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ORIGINAL PAPER

Isolation of pyrene degrading *Achromobacter xylooxidans* and characterization of metabolic product

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Abstract Polycyclic aromatic hydrocarbons (PAHs) are common ubiquitous pollutants existing in nature with high recalcitrance and toxicity. In this study a bacterium capable of aerobic degradation of high molecular weight PAHs (with special reference to pyrene) was isolated by selective enrichment culture technique from oil refinery effluent sludge. The isolate was characterized as *Achromobacter xylooxidans* by 16S rRNA gene sequence analysis technique. For the first time it is hereby reported a bacterium capable of effectively degrading pyrene (up to 80%), as evident by reverse phase high performance liquid chromatographic analysis (RP-HPLC). After incubation of *Achromobacter xylooxidans* in minimal salt medium (MSM) containing pyrene, at concentration of 200 mg/L, as sole source of carbon and energy, there was decrease in pyrene concentration concomitant with increase in bacterial cell protein concentration. RP-HPLC analysis revealed that pyrene was degraded into three metabolites viz. I, II and III. The RP-HPLC eluent fraction were collected from 2.5 to 32.0 min by repeated injection of degraded sample, concentrated and analyzed on gas chromatography mass spectroscopy (GC-MS) for metabolite identification. The fraction shows 1-hydroxypyrene, 1-hydroxy-6-methoxypyrene and 1,6dimethoxypyrene as metabolic product of pyrene degradation, on the basis of their *m*/*z* values. On contrary to the reported PAH degradation with reference to pyrene by different isolates till date; the efficient degradation, as evident by RP-HPLC, by this isolate holds a promising potential for planning of bioremediation strategies of contaminated sites.

Keywords Oil refinery · *Achromobacter xylooxidans* · 1-hydroxypyrene · 1-hydroxy-6-methoxypyrene · 1,6-dimethoxypyrene

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Introduction

Pyrene along with other polycyclic aromatic hydrocarbons (PAHs) is ubiquitous pollutants as a result of both natural and anthropogenic production, these compounds persist in the environment, due to their hydrophobicity, deposited in soil and sediment (Ravindra et al. 2008). PAHs constitute a major environmental concern on ecosystems because of their adverse health effects on organisms due to lipophilic, biomagnified, bind covalently with cellular DNA, shows carcinogenesis (Malik et al. 2004) and endocrine disrupting activity (Zaghden et al. 2007). PAHs are included in the "priority pollutants" lists developed by the United States Environmental Protection Agency (US EPA). Degradation of PAHs from contaminated sites occurs through chemical, photochemical processes and bioremediation. Bioremediation is an economic, safe, ecofriendly and efficient method therefore researchers have been focusing on bioremediation of PAHs. PAHs with high molecular weight (i.e., 4–6 ring) was less significant to biodegradation due to their low

water solubility and strong absorption onto the particle surface than PAHs with low molecular weight and aliphatic (i.e., 2–4 ring) (Kanaly and Harayama 2000; Cerniglia 1992). The microbial biodegradation of two- and three-ring PAHs has been extensively reviewed (Widada et al. 2002), Using culture dependent and independent isolation techniques, different bacterial genera have been characterized from hydrocarbon polluted soil in different geographical and ecological contexts (Hamamura et al. 2006). Low water solubility, production of toxic or dead-end metabolites, metabolite repression, and presence of preferred substrates, lack of cometabolic or inducer and physical condition (temperature, oxygen, pH value, and water content in soils) are often found to affect biodegradation (Wong et al. 2002; Molina et al. 1999). Biological degradation of PAHs compounds often results, incomplete degradation, production and accumulation of intermediate products. Intermediate metabolites including dihydrodiols, phenols and areneoxides have been identified as carcinogen and mutagen pose greater risk to the environment than the parent compounds due to their increased polarity, solubility and mobility (Datta and Samanta 1988). In India, oil refineries environment contaminated with PAHs due to poor management, faulty equipment, vehicle exhaust and lack of environmental controls may have significant impact on surrounding environment. Our present research goal is to isolate in situ pyrene tolerant bacteria from oil refinery Mathura, sludge to degrade solid pyrene as a model PAHs, identify possible metabolites and determine metabolic pathways, to predict the fate of PAHs compounds in the environment and how they are detoxified.

Materials and methods

Pyrene, Naphthalene, Phenanthrene, Chrysene and dibenzo(a,h) anthracene (purity 97.0%), were purchased from Aldrich (St. Louis, MO, USA). All other solvent (ethyl acetate & methanol), hydrogen peroxide, anhydrous sodium sulphate and other required chemicals were purchase from MERCK, Germany.

Enrichment and isolation of the pyrene degrading microbial consortium

Isolation of pyrene degrading bacteria and characterization

The bacterial strains were isolated by the enrichment culture technique oily sludge procured from oil refinery, Mathura. 5.0 g sample of oil refinery effluent sludge was inoculated into 100 mL of MSM (KH_2PO_4, 170 mg; Na_2HPO_4, 980 mg; $(NH_4)_2SO_4$, 100 mg; $MgSO_4$, 4.87 mg; $FeSO_4$, 0.05 mg; $CaCO_3$, 0.20 mg; $ZnSO_4$, 0.08 mg; $CuSO_4{\cdot}5H_2O$, 0.016 mg; and H_3BO_4, 0.006 mg) containing pyrene (200 mg/L) as the carbon source and incubated at 30 °C on a rotary shaker (200 rpm) for 10 days. After five cycles of such enrichment, 1 mL of the culture was diluted up to tenfolds, dilutions was plated on minimal salt (MS) agar plates and sprayed with pyrene (5.0 mg/mL dissolved in diethyl ether), incubated at 30 °C. The bacterial colonies obtained were further purified MS agar plates containing pyrene (1%, w/v) as the carbon source. The strains showing extensive pyrene degradation were routinely cultured on MSM containing pyrene (1%, w/v) and stored as frozen stock cultures in 25% glycerol at −80 °C. This bacterial strains also examined for its ability to degrade naphthalene, phenanthrene, chrysene and dibenzo(a,h) anthracene when used separately as a sole source of carbon and incubated at 30 °C on a rotary shaker (200 rpm) for 10 days. Bacterial isolate was examined for its ability to grow in saline condition, biochemical test was performed for fructose, sucrose, glucose, xylose, lactose, gram staining and urease. 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.

Isolation of genomic DNA

The genomic DNA was isolated from cells grown in MS medium using Wizard Genomic DNA Purification kit (Promega, USA). 1 mL of the culture was centrifuged at 13,000×*g* for 2 min. The supernatant was discarded and the cell pellet was resuspended in 600 μL of Nuclei Lysis solution. Incubated for 5 min at 80 °C, and then cooled to room temperature. 3 μL of RNase was added and incubated at 37 °C for 15–60 min. Added 200 μL of protein precipitation solution (Promega kit, USA), vortexed and incubated on ice for 5 min. Centrifuged at 13,000×*g* for 3 min and the supernatant was transferred to a clean tube containing 600 μL of room temperature isopropanol. Mixed well and centrifuged at 13,000×*g* for 2 min. To the pellet, added 600 μL of 70% ethanol and centrifuged at 13,000×*g* for 2 min. The ethanol was decanted and the pellet was air-dried. The DNA was dissolved in TE (Tris–EDTA) buffer at 4 °C, overnight. The genomic DNA obtained was visualized by agarose gel electrophoresis.

PCR amplification and sequencing of 16S rRNA gene

Using enrichment technique, pyrene-degrading bacteria were isolated from oil refinery sludge. To identify, 16S rRNA gene was amplified by the polymerase chain reaction (PCR) using genomic DNA as template. The primers used for the amplification and sequencing of the 16S rRNA gene

is 27F (5′- AGAGTTTGATCCTGGCTCAG-3′) 1492R (5′- TACGGATACCTTGTTACGACT T-3′). The PCR programme (Thermocycler MJ Research, USA) was started with an initial 5 min denaturation step at 94 °C. 34 cycles of 94 °C for 30 s, 58 °C for 30 s for annealing of primers to the DNA template, extension at 72 °C for 30 s and final extension at 72 °C for 7 min. All the reaction mixtures were held at 4 °C until analyzed. After completion of the reaction, the samples were loaded on 0.8% agarose gel to check the amplified genes. The amplified genes were purified using PCR Clean-Up kit (MoBio, USA) to remove the unutilized primers, dNTPs etc. The purified DNA was recovered in 50 µL of demonized water. The amplified 16S rDNA was subjected to cycle sequencing with the Microseq 500 16S rDNA sequencing module.

Utilization of pyrene as a sole source of carbon and energy

Achromobacter was grown in batch cultures in 250 mL flasks containing 100 mL of MSM supplemented with pyrene (200 mg/L) as the sole carbon source. The flasks were inoculated with 1% (v/v) of bacterial culture and incubated at 30 °C with continuos shaking on rotary shaker (200 rpm) for 21 days. At specific time intervals, three flasks were taken out and 1 mL of culture from each flask was withdrawn for protein estimation (Lowry et al. 1951). Two flasks uninoculated and without pyrene served as controls. Pyrene and protein concentrations were measured at 72-h intervals over 21 days. Controls were analyzed at 0, 7, 14 and 21 days. The residual pyrene from the same culture flask was extracted three times with an equal volume of ethyl acetate. Evaporation of solvents was done under a gentle nitrogen stream in a fume hood. The residual pyrene was dissolved in 5 mL of acetone and 20 µL of the resultant solution was analyzed by RP-HPLC.

Characterization of metabolites

For analysis and identification of pyrene metabolites, bacterial cells and culture medium was extracted with six equal volumes of ethyl acetate. The extracts were pooled, dried with anhydrous Na_2SO_4, and evaporated with rotatory evaporator at 40 °C to 10 mL. The extracts were transferred to calibrated glass vials and evaporated to dryness under a gentle stream of dry nitrogen. The RP-HPLC system (Water's, Miliford, MA, USA), which consists of Waters-515 binary pump, rheodyne injector-7725 with 20 µL loop, waters column C-18 (250 × 4 mm, particle size 5 µm) and Water's UV detector-2487 at 254 nm. Analysis were performed on RP-HPLC and separation was achieved using solvent system, methanol–water (70: 30, v/v) for metabolic product determination at isocratic condition with a flow rate of 1.5 mL/min. GC-MS analysis were performed with a Auto XL gas chromatograph and Turbo mass spectrophotometer (Perkin Elmer, USA). Chromatographic resolution was achieved with 30 m × 250 µm, DB-5 capillary columns with 0.25 µm film thickness and helium was used as carrier gas with flow rate of 1 mL/min. The column temperature was kept at 50 °C for 1 min and then programmed to 280 °C at a rate of 10 °C/min. The mass spectrometer was operated at 70 eV of electron ionization energy. Injector, source and transferline temperatures were set at 290, 230 and 280 °C, respectively. The metabolites were compared with that of the mass spectra available in National Institute of Standards and Technology (NIST) library.

Results

Isolation and characterization

It was accepted that screened indigenous microorganisms from polluted soils were often more effective to metabolize PAHs than organisms obtained from elsewhere in bioremediation. Non-sterile sludge from oil refinery effluents was used to screened indigenous pyrene degrading bacteria was isolated from oil refinery sludge after 4 weeks of selective enrichment in MS medium containing pyrene as sole source of carbon by repeatedly subcultures. The ability to degrade pyrene was confirmed by clearance assay, culture was streaked on MS agar plates and sprayed with pyrene conc. at 5 mg/mL dissolved in diethyl ether, bacteria shown zone of clearance around streak area (Fig. 1).

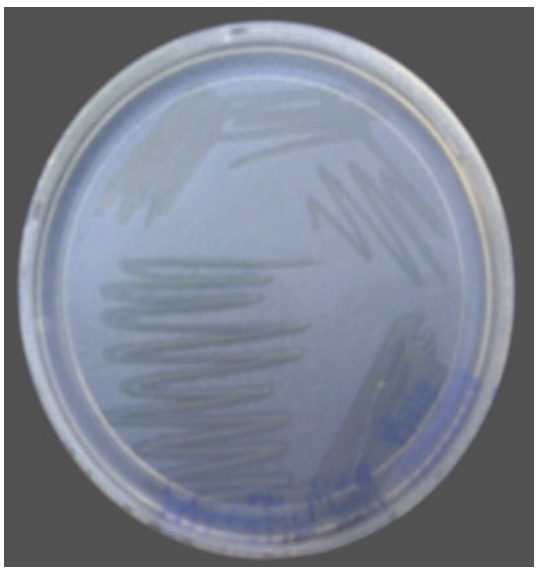

Fig. 1 Zones of clearance as shown by *Achromobacter xylooxidans* streaked on minimal salt (MS) agar plate. Pyrene at the conc. of 5.0 mg/mL, dissolved in diethyl ether was sprayed on streaked MS agar plate and was incubated at 30 °C for 96 h

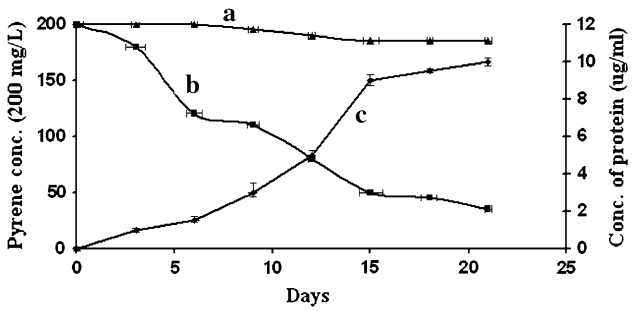

Fig. 2 Utilization of pyrene by *Achromobacter xylooxidans* in liquid mineral medium with pyrene as the sole source (200 mg/L) of carbon and energy at 30 °C and 200 rpm. **a** Uninoculated controls **b** pyrene concentrations as determined by RP-HPLC analysis of organic extracts from cultures **c** growth of isolate is shown as an increase in the level of cellular protein

The characterization of isolate was done by polyphasic approach. The isolate when plated on nutrient rich medium grow aerobically as pale yellow colonies with oval margins. Biochemically, the isolate was found to be, gram positive, catalase positive, urease positive, did not show growth in 10% NaCl and negative for fructose, sucrose, glucose, xylose and lactose. Full-length size of 16S rRNA genes of the isolated bacteria was 1,540 bp (base pair) and alignment of the gene sequences of the isolates was analyzed by Basic Local Alignment Search Tool (BLAST) search of the National Center for Biotechnology Information (NCBI). BLAST analysis of lab isolate showed 99.0% identity to *Achromobacter xylooxidans* (Accession No. AY189752.1.)

Utilization of pyrene as a sole source of carbon and energy

Utilization of pyrene as a sole source of carbon and energy by *Achromobacter xylooxidans* was confirmed by its removal from pyrene in MSM, with a corresponding increase in bacterial protein (Fig. 2). The concentration of pyrene in the incubated culture decreased from 200 to 35 mg/L, after 21 days of incubation. The degradation of pyrene up to 80% was evident from increase in bacterial cellular protein from 0.4 μg/mL at the time of inoculation to 10.0 μg/mL after 21 days of incubation (Fig. 2). A study has also been conducted to see the effect of incubation temperature (25 and 30 °C) on the degradation of pyrene and we found that the incubation at 30 °C has shown more degradation. Similarly, the degradation of phenanthrene up to 73.2% by to *Achromobacter xylooxidans* after 21 days of incubation was also observed. But, naphthalene, chrysene, dibenzo (a,h) anthracene degradation was not observed by this bacteria. The retention time of pyrene in RP-HPLC analysis was found to be at a retention time of 19.5 min (Fig. 3).

Characterization of metabolites

RP-HPLC analysis revealed that after 21 days of incubation, pyrene degraded into three metabolites III, II and I with retention time 3.0, 15.2 and 29.0 min, respectively (Fig. 3). We have collected eluent from 2.5 to 32.0 min of repeated injection of degraded sample on RP-HPLC and

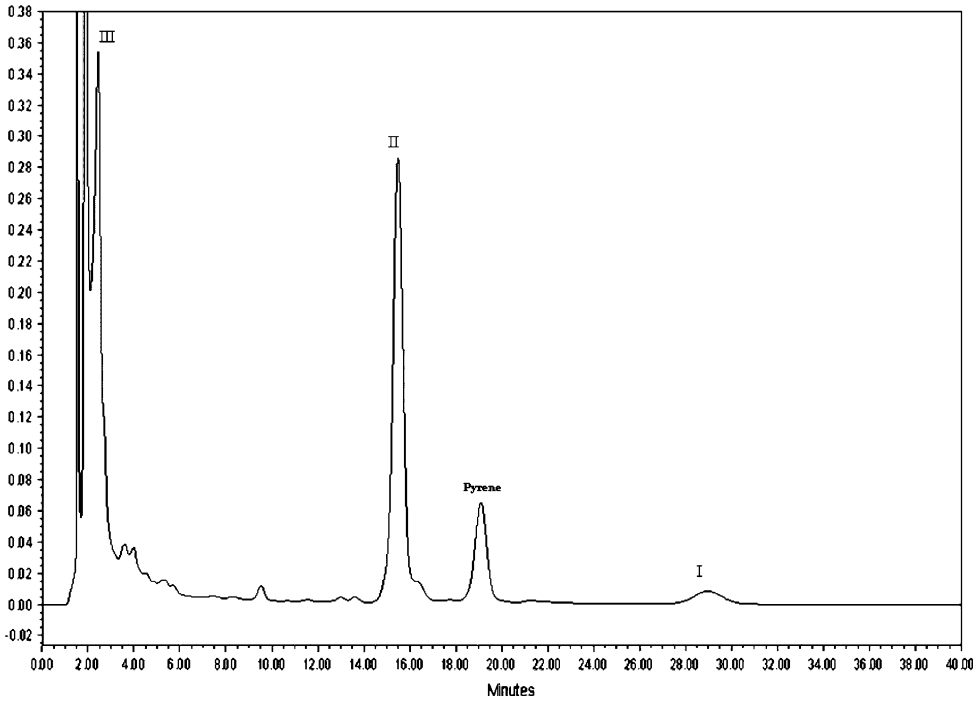

Fig. 3 Reversed-phase HPLC elution profile of organic extract from pyrene degrading culture of *Achromobacter xylooxidans* chromatogram. Organic extract was obtained by using ethyl acetate and RP-HPLC elution was done by using methanol–water (70: 30, v/v) isocratic solvent system at the flow rate of 1.5 mL/min

Table 1 The compounds identified through GC-MS analysis along with their retention times (RT) and their mass fragmentation pattern formed by *Achromobacter xylooxidans* after 21 days of incubation in MSM containing pyrene (400 mg/L)

Identified compound[a]	RT (min)	*m/z* values of identified compound
1-hydroxypyrene	20.4	219(10),218(100), 190(5), 189(45), 163(7), 109(18), 94(20), 81(10)
1-hydroxy-6-methoxypyrene	24.6	248(60), 204(100), 176(45), 150(10), 88(40)
1,6-dimethoxypyrene	25.2	262(100), 247(95), 232(23), 204(21), 187(11), 176(38), 149(15), 131(17), 64(16), 46(46)

concentrated up to 100 μL for metabolites identification on GC-MS. GC-MS analysis of collected fraction shows pyrene at 19.7 min and three metabolic products, 1-hydroxypyrene at 20.4 min, 1-hydroxy-6-methoxypyrene at 24.6 min and 1,6 dimethoxypyrene at 25.2 min. These metabolic products were identified on the basis of their *m/z* values and the comparison of their fragmentation pattern with NIST library that available in GC-MS. The *m/z* values of metabolite products obtained after pyrene degradation were depicted in Table 1.

Discussion

Previously shown *Mycobacterium* sp. strains PYR1 (Heitkamp et al. 1988) and RJGII 135 (Schneider et al. 1996) mineralized approximately 50% of added pyrene, while *Rhodococcus* sp. strain UW1 (Walter et al. 1991) mineralized 72% of added pyrene after 14 days incubation while isolated *Achromobacter xylooxidans* for the first time it is hereby reported effectively degrading pyrene (up to 80%), as evident by RP-HPLC. Bacteria having an enzyme, NADH-linked dioxygenase, stereo and regio specific for PAH-cis-dihydrodiol production and NAD (P)H-linked cytochrome P450 monooxygenase, less stereo and region specificity than the dioxygenase. P450 can be involved in the production of various PAH epoxides and PAH phenols from PAH degradation. PAH epoxides are hydrolyzed to PAH-trans-dihydrodiols by an epoxidehydrolase and the corresponding PAH catechols are produced by dihydrodiols-dehydrogenase activity. Various studies suggesting existence of several cytochromes and monooxygenase enzyme systems for PAH degradation (Brezna et al. 2006), monoxygenation of fluorine at C-9 position by bacterial strains (Kasuga et al. 2001). 1-hydroxypyrene as an intermediate product is reported under varied research interest viz pyrene metabolism by gut microflora (Wiele et al. 2005), from urine sample of animals exposed orally to pyrene (Buratti et al. 2000), during mammalian pyrene degradation (Buratti et al. 2000; Costera et al. 2009) pyrene mineralization by gram-negative bacteria *Leclercia adecarboxylata* PS4040 (Sarma et al. 2009) and during pyrene metabolism by fungus *Penicillium glabrum* strain TW 9424 (Wunder et al.1997). We detected 1-hydroxypyrene as an initial ring oxidation product. Identification of 1-hydroxypyrene during growth shows that bacterium initiates its attack on pyrene at C-1 position by monoxygenation, there was no detection of cis or trans pyrene dihydrodiols. 1-

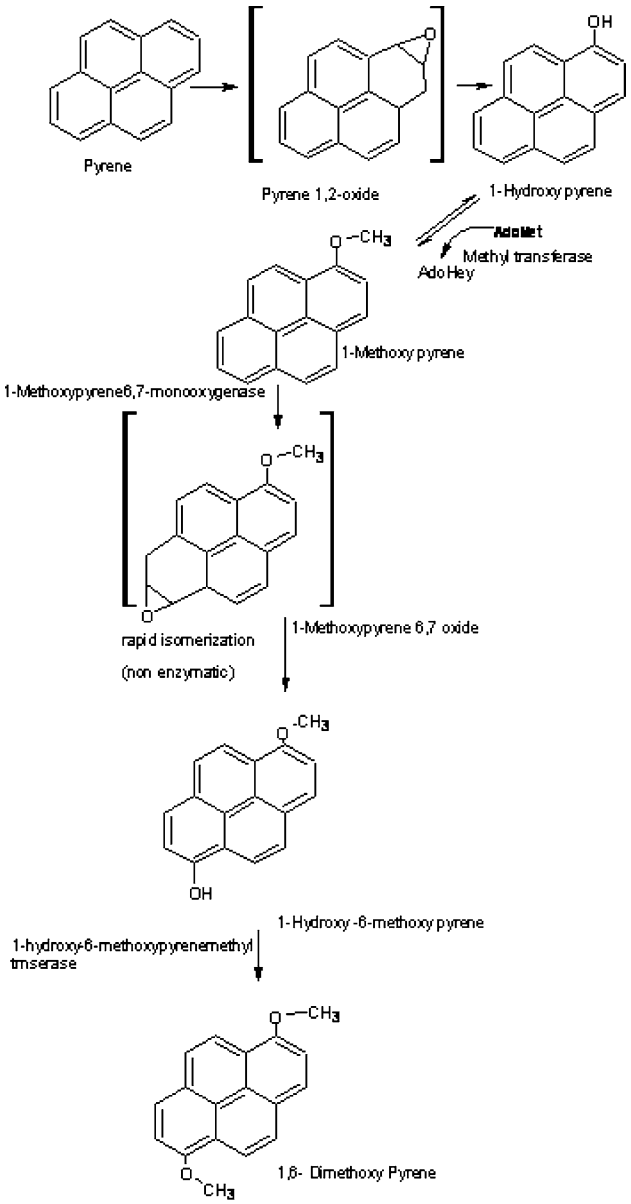

Fig. 4 Proposed metabolic pathway of pyrene by *Achromobacter xylooxidans* on the basis of identified metabolites

Table 2 Bacteria that have been used to degrade pyrene and metabolites identified thereof

Bacteria	Metabolites	References
Coriolopsis gallica	1,2- and 4,5-dihydroxypyrene	Bogan et al. (2003)
Mycobacterium sp. strain AP1	*cis*- and *trans*-4,5-Pyrene dihydrodiols, Phenanthrene 4-carboxylic acid, Phenanthrene 4,5- dicarboxylic acid Phthalic acid	Vila et al. (2001)
Mycobacterium vanbaalenii PYR-1	Pyrene *cis*-4,5-dihydrodiol, 4,5-dihydroxypyrene, phenanthrene-4,5-dicarboxylate, phenanthrene-4-carboxylate and *cis*- 3,4-dihydroxyphenanthrene-4-carboxylate. 1-methoxypyrene, 1-hydroxy-2-methoxypyrene and 1,2-dimethoxypyrene.	Kim et al. (2007)
Mycobacterium sp. strain KMS	4,5-dihydroxypyrene, phenanthrene-4,5-dicarboxylate and 4- phenanthroic acid	Yanna et al. (2006)
Pseudomonas saccharophila P15	*cis*-4,5-dihydro-4,5-dihydroxypyrene, pyrene-4,5-dione, *cis*-4,5-dihydro- 4,5-dihydroxypyrene and 4,5-dihydroxypyrene	Kazunga and Atiken (2000)
Leclercia adecarboxylata PS4040	1-hydroxypyrene	(Sarma et al. 2009)
Achromobacter xylooxidans	1-hydroxypyrene, 1-hydroxy-6-methoxypyrene and 1,6-dimethoxypyrene	This study

hydroxypyrene is also extensively used as a biomarker and for biomonitoring of pyrene exposure to mammals including humans (Buratti et al. 2000; Wiele et al. 2005; Costera et al. 2009). *Achromobacter xylooxidans* may have a catechol-O-methyl transferase (COMT) enzyme that catalyzes the conversion of potentially toxic catechol compounds to less active methylated forms. This bacterium may transformed 1-hydroxy pyrene to 1-hydroxy-6-methoxypyrene and 1,6-dimethoxypyrene. The formation of the 1,6-dimethylated derivative most likely precedes via hydroxylation of 1- methoxypyrene at the C-6 position with subsequent methylation of the second hydroxyl group (Fig. 4). *Mycobacterium vanbaalenii* PYR-1 have COMT enzyme, metabolize pyrene and 1-hydroxypyrene to 1-methoxypyrene, 1-methoxy-2-hydroxypyrene, 1-hydroxy-2-methoxypyrene and 1,2-dimethoxypyrene (Kim et al. 2004). *Mycobacterium vanbaalenii* PYR-1 uses two possible pathways, NADH-linked dioxygenase and NAD (P)H-linked cytochrome P450 monooxygenase to produce PAH-dihydrodiols, 1-methoxypyrene and 1,2-dimethoxypyrene (Kim et al. 2004; Heitkamp et al. 1988). Wunder et al. (1997) also found that fungus *Penicillium glabrum* strain TW 9424 metabolized pyrene and 1-hydroxypyrene to 1-methoxypyrene and 1,6-dimethoxypyrene by COMT activity. Narro et al. (1992) reported that the O-methylation of hydroxyphenanthrene derived from phenanthrene degradation by a marine cyanobacterium, *Agmenellum quadruplicatum* PR-6, significantly reduced the toxicity. Most reports suggest *cis* and *trans*-4,5-pyrene dihydrodiols, 4,5-dihydroxypyrene, phenanthrene-4,5-dicarboxylate, 4- phenanthroic acid, 2-hydroxy-2-(phenanthren-5-one-4- enyl) acetic acid etc. as metabolic products of different bacteria while 1-hydroxypyrene, 1-hydroxy-6-methoxypyrene and 1,6 dimethoxypyrene were identified as unique metabolic products of pyrene degradation by *Achromobacter xylooxidans* (Table 2). Summarily it can be stated that novel isolate namely *Achromobacter xylooxidans* is hereby reported for the first time for efficient degradation of pyrene (up to 80%) with formations of 3 metabolites. The efficient degradation by this isolate further demands the outlook into enzymatic and genetic studies like identification and characterization of efficient degradative genes and enzymes which will open up new avenues for the planning of better bioremediation strategies. Over all further studies will give new insights into the understanding of environmental fate of PAHs with reference relation to their toxicity and persistence in nature.

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References

Bogan BW, Lahner LM, Sullivan WR, Paterek JR (2003) Degradation of straight-chain aliphatic and high-molecular-weight polycyclic aromatic hydrocarbons by a strain of *Mycobacterium austroafricanum*. J Appl Microbiol 94:230–239

Brezna B, Kweon O, Stingley RL, Freeman JP, Khan AA, Polek B, Jones RC, Cerniglia CE (2006) Molecular characterization of cytochrome P450 genes in the polycyclic aromatic hydrocarbon degrading *Mycobacterium vanbaalenii* PYR-1. Appl Microbiol Biotechnol 71:522–532

Buratti M, Pellegrino O, Brambilla G, Colombi A (2000) Urinary excretion of 1-hydroxypyrene as a biomarker of exposure to polycyclic aromatic hydrocarbons from different sources. Biomarkers 5:368–372

Cerniglia CE (1992) Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation 3:351–368

Costera A, Feidt C, Dziurla MA, Monteau F, Bizec BL, Rychen G (2009) Bioavailability of polycyclic aromatic hydrocarbons

(PAHs) from coil and hay matrices in lactating goats. J Agric Food Chem 57:5352–5357

Datta D, Samanta TB (1988) Effects of inducers on metabolism of benzo[a]pyrene in vivo and in vitro: analysis by high pressure liquid chromatography. Biochem Biophys Res Comm 155: 493–502

Hamamura N, Olson SH, Ward DM, Inskeep WP (2006) Microbial population dynamics associated with crude oil biodegradation in diverse soils. Appl Environ Microbiol 72:6316–6324

Heitkamp MA, Freeman JP, Miller DW, Cerniglia CE (1988) Pyrene degradation by a *Mycobacterium* sp.: identification of ring oxidation and ring fission products. Appl Environ Microbiol 54:2556–2565

Kanaly RA, Harayama S (2000) Biodegradation of high-molecular weight polycyclic aromatic hydrocarbons by bacteria. J Bacteriol 182:2059–2067

Kasuga K, Habe H, Chung JS, Yoshida T, Nojiri H, Yamane H, Omori T (2001) Isolation and characterization of the genes encoding a novel oxygenase component of angular dioxygenase from the gram-positive dibenzofuran-degrader Terrabacter sp. strain DBF63. Biochem Biophys Res Commun 283:195–204

Kazunga C, Aitken MD (2000) Products from the incomplete metabolism of pyrene by polycyclic aromatic hydrocarbon degrading bacteria. Appl Environ Microbiol 66:1917–1922

Kim YH, Moody JD, Freeman JP, Brezna B, Engesser KH, Cerniglia CE (2004) Evidence for the existence of PAH-quinone reductase and catechol- *O*-methyltransferase in *Mycobacterium Vanbaalenii* PYR-1. J Ind Microbiol Biotechnol 31:507–516

Kim SJ, Kweon O, Jones RC, Edmondson RD, Cerniglia CE (2007) Complete and integrated pyrene degradation pathway in *Mycobacterium vanbaalenii* PYR-1 based on system biology. J Bacteriol 189:464–472

Lowry OH, Roserough NJ, Farr AL, Randall RJ (1951) Protein measurement with folin phenol reagent. J Biol Chem 193:265

Malik A, Singh KP, Mohan D, Patel DK (2004) Distribution of polycyclic aromatic hydrocarbons in Gomti river system, India. Bull Environ Contam Toxicol 72:1211–1218

Molina M, Araujo R, Hodson RE (1999) Cross-induction of Pyrene and phenanthrene in a Mycobacterium sp. isolated from polycyclic aromatic hydrocarbon contaminated river sediments. Can J Microbiol 45:520–529

Narro ML, Cerniglia CE, Van Baalen C, Gibson DT (1992) Metabolism of phenanthrene by the marine cyanobacterium *Agmenellum quadruplicatum* PR-6. Appl Environ Microbiol 58:1351–1359

Ravindra K, Sokhi R, Van Grieken R (2008) Review: atmospheric polycyclic aromatic hydrocarbons: source attribution, emission factors and regulation. Atmos Environ 42:2895–2921

Sarma PM, Duraja P, Deshpande S, Lal B (2009) Degradation of pyrene by an enteric bacterium, *Leclercia adecarboxylata* PS4040. Biodegradation 21:59–69. doi:10.1007/s10532-009-9281-z

Schneider J, Grosser R, Jayasimhulu K, Xue W, Warshawsky D (1996) Degradation of pyrene, benz[a]anthracene and benzo[a]-pyrene by *Mycobacterium* sp. strain RJGII-135, isolated from a former coal gasification site. Appl Environ Microbiol 62:13–19

Vila J, Lopez Z, Sabate J, Minguillon C, Solanas AM, Grifoll M (2001) Identification of a novel metabolite in the degradation of pyrene by *Mycobacterium* sp. strain AP1: actions of the isolate on two and three-ring polycyclic aromatic hydrocarbons. Appl Environ Microbiol 67:5497–5505

Walter U, Beyer M, Klein J, Rehm HJ (1991) Degradation of pyrene by *Rhodococcus* sp. UW1. Appl Microbiol Biotechnol 34:671–676

Widada J, Nojiri H, Kasuga K, Yoshida T, Habe H, Omori T (2002) Molecular detection and diversity of polycyclic aromatic hydrocarbon degrading bacteria isolated from geographically diverse sites. Appl Microbiol Biotechnol 58:202–209

Wiele T, Vanhaecke VL, Boeckaert C, Peru K, Headley J, Verstraete W, Siciliano S (2005) Human colon microbiota transform polycyclic aromatic hydrocarbons to estrogenic metabolites. Environ Health Perspect 113:6–10

Wong JWC, Lai KM, Wan CK, Ma KK, Fang M (2002) Isolation and optimization of PAH-degradation bacteria from contaminated soil for PAHs bioremediation. Water Air Soil Pollut 139:1–13

Wunder T, Marr J, Kremer S, Sterner O, Anke H (1997) 1- Methoxypyrene and 1, 6-methoxypyrene: two novel metabolites in fungal metabolism of polycyclic aromatic hydrocarbons. Arch Microbiol 167:310–331

Yanna L, Gardner DR, Chen D, Anderson AJ, Weimer BC, Sims RC (2006) Study of biochemical pathways and enzymes involved in pyrene degradation by *Mycobacterium* sp. Strain KMS. Appl Environ Microbiol 72:7821–7828

Zaghden H, Kallel M, Elleuch B, Oudot J, Saliot A (2007) Sources and distribution of aliphatic and polyaromatic hydrocarbons in sediments of Sfax, Tunisia, Mediterranean Sea. Mar Chem 105: 70–89