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Anthracene degradation by an oligotrophic bacterium isolated from refinery soil

I. Amini[1*], A. Tahmourespour[2], A. Abdollahi[3]

[1]Department of Microbiology, Islamic Azad University, Falavarjan Branch, Falavarjan, Isfahan, Iran
[2]Department of Basic Medical Sciences, Islamic Azad University, Khorasgan (Isfahan) Branch, Isfahan, Iran
[3]Department of Basic Sciences, Islamic Azad University, Khorasgan (Isfahan) Branch, Isfahan, Iran

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Abstract

Anthracene is a widespread environmental pollutant with carcinogenic and genotoxic properties. Biodegradation is a simple, cost-effective and safe technique to clean-up contaminated environments. The aims of this study are isolation and characterization of an oligotrophic bacterium with the ability to degrade anthracene and assessment of in vitro biodegradation process. For this purpose petroleum contaminated soil was collected from Isfahan refinery. Soil sample was found to be neutral and slightly saline with high concentration of anthracene. The population of total heterotrophic bacteria and anthracene degrading bacteria were 2.50×10^3 and 1.96×10^3 CFU/g, respectively. Seven anthracene degrading bacteria were isolated using enrichment culture technique in Basal Salt Medium (BSM) supplemented with 50 mg/L anthracene. An oligotrophic bacterium was selected based on growth on carbon free medium. This isolate was identified as *Methylobacterium rhodesianum* ATAI15, submitted to GenBank under accession number of KC469989.1. The biodegradation rate of 50 mg/L anthracene by this isolate during 9 days was 41.2%, determined by gas chromatography. This bacterium was adapted to survive in nutrient deficient medium and high concentration of anthracene (up to 12000 mg/l). Use of this isolate with other PAH degrading bacteria can be effective in bioremediation of polluted environments.

Keywords: Oligotrophic bacterium, *Methylobacterium rhodesianum,* Anthracene, Bioremediation

*Corresponding author; imaneh.a.66@gmail.com

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of organic contaminants with two or more fused benzene rings. They are ubiquitously found in the environment (atmosphere, water and soil) (Bayoumi, 2009; Haritash and Kaushik, 2009). They have relatively low bioavailability, due to their hydrophobicity. Based on molecular weight, these compounds are classified as low molecular weight PAHs (LMW PAHs) with two or three benzene rings that easily degrade by bacteria and high molecular weight PAHs (HMW PAHs) with four or more benzene rings that persist in the environment (Bamforth and Singleton, 2005; Wick *et al.*, 2011; Lu *et al.*, 2011). Two main sources of environmental PAHs are natural (biogenic and geochemical) and anthropogenic (pyrogenic and petrogenic) activities (Bamforth and Singleton, 2005). These chemical compounds exhibit genotoxic, mutagenic and carcinogenic properties (Arulazhagan *et al.*, 2010). Among several treatment strategies for PAHs contaminated soils, biodegradation is a cost effective, simple and environmentally benign clean-up technique (Jain and Bajpai, 2012). Anthracene is a LMW PAH with three benzene rings. It is a natural constituent of fossil fuels that is widely distributed throughout the environment. Anthracene is not a genotoxic compound by itself but its structure is similar to carcinogenic PAHs such as benzo [a] anthracene (Mrozik *et al.*, 2003). This compound is one of the 16 priority pollutants in United States Environmental Protection Agency (USEPA) list. It causes damage to skin, headaches and inflammation of the stomach and intestines (Wick *et al.*, 2011). Some bacterial genera belonging to *Pseudomonas, Sphingomonas, Nocardia, Beijerinkia, Rhodococcus* and *Mycobacterium* have been reported as anthracene degrading strains (Mrozik *et al.*, 2003). The biodegradative potential of methylotrophic bacteria toward different polluted compounds, such as explosive, methyl tert-butyl ether (MTBE), and PAHs, has been reported (Van Aken *et al.*, 2004; Li and Gu, 2007). van Aken *et al.* (2004) isolated a hydrocarbon-degrading Methylobacterium strain from the rhizosphere of poplar trees. This strain improved plant growth and remediation of different hydrocarbons. The aims of the present study are isolation and identification of bacteria capable of growth in carbon free medium as oligotrophic bacteria. Then, the ability of in vitro degradation of anthracene by these isolates will be determined.

Material and methods

Chemicals

Anthracene (96%), dichloromethane and salts of basal salt medium were purchased from Merck-Germany. Bacteriological culture media were purchased from Quelab-Canada.

Soil sampling and chemical analyses

Soil sample was collected at a depth of 10-20 cm from petroleum refinery in Isfahan, Iran and stored in sterile container at 4°C prior to analysis. Samples were air-dried and sieved (< 2mm) before chemical analysis. The chemical analyses of soil sample were performed as described by Winter and Behan-Pelletier (2007). Anthracene concentration in sample was measured by gas chromatography (GC Agilent 6890N), equipped with FID detector, HP-5 capillary column (30cm×0.25mm×1 µm) and an autosampler (7683B). Soil sample was soxhlet extracted with dichloromethane (DCM) for 12 hrs. The extract was concentrated in a rotary evaporator (IKA RV10) then passed through a silica column for clean-up and analysed by GC. External standards of anthracene were prepared (10-40 mg/L), then internal standard (9,10-Dihydroanthracene) was added to both extracts and external standards (with the same concentration) before GC analysis. Nitrogen was used as the carrier gas at a constant flow rate of 1.5 ml/min. Splitless injection of 1 µl of the sample was automatically conducted. The GC oven temperature was programmed from 70 to 272 °C with a rate of 5 °C/min. The injector and detector temperatures were 270 °C and

total run time was 40 min. (Rasdy *et al.*, 2008).

Enumeration of bacterial population

The enumeration of Total Heterotrophic Bacteria (THB) and anthracene degrading bacteria was completed by spread plate technique on nutrient agar and solid Basal Salt Medium (BSM) supplemented with anthracene, respectively (Mhamane *et al.*, 2013) The BSM composed of KH_2PO_4 1.0g, $Na_2HPO_4.12H_2O$ 1.25g, $(NH_4)_2SO_4$ 1.0 g, $MgSO_4.7H_2O$ 0.5g, $CaCl_2.6H_2O$ 0.05g, $FeSO_4.7H_2O$ 0.005g, 15g agar, dH_2O 1000ml, pH 7.0 (pH meter, Metrohm 827), autoclaved at 121 °C for 20 minutes (Naama *et al.*, 2010). Anthracene was dissolved in dichloromethane (DCM) at a final concentration of 0.5 mg/ml, transferred through a 0.22 µm syringe filter. Stock solution (0.5 ml) was sprayed onto the BSM agar, that allowed the solvent evaporated before inoculation. The plates were incubated at 30°C for 3-5 days (Tian *et al.*, 2008). The experiments were carried out in triplicate and the number of bacteria was expressed as CFU/g.

Enrichment and isolation of oligotrophic anthracene degrading bacteria

Anthracene degrading bacteria were isolated from contaminated soil using enrichment culture technique in BSM supplemented with 50mg/l anthracene as the sole source of carbon and energy. A stock solution of anthracene was added to sterilized flask at a final concentration of 50mg/l. After solvent evaporation, a thin layer of anthracene formed on the flask bottom. Sterilized BSM was added to the flask and 1g of contaminated soil was suspended in this medium. The experiment was carried out in triplicate. Flasks were incubated for 7 days at 30°C on rotary shaker incubator (Vision 8480 SFN) at 150 rpm. At the end of each week, 10% of cultured medium was transferred to fresh medium until five such transfers. After 5 weeks of incubation, 0.5ml of the last enrichment culture was spread on nutrient agar. Purification of isolates was conducted by several sub culturing on nutrient agar through streaking plate method (Bin *et al.*, 2010; Naama *et al.*, 2010; Tian *et al.*, 2008). The purified colonies were inoculated to both BSM agar with 50mg/l anthracene and control BSM without anthracene. Colonies that grew on control medium were considered as oligotrophic (John *et al.*, 2012).

Phenotypic characterization of isolate

Oligotrophic isolate was identified based on cultural, microscopic, morphological and biochemical characteristics as described previously (Garrity *et al.*, 2005). Gram staining, catalase, oxidase, urease, IMVIC tests, nitrate reduction, and acid formation from some carbohydrates were carried out for this purpose.

SrDNA gene amplification and sequencing

Bacterial genomic DNA was extracted using the CTAB method. Briefly, a loopful of bacterial colonies was placed into a sterile microtube containing CTAB buffer (2% CTAB, 100 mM Tris-HCl, 20 mM EDTA, 1.4 M NaCl, 2% β mercaptoethanol) incubated in 65°C water bath for 10 min with inverting every 15 mins. An amount of 800 µl of chloroform-isoamylalcohol solution (24:1) was added and centrifuged for 5 min. Supernatant was transferred into a new sterile microtube, then 600 µl of cold isopropanol was added, mixed gently and placed into -20°C freezer for 1 hour or more. The microtube was centrifuged, the supernatant was removed and the precipitate washed with 70% ethanol. The supernatant was removed again and the precipitate dried at 36°C. The pellet was resuspended in sterile dH_2O (Nishiguchi *et al.*, 2002). The 16SrRNA gene was amplified by PCR (Thermocycler , Eppendorph 632500) using universal primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The PCR program followed by: initial denaturation step at 95°C for 2 min, 30 cycles of 95°C for 1 min, 60°C for 30 Sec, 72°C for 35 and the final extension step was carried out at 72 °C for 5 min (Madueno *et al.*, 2011). PCR product was then sequenced and compared with the National

Center for Biotechnology Information (NCBI) database using the BLAST search available through the center's website (http://www.ncbi.nlm.nih.gov/BLAST).
The 16S rDNA sequence was then submitted to the Gene Bank using the BankIt service.

Determination of Maximum Tolerable Concentration (MTC)

Tolerance to Anthracene was assayed in both liquid and solid BSM by increasing anthracene concentration from initial concentration (50 mg/l) to concentrations that inhibit bacterial growth (Bennett *et al.*, 2012).

Growth rate and Biodegradation Experiments

Standardized bacterial cells equivalent to 0.5 Mc Farland were inoculated in liquid BSM supplemented with 50 mg/l anthracene, incubated at 30°C on a rotary shaker at 150 rpm. The medium devoid of anthracene (nutrient broth) was served as control. The optical density of cultures were measured at 24 hrs intervals using a spectrophotometer (CE/WPA Biowave II) at 600 nm wave length (Bin *et al.*, 2011). For biodegradation experiment, the residual concentration of anthracene was extracted from culture medium after 9 days, using liquid-liquid extraction method. BSM containing 50 mg/l anthracene without inoculation was served as control. The extraction was performed twice by 5ml DCM. After dehydration by anhydrous sodium sulfate, the extracts were cold-dried by flow of nitrogen gas and diluted in DCM (Shokrollahzadeh *et al.*, 2012). External standards of anthracene were prepared (125-250 mg/L), then internal standard (Acenaphthene) was added to both extracts and external standards (with the same concentration) before GC analysis. Splitless injection of 1 µl of the sample was automatically conducted. Nitrogen flow rate was 1.6 ml/min for anthracene and phenanthrene and 1.7 ml/min for pyrene. The GC oven temperature was programmed from 100 °C to 250 °C with the rate of 7 °C/min. The injector and detector temperatures were 250 °C and the total run time was 20 min.

Statistical Analysis

Microsoft Excel software was applied to process all the experimental data. SPSS 19.0 was applied for the statistical analysis, and $p < 0.05$ was considered as a significant difference.

Results

Soil properties

Chemical properties of soil sample such as pH, electrical conductivity (EC), organic matter and anthracene concentration are given in Table1. The population of total heterotrophic bacteria and anthracene degrading bacteria were 2.50×10^3 and 1.96×10^3 CFU/g, respectively (Figure 1).

Isolation and purification of anthracene degrading bacteria

In the present study, seven bacterial isolates (including both gram negative and positive bacilli) were isolated from contaminated soil by enrichment culture technique in the presence of 50 mg/l anthracene as sole source of carbon and energy. One isolate was selected based on the ability to grow on carbon free medium and was taken as oligotrophic.

Table 1. Chemical characteristics of soil sample

Characteristics	Results
pH	7.34
EC (dS/m)	3.26
OM%	5.04
Anthracene concentration (mg/kg)	18.48

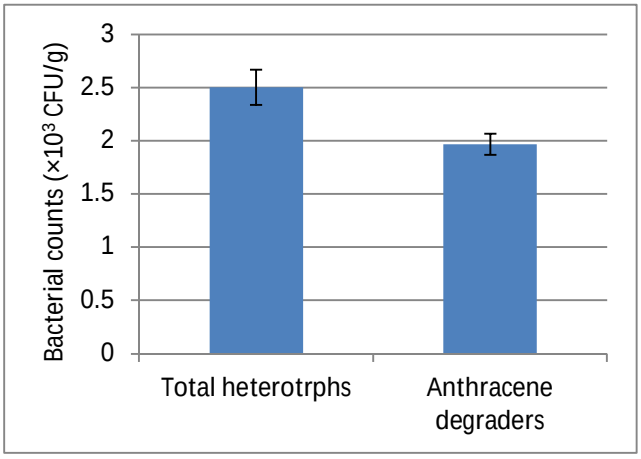

Figure 1. The bacterial population in soil sample

Identification of oligotrophic isolate

The gram negative (variable), pink pigmented bacterium was identified by further biochemical tests (Figure 2- a and b). Morphological and biochemical characteristics of this isolate were listed in Table 2. This isolate was identified by physiological method and the 16S rDNA gene sequencing as *Methylobacterium rhodesianum* strain ATAI15. PCR amplification Product of the 16S rDNA gene and phylogenetic three of *Methylobacterium rhodesianum* strain ATA115 is shown in Figure 3. The 16S rDNA gene sequence of strain ATAI15 was submitted to GenBank and the accession number assigned to it is KC469989.1.

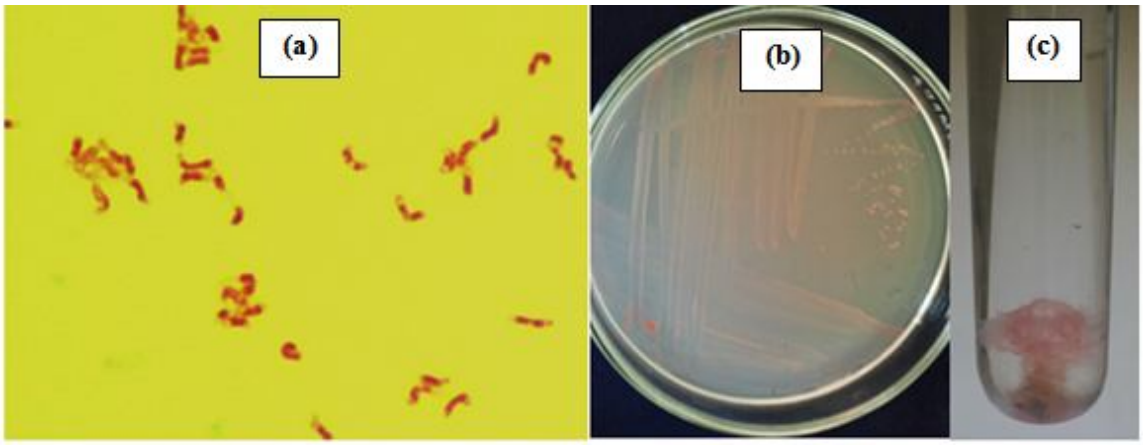

Figure 2. Gram stain (a), macroscopic appearance (b) and bulky mass of *strain ATAI15* growth in BSM broth (c)

The assessment of bacterial growth and resistance to the Anthracene

This strain tolerated high concentrations of anthracene (up to12000mg/l) but the best growth was observed in 50mg/l anthracene. The optical density in both BSM with anthracene and control medium was measured during 11 days at 600 nm and the growth curve of this strain was drawn. During a lag phase of 24 hrs., the cell number increases very slowly. The initial growth phase of strain ATAI15 in BSM supplemented with anthracene occurred within one day of incubation. Between days 1 to 9 the logarithmic phase was observed in both media. This isolate showed maximum growth at day 8. The growth of isolate in this medium was accomplished with lower slope rather than control medium (Figure 4). Also, production of pink ropy masses of growth was observed in both mediums after 7 days (Figure 2-c).

Table 2. Phenotypic characteristics of the isolate

Characteristics	Results
Morphological characteristics:	
Macroscopic character	Convex, Round, Mucoid, Pink pigmented colony
Gram stain	Gram variable bacilli
Biochemical characteristics:	
KOH test	+
Catalase	+
Oxidase	-
Urease	+
Citrate	-
Nitrate reduction	+
Acid production from:	
Glucose	+
Fructose	+
Mannose	+
Mannitol	+
Sucrose	(Orange) +/-
Lactose	(Orange) +/-
MR-VP	-/-
SIM	-/-/-

Figure 3. Product of PCR amplification of the 16 SrDNA gene (a) and Phylogenetic analysis of *Methylobacterium rhodesianum* strain ATA115 (b)

Biodegradation of anthracen by selected isolate

Biodegradation efficiency of this isolate was determined during 9 days by GC. The concentration of anthracene decreased from initial concentration to about 37 mg/l during 3 days. This amount remained constant up to 3 days and then decreased to about 29 mg/l at day 9. After incubation period, 41.2% of anthracene with initial concentration of 50 mg/l was degraded by strain ATAI15. As the bacterial growth increased, the anthracene concentration decreased showing an inverse relationship (Figure 5). Abiotic loss of anthracene in this study was found negligible (8.04%).

Discussions and Conclusions

The pH of soil was found to be neutral and according to Richards (1954) it was very slightly saline. The concentration of anthracene in soil sample (Table 1) was more than standard level (1-3 mg/kg) reported by Hertel *et al.*, (1998), so this sample was considered as contaminated soil. Acoording to results, anthracene degrading bacterial population was 78.40% of the total heterotrophic bacterial

population in the soil sample (Figure1). Among seven anthracene degrading bacteria isolated after 5 weeks of enrichment period, only one isolate was oligotrophic bacterium. Oligotrophic bacteria are a type of bacteria that survive in extreme environment such as oceans and nutrient deficient soils. In a study by Pan *et al.* (Pan *et al.*, 2007), an oligotrophic bacterium with the ability to tolerate extreme condition of carbon source, temperature and pH was isolated from the biological soil crust under layer in the Xinjiang Gurbantunggut Desert. In the environment and in food-manufacturing plants, pink-pigmented oligotrophic bacteria are often isolated (Kato *et al.*, 2008). In the present study, an oligotrophic isolate was identified as *Methylobacterium rhodesianum* strain ATAI15.

Methylobacterium rhodesianum is a rod shape, gram variable, pink pigmented (due to production of carotenoid pigment), non-spore forming bacterium. Members of this genus are slow grower, widely distributed in the environment and can survive in poor carbon sources (Gallego *et al.* 2005; Green *et al.* 1988; Kato *et al.* 2005; Van Aken *et al.* 2004; Cao *et al.* 2011). Comparison of 16S rDNA gene sequences revealed that ATAI15 showed about 99% similarity to *Methylobacterium rhodesianum* strain DSM 5687 and also strain NCIMB 12249 (Figure 3-b).

The growth of *Methylobacterium rhodesianum* strain ATAI15 in mediums was accomplished with the production of pink ropy masses after 7 days of incubation (Figure 2-c). Previously, production of pink ropy masses by *Methylobacterium* sp was reported in some human made environments such as bathrooms and washstands (Gallego *et al.*, 2005; Hiraishi *et al.*, 1995). In the current study, strain ATAI15 showed the ability to survive at high concentration of anthracene. This is perhaps due to membrane alterations such as increasing membrane rigidity or the presence of efflux pumps (proteins involved in transport of aromatic compounds across the cell) (Van Hamme *et al.*, 2003).

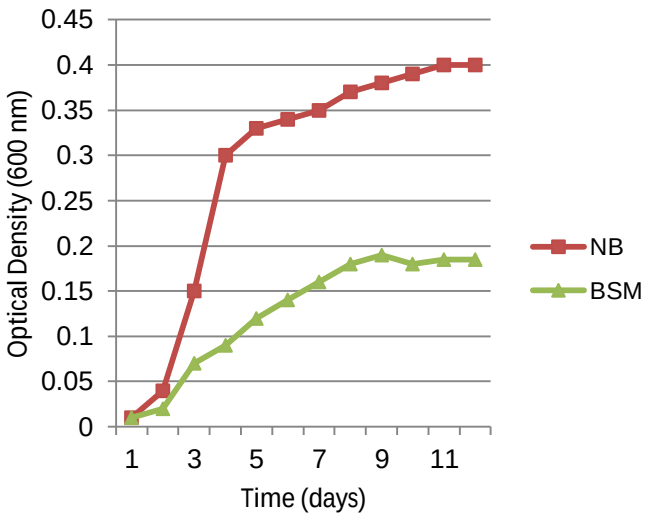

Figure 4. Growth curve of *strain ATAI15* in both control and BSM mediums

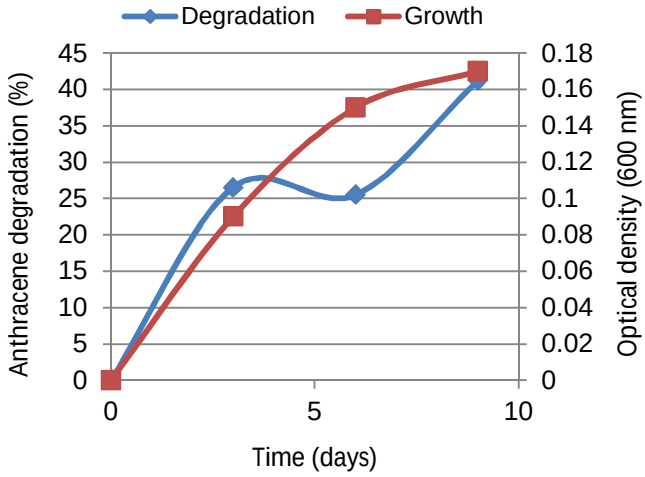

Figure 5. Analysis of growth and anthracene degradation by strain ATAI15during 9 days

The present study offers the first report demonstrating the utilization of anthracene by *Methylobacterium rhodesianum*. In a similar study, Methylobacterium populi VP2 was selected as the best putative PAH-degrading strain (Ventorino *et al.*, 2014). The ability of *Methylobacterium* sp. to utilize PAHs was also confirmed by previous studies (Bodour *et al.*, 2003; Andreoni *et al.*, 2004; Bin *et al.*, 2010). Previously, isolation and identification of anthracene degraders such as *Mycobacterium* Sp. GIPAH-01 (Bennett *et al.*, 2012), *Pseudomonas aeruginosa*, *Alcaligenes eutrophus*, *Bacillus subtilis* and *Micrococcus luteus* (Ilori *et al.*, 2000) and an alkalophilic *Bacillus badius* D1 (Ahmed *et al.*, 2012) were reported from different environments. In this study, 41.2% of anthracene with initial concentration of 50 mg/l was degraded by strain ATAI15 after 9 days of incubation (Figure 5). In a study by Bisht *et al.* (2010), the degradation rate of anthracene (with concentration of 0.5 to 1.0 mg/50 ml) by *Bacillus circulans* SBA12 and *Kurthia* SBA4 was 87.5 and 86.6% respectively. In another study, *Pseudomonas* sp. Strain E was isolated from oil contaminated soil of filling station in Himachal Pradesh, India that degraded 74.8% of anthracene (with concentration of 0.1%) (Kumar *et al.*, 2010). Also, the ability of *Corynebacterium* sp. and *Pseudomonas putida* in anthracene degradation was studied by Azeez *et al.* (2012). These bacteria degraded 95.2 and 93.5% of anthracene, respectively. The differences between anthracene biodegradation rate in this study and other studies are anthracene concentration and using the cosubstrate or optimal temperature and pH in other studies.

The present study provided information regarding the PAH-degrading ability of an oligotrophic bacterium *Methylobacterium rhodesianum* strain ATAI15 from refinery soil. Although anthracene degradation rate by strain ATAI15 was lower than some other reports, the cooperation of this strain with indigenous bacterial communities in contaminated environments could be useful for remediation of such pollutants even in extreme conditions.

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