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ISOLATION AND CHARACTERIZATION OF THREE AND FOUR RING PAHS DEGRADING BACTERIA FROM CONTAMINATED SITES, ANKLESHWAR, GUJARAT, INDIA

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Abstract

Polycyclic aromatic hydrocarbon (PAH)-degrading bacteria were isolated from prolong contaminated Amalakhadi sediment and crude oil polluted soil Telva, near Ankleshwar Gujarat India. Organisms were treated with two-model PAHs compound Anthracene (ANT), and Pyrene (PYR) as the sole source of carbon and energy. Identification of the isolates was carried out based on their morphological and partial 16S rRNA gene sequences, which revealed that the isolates belong to two main bacterial groups: gram-negative *pseudomonas indoxyladons* and gram-positive, spore-forming group, *Bacillus benzoevorans*. GC-MS based degradation study demonstrated that *P. indoxyladons* efficiently degrade 98% of ANT and PYR by 93.2 % when treated with 250 mg L^{-1}. However, *B. benzoevorans* could tolerate to 200 mg $L^{-1}$of PYR. Thus, the findings of the study provide novel bacterial sp. having different capacity to degrade model PAHs compounds and further could be utilized for the standardization of bioremediation protocols for *ex situ* and *in situ* studies in aquatic as well as terrestrial ecosystem.

Key words: PAHs contaminated soil; isolated Bacteria; Biodegradation of PAHs

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous compounds found worldwide in soils and sediments because of both natural and anthropogenic production (Chung et al., 2001). PAHs are major components of fossil fuels and widely used as solvents in pesticides, paints and many other industrial products. They can be formed because of the incomplete combustion of organic material, and are thus ubiquitous pollutants found in soil, oil contaminated sediments, and both industrial and urban wastewater (Nigam et al., 1998). PAHs have been shown to be highly toxic, carcinogenic, and mutagenic, so they present great environmental concerns (Sato and Aoki, 2002). The US Environmental Protection Agency (USEPA) has designated PAHs as priority pollutants in ecosystems since the 1970s (Mazzera et al., 1999). The PAH have low bioavailability and high hydrophobicity which increase as molecular size increase, making difficult its treatment and depletion. Fortunately, the degradation of these PAHs in the environment is possible through several techniques: physical (Costes, and Druelle., 1997), chemical (Chu and Kwan. 2003) or biological (Mittal and Singh. 2009; Malatova 2005). The technology commonly used for the soil remediation includes mechanical, burying, evaporation, dispersion, and washing. However, these technologies are expensive and can lead to incomplete decomposition of contaminants. 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 (Ulrici, 2000). Among the microorganisms able to grow on hydrocarbons, bacteria remain qualitatively and quantitatively the most active agents (Das and Chandran, 2011; Bertrand and Mille, 1989; Brooijmans et al., 2009).

Moreover, microorganisms can play an important role in the degradation of PAH in both terrestrial and aquatic ecosystems and they have a potential role in biorestoration of polluted sites (Bossert et al. 1984). Moreover, PAHs such as anthracene, and pyrene degradation by cyanobacterial species NOSTOC MUSCORUM, ANABAENA FERTILISSIMA, AND SYNECHOCYSTIS SP., was carried out (Jignasha et al., 2013; Nirmal kumar et al., 2014). However, in India, scanty literature available on microbial degradation of polycyclic aromatic hydrocarbons therefore, the present study was focused on isolation of bacteria from artificially enriched anthracene and pyrene of the contaminated soils and evaluated their PAHs degradation efficacy.

Materials & methods

Media and chemicals

Bushnell Haas medium (BHM; Hi-Media, Mumbai, India) supplemented with ANT and PYR was used for the enrichment of PAHs degrading bacteria. Pure PAHs compounds were procured from Sigma–Aldrich (Steinheim, Germany) (98% purity) and other chemicals used under study were of analytical grade such as Hi-media (Mumbai, India) and Merck (Darmstadt Hesse, Germany).

Soil sample collection for bacterial isolation

The soil samples were collected from the sampling point of common industrial effluent canal Amalakhadi, Ankleshwar (site-A) and from crude oil polluted site near Telva (site B), Gujarat, India where soil, sediment, and water have been contaminated by PAHs and many other xenobiotic compounds through industrial effluent from many decades. Figure 1 shows satellite image of study site. Sediment / soil from 0-15 cm in depth were collected and stored in ice at 4°C until further analysis.

Enrichment, isolation, and selection of potential PAHs tolerant strains

About 10 g of soil from each site was inoculated in 100 ml BHM amended with 50 mg L^{-1} of model PAHs compounds separately as sole carbon source and incubated at 37 ^{o}C under shaking conditions (150 rpm). After one week, 10% of this culture was used as inoculum in fresh BHM amended with same amount of PAHs separately (Patel et al., 2011). Repeated transfers were carried out in fresh BHM amended with PAHs, until stable PAHs degrading bacterial consortium was obtained showing consistent growth and selected PAHs tolerance. The appropriate dilutions of the enriched bacteria were spread on BHM agar plate and incubated at 37^{o}C for bacterial growth. Grams staining, capsule staining and endospore staining was performed to 16 different bacterial colonies.

In order to select the potential and efficient degrading strains, amongst 16 different isolated bacteria, single colony was transfer in 50 ml of BHM media supplemented with the range of 20 to 250 mg L^{-1} of each selected model PAHs. The treated cultures were incubated at 37^{o}C temperature 150 rpm in incubator with shaker (Remi make). After intervals of 48 hours, 5.0 ml sample was collected from each Erlenmeyer flask and monitored in a spectrophotometer by measuring absorbance at A_{600} nm for bacterial growth. The strain with good growth with high O.D. proved to be the best tolerance species, which were further identified and confirmed by 16S rDNA analysis.

Genomic DNA isolation and Amplification of 16S rDNA

Genomic DNA of two best tolerant strains out of 16 strains was extracted by the modified method of SDS-CTAB, as previously described by Wilson (1999).Universal bacterial primers (GAG AGT TTG ATC CTG GCT CAG) and 1495R (CTA CGG CTA CCT TGT TAC GA) were used for amplification of 16S rDNA (Studholme D J et al, 1999). 16S rDNA was amplified in an NYXTECHNIX – Master cycler gradient. Products of amplification were analysed by electrophoresis through 1% agarose gel. Primers and unincorporated dNTPs were removed from the amplified PCR product using a commercially available purification kit (Banglore GeNei, India). The purified PCR products were sent for sequencing at Chromous biotech Bangalore, India. The basic local alignment search tool-BLAST was used to classify and identify closely related bacterial sequences using sequence similarity tools and further submitted to NCBI Gene Bank, USA.

PAHs degradation by selected bacteria

After isolating the most PAHs tolerable strains, the biodegradation experiment was carried out to determine their degradation capacity towards selected PAHs using GC-MS analysis. The bacterial strains were treated with 250 and 200 mg L^{-1} of ANT and PYR further incubated for 48h at 37^{o}C temperature and 150 rpm agitation conditions. Entire content (50 ml) of the flask was taken for the determination of residual PAHs in all the experiments. PAHs were extracted using soxhlet apparatus in methanol: dichloromethane (1:2, v/v) of sample aliquots of about 1 g at 40 ^{o}C. These extracts were concentrated to small volumes after addition of sodium sulphate to remove water fraction.

GC-MS instrumental analysis for degradation studies

An autosystem XL GC apparatus (Perkin Elmer, USA) was used to detect the remaining PAHs. The column temperature was initially 80ºC , held for 5 min, then ramped from 80ºC- 290ºC at 10ºC / min. Helium (1.0 ml / min) was used as the carrier gas. The gas chromatogram as reproduced by the mass spectrometer identified the mass spectra scanned at each GC peak maximum. Data was obtained by comparing the mass spectra to those in the Wiley NIST/ EPA/ NIH Mass Spectral Library 2005. The percentage of PAHs loss (% D) was given by the formula of Xiaojun et al. (2007).

Results and Discussion

Enrichment Isolation and characterization of selected PAHS degrading bacteria

Ankleshwar industrial estate (Gujarat, India) consists of nearly 3000 industrialized units. Majority of the industries manufacture chemicals, dyes, paints, fertilizers, pharmaceuticals, pesticides, and other xenobiotic compounds. Figure 1 shows satellite image of the study site. Ankleshwar common industrial effluent canal (Amlakhadi) is increasingly being exposed to anthropogenic pollutants such as PAHs and heavy metals. The treated and untreated contaminated effluents from various industries of Ankleshwar industrial estate are finally released in Amlakhadi (Site A) which is further ends up in Narmada estuary that enters the Arabian Sea, Gujarat. Kathuria (2007) reported that many of these pollutants (PAHs) adsorb on to particulate matter due to their hydrophobic nature and settle down as sediment. Therefore, sediment of this canal has become sinking of recalcitrant compounds and poses a major threat to the ecosystem. Moreover, Site B (Telva) was located near ONGC plant of natural gas and having underground pipeline of crude oil. The site was polluted with crude oil from more than 50 years therefore the nearby agricultural land become very unfertile. Hence, this site was chosen in the present study with an aim to screen for bacteria that can degrade PAHs completely and rapidly with good adaptability.

Figure 1 Satellite image of study area; Site A- Amalakhadi (Common industrial effluent canal), Ankleshwar and Site-B-Telva (Crude oil polluted site) near Ankleshwar Gujarat, India.

A mixed microbial population was obtained from both the contaminated site soil when the soil cultures were enriched using selected PAHs as a sole source of carbon and energy in BHM. Each successive transfer of the enrichment culture was found to contain both bacteria and fungi. By inhibiting fungal growth with cycloheximide and ketoconazole a consortium of bacteria which grew on selected PAHs was isolated. Each individual bacterium was further screened for their ability to tolerate selected PAHs at various ranged concentration of 20-250 mg L^{-1}. Based upon colony morphology as well as different staining method 16 different bacterial strains were isolated (Table: 1).

Table 1 Differential Staining method for morphological identification.

Bacterial strain	Gram's staining	Capsule staining	Endospore staining
AX1	-ve	Non capsulated	Non sporulated
AX2	-ve	Capsulated	Non sporulated
AX3	-ve	Capsulated	Non sporulated
AX5	-ve	Capsulated	Non sporulated
AX7	+ve	Capsulated	Sporulated
AX8	-ve	Capsulated	Non sporulated
BX2	-ve	Capsulated	Non sporulated
BX3	+ve	Non Capsulated	Non sporulated
BX4	+ve	Capsulated	Non sporulated
BX6	+ve	Non capsulated	Non sporulated
BX9	+ve	Non capsulated	Non sporulated
BX11	-ve	Capsulated	Sporulated
BX16	+ve	Non Capsulated	Non sporulated
BX17	+ve	Capsulated	Non sporulated
BX18	+ve	Non Capsulated	Non sporulated
BX22	+ve	Non capsulated	Non sporulated

*AX strains were isolated from Site-A and BX strains isolated from Site-B

Effect of ANT and PYR concentrations on isolates

The strain AX1 grew poorly and having A_{600} = 0.002 towards PYR as compared to other strains. Each isolate was tested for the ability to utilize PAHs up to 250 mg L^{-1}. Figure (2-4) shows the growth pattern of chief degraders under different model PAHs concentrations (20 - 250 mg L^{-1}). Amongst them six, most powerful strains were selected having good capacity to tolerate to PAHs compared to other strain. Growth on ANT and PYR treatment showed that the strains BX2, and AX7 grew more efficiently than any other isolated strains and most pronounced growth of these strains was detected for ANT and PYR at 250 mg L^{-1} and 200 mg L^{-1} respectively besides these other strains AX2, BX4, BX6 and BX9 were also show growth on ANT and PYR. On the contrary, poor growth of isolate BX22 was registered as 0.05 at 20 mg L^{-1} while the highest growth was noted for BX2 i.e. 0.45 when ANT level was 75 mg L^{-1}. Similarly Patel et al (2011) suggested the *Pseudxanthomonas* strain from Ankleshwar was capable for degrading 300 mg L^{-1} of Phenanthrene. The results demonstrated that strains isolated from site B were able to grow better on the selected PAHs than site-A might be due to prolong exposure of complex hydrocarbon mixture of crude oil. However bacterial strains isolated from Site-A were shown pronounce growth in response to ANT treatment was due to continuous exposure of industrial effluent containing dye as well as pesticides in the effluents. The growth of the isolates on selected PAHs was concentration dependent.

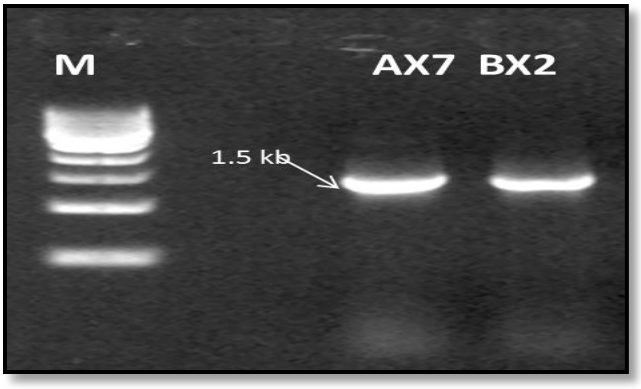

Figure 2 Gel electrophoresis of amplified 16s rDNA of PAHs degrading isolates (AX7 and BX2) where, M=500bp marker.

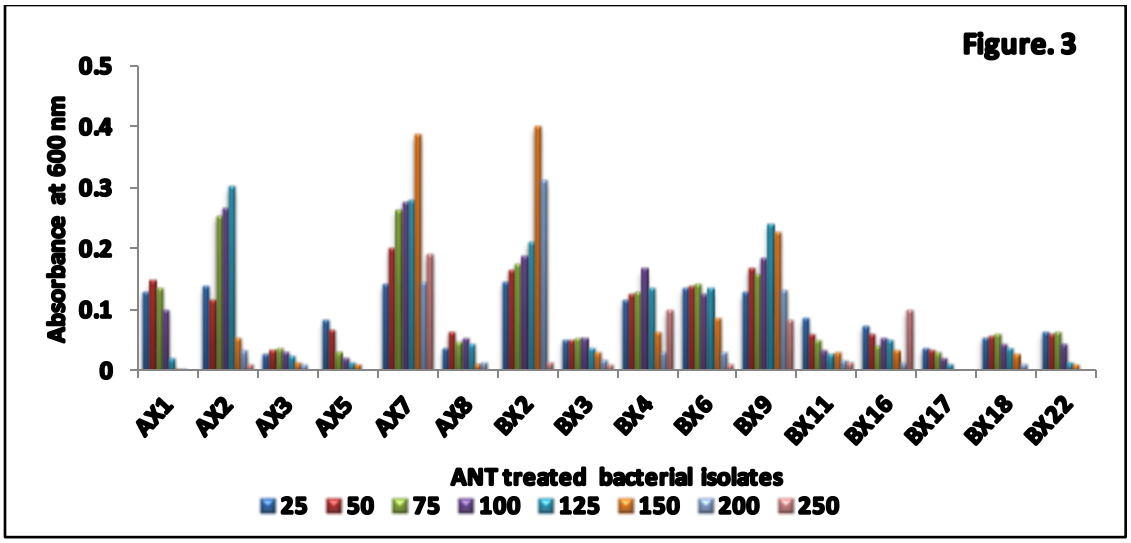

Figure 3 Tolerance level of isolated bacterial strains to different ANT concentration at 37^0C temp 150 rpm shaking condition after 48 hours of interval.

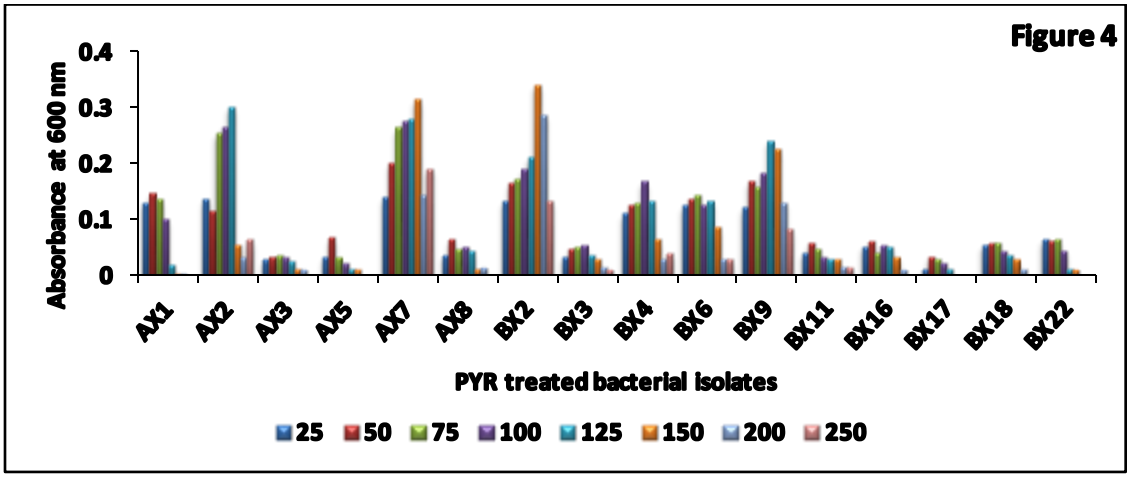

Figure 4 Tolerance level of isolated bacterial strains to different PYR concentration at 37^0C temp 150 rpm shaking condition after 48 hours of interval

Identification of Bacteria

The isolates were characterized using a variety of morphological, staining, and molecular properties. Morphological as well as Grams staining observation revealed that strain AX7 was gram- positive, spore forming, colonies were circular flat and opaque whereas strain BX2 was gram-negative, non-spore forming, and short rods. The colonies of BX2 are deep brown pigmented, circular, opaque, and convex, with a smooth margin. Sequences obtained from BLAST were further confirmed using NCBI sequence similarity search tool based on 16S rRNA conserved region, AX7 shows 98% similarity to *Bacillus benzoevorans* (AX7-1346516*)* however, BX2 shows 99 % similarity with *Pseudomonas indoxyladons* (BX2-236735*)*.

PAHs degradation by bacterial isolates

Amongst 16 isolated bacterial strains, six most powerful PAHs tolerable strains were selected and further inoculated in BHM for their individual PAHs degradation capacity. The degradation study revealed that strain BX4 degraded 250 mg L^{-1} of ANT by 51% followed by 72.4% and 38. 2 % by strain AX2 and BX6 respectively (Figure 5). Moreover strain AX2 could degrade 46% of 200 mg L^{-1} PYR followed by strain BX6 (62%) and BX9 (71%) (Figure 6). However, strain BX2 and AX7 degrade up to 98.4% and 94% of ANT within 48 hours of incubation period. Thus genus *Pseudomonas* is one of the most studied genera for the degradation of PAHs and other xenobiotic compounds out of which *P. putida* and *P. aeruginosa* are the species for PAH degradation specifically. Jacques et al (2005) studied anthracene degradation by strains *P. aeruginosa* and *P. citronellolis* isolated from petrochemical sludge as well as biosurfactants production, which increased solubilisation of anthracene in medium. Said et al (2007) isolated PAH degrading *P. xylosoxidans* and many other strains of *Pseudomonas* genera from Bizerte lagoon sediment. Moreover, Luo et al (2009) reported various communities that belong to *Bacillus sp.* in biodegrdation of benzo (a) pyrene.

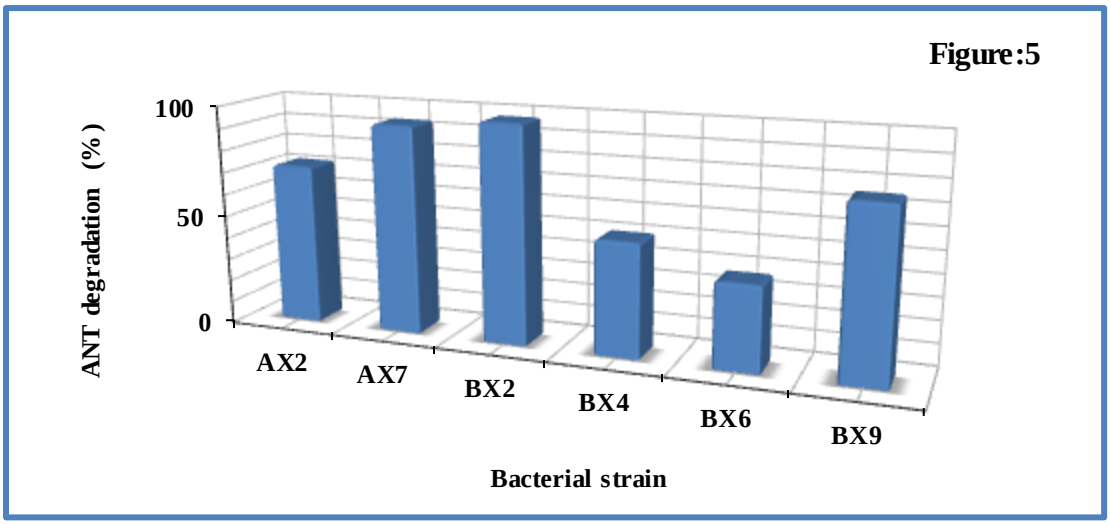

Figure 5 Percent degradation of selected bacterial isolates to 250mg L^{-1} of ANT

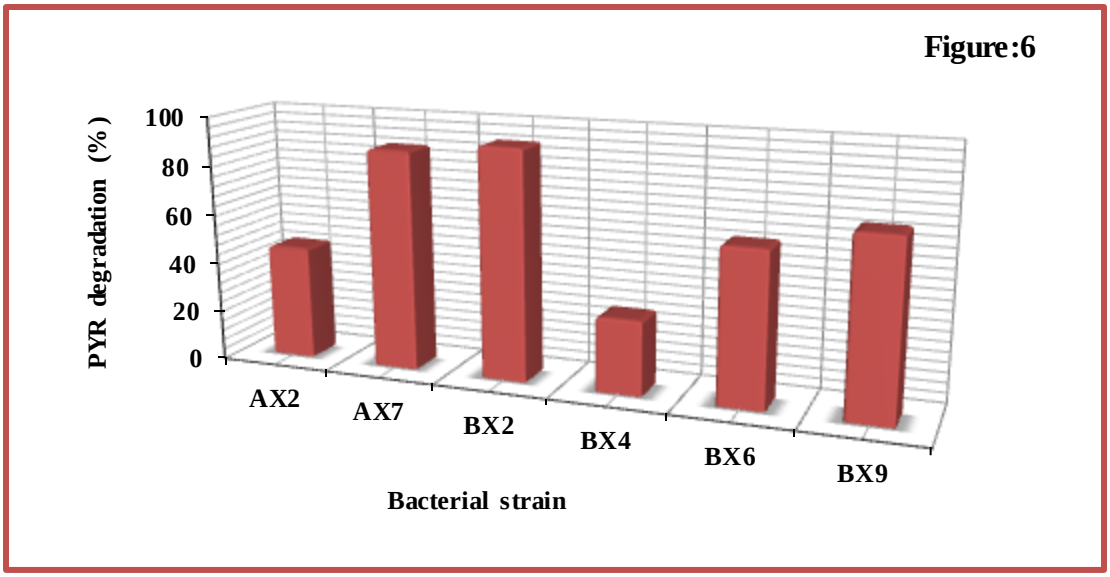

Figure 6 Percentage degradation of selected bacterial isolates to 200 mg L^{-1} of PYR

Conclusion

The present study suggested that the prolonged exposure of PAHs would increase PAHs degradation efficacy by the isolated two bacterial strains as compared to other strains. Therefore, the findings of the study could be utilized for the standardization of bioremediation protocols for ex situ studies.

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