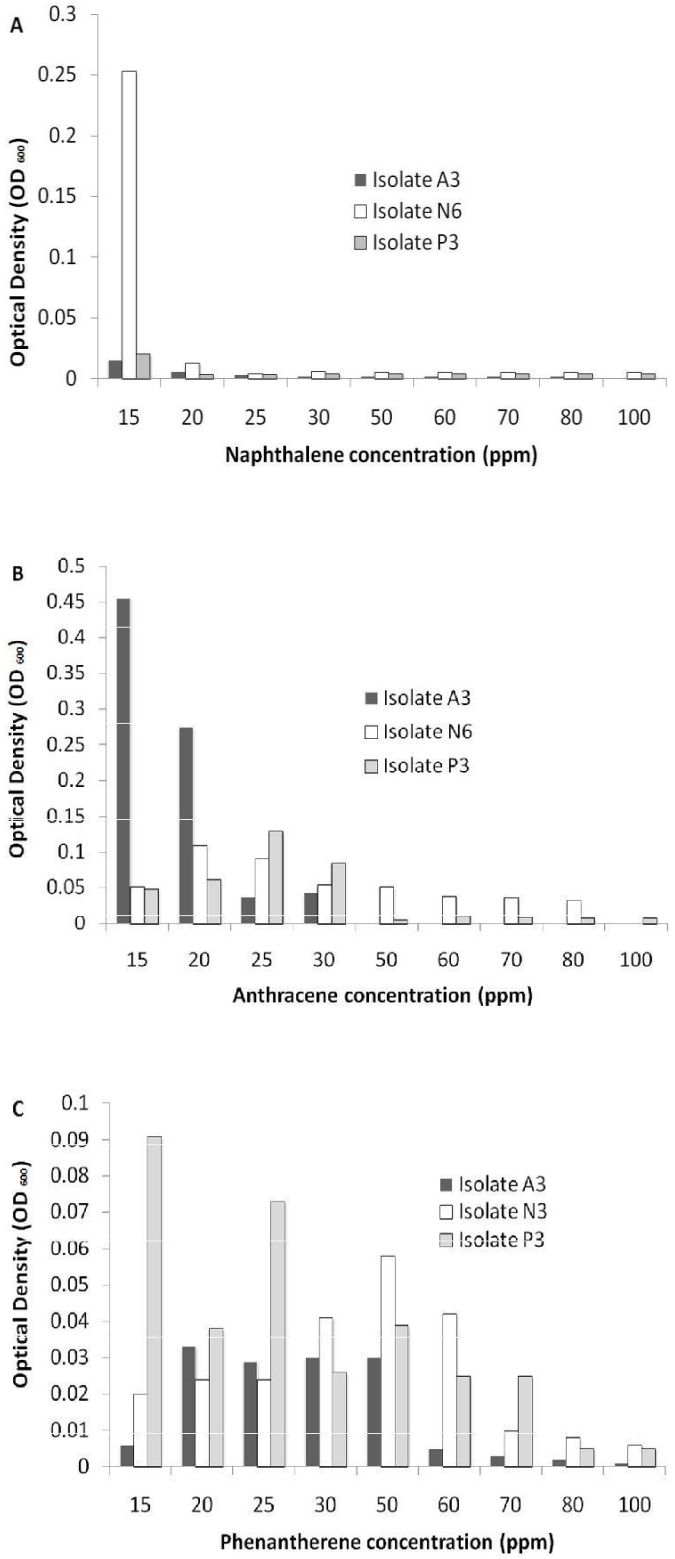

Figure 1. Effect of different concentrations of naphthalene (A), anthracene (B) and phenanthrene (C) on the growth of the isolates A3, P3 and N6.

DNA (~500 bp) of the isolates revealed that two of them N6 and P3 are closely related to the genus of *Achromobacter* exhibiting similarity values ranging from 95 - 97% (Figure 2). However, isolate A3 was closely related to *Pseudomonas geniculata* (98% similarity).

DISCUSSION

Because of the methodological differences used to enumerate the widely distributed microbes in nature, reports of actual numbers present are confusing (Atlas and Bartha, 1998). However, a small fraction (<1 to 10%) of indigenous microorganisms inhabiting different soils can be isolated and cultivated on laboratory media, since microorganisms are extremely diverse and the growth requirements for many strains are unknown (Amman et al., 1995; Atlas and Bartha, 1998).

Different methods to enumerate microorganisms are nowadays available. However, each has its advantages and limitations (Atlas, 1982). Although the viable count approach is widely used to enumerate cells and to gain information on their biodegradation potential (Lorch et al., 1995), it has been criticized as underestimating the total culturable bacteria inhabiting soils (Grigorova and Norris, 1990). In the present study enrichment culture-based approach was used to detect PAH-degrading bacteria.

Although culture- based microbiological methods have provided important, though limited, information about the microbial diversity of natural samples, novel molecular techniques have been extremely valuable in exploring the diversity of indigenous microbiota involved in natural attenuation of soil contaminants (Demnerova´ et al., 2005). The tentative taxa and phylogenetic affiliation of the purified bacterial isolates were studied by amplification and partial sequencing of about 500 bp of their 16S rDNA. Gene comparison studies have shown that 16S rRNA is highly conserved within a species and among species of the same genus (Abd-El-Haleem et al., 2000, 2002). Partial 16S rDNA gene sequencing and database homology search for the eubacterial isolates revealed their tentative close relationship to members of *Achromobacter xylosoxidans* and *P. geniculata*.

Recently, members of the *A. xylosoxidans* able to degrade PAHs were isolated from wetland sediment (Wan et al., 2006). *A. xylosoxidans*, first described by Yabuuchi et al. (1974), was previously listed under the name *Alcaligenes denitrificans* subspecies *xylosoxidans* and oligotrophic bacteria. The most applied bacteria fall under the genders of *Pseudomonas, Arthrobacter, Acinetobacter, Flavobacterium, Alcaligenes, Micrococcus* and *Corynebacterium* (Masák, 1992). Intense research in this area confirms that besides bacteria, other microorganisms, including fungi and algae, can be used. The results of PAH -dependent bacterial growth in the present study provide evidence for the degradation of PAH compounds. The degradation and utilization of these compounds offer the carbon required for the increase in optical density and hence the cell mass of these cultures.

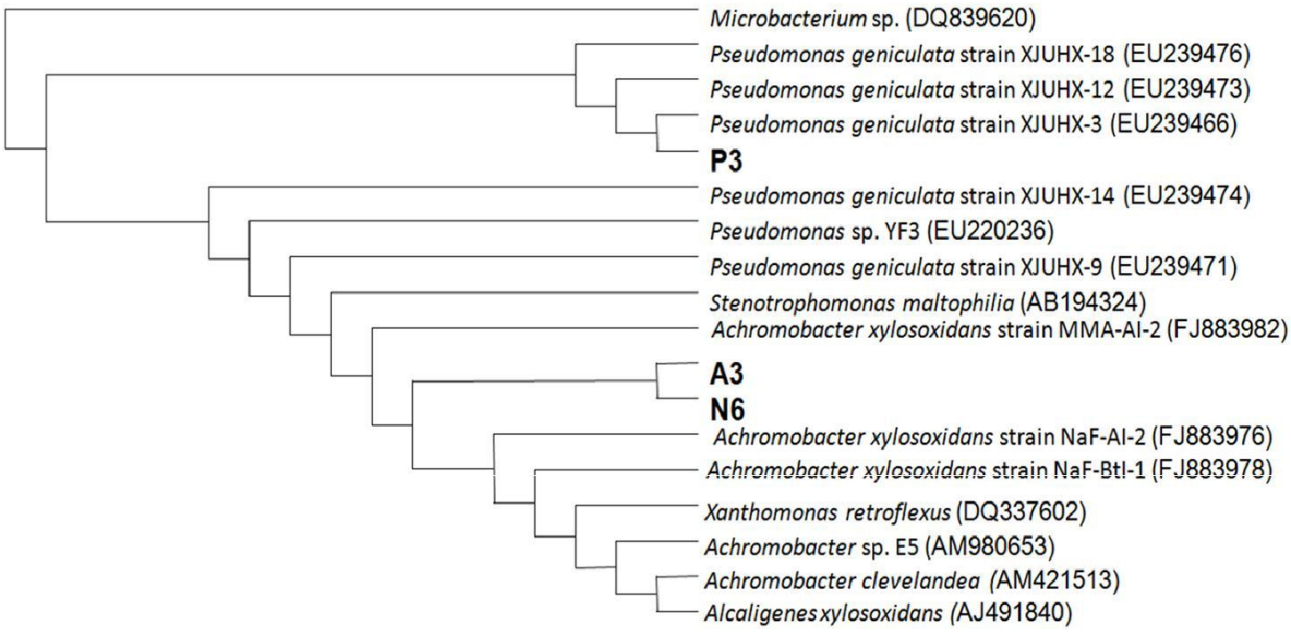

Figure 2. Phylogenetic tree showing the relationships among the selected isolates A3, P3 and N6 and other closely related sequences collected from the Gene Bank.

Furthermore, it seems likely that the degradation of individual PAH compounds by the isolated bacteria proceeds via independent pathways (Bressler and Fedorak, 2000; Van Hamme et al., 2003). Isolate A3 and N6 were only resisting to both anthracene and naphthalene up to concentration 30 and 20 ppm, respectively. However, the phenanthrene utilizing bacterium P3 was able to utilize other two tested PAH-compounds.

This results are agree with the statement of Alexander (1999) that the acclimation of a microbial community to one substrate frequently results in the simultaneous acclimation to some, but not all structurally related molecules. Also, individual microbial species have the ability to act on several structurally similar substrates and therefore more easily act on their analogues after the first addition (Bauer and Capone, 1985; Mitchell and Cain, 1996). It is therefore not surprising that the P3 isolate grew well on these other organic aromatic compounds, considering that they are all commonly composed of benzene rings as phenanthrene.

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