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ORIGINAL PAPER

# Degradation of pyrene by an enteric bacterium, *Leclercia adecarboxylata* PS4040

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**Abstract** A newly discovered enteric bacterium *Leclercia adecarboxylata* PS4040, isolated from oily sludge contaminated soil sample was reported for degradation of polycyclic aromatic hydrocarbons (Appl Environ Microbiol 70:3163–3166, 2004a). This strain could degrade 61.5% of pyrene within 20 days when used as sole source of carbon and energy. The time course degradation experiment detected several intermediate products and the metabolites were identified by gas chromatography mass spectrometry analysis. Metabolite I was the detected on the 5th day and was identified as 1-hydroxypyrene and was detected till 10th day. Metabolite II which was detected on 10th day was identified as 1,2-phenanthrenedicarboxylic acid. Metabolite III and Metabolite IV were identified as 2-carboxy benzaldehyde and *ortho*-phthalic acid, respectively and were detected in the culture broth on 10th and 15th day. 1,2-benzene diol (catechol) was the fifth metabolite detected in the culture extracts on the 15th day and was subsequently reduced on day 20. Identification of Metabolite I as 1-hydroxypyrene was further investigated as this intermediate was not previously reported as a ring oxidation product for degradation of pyrene by bacterial strains. Purification by preparative high performance liquid chromatography and nuclear magnetic resonance spectroscopy, confirmed the identification of Metabolite I as 1-hydroxypyrene. *L. adecarboxylata* PS4040 could also use 1-hydroxypyrene as a sole source of carbon and energy. Thus a probable pathway for degradation of pyrene by enteric bacterium is proposed in this study, with 1-hydroxypyrene as initial ring oxidation product.

**Keywords** Pyrene degradation · Enteric bacteria · *Leclercia adecarboxylata*

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## Introduction

Polycyclic aromatic hydrocarbons (PAHs) are environmental pollutants of serious concern because of their toxic and carcinogenic potentials. They represent a unique class of petroleum hydrocarbons due to its

pyrogenic nature and the complexity of the assemblages in which it occurs (Kanaly and Harayama 2000). Oily sludge, a major petroleum refinery effluent, contains considerable amounts of PAH compounds (Seo et al. 2009; Silva et al. 2009). Accidental spillage and improper disposal of these materials have resulted in a number of contaminated sites presenting serious health and ecological risks (Mishra et al. 2004; Tam and Wong 2008). Bioremediation technologies have increasingly been proposed to decontaminate these sites (Bhattacharya et al. 2003; Silva et al. 2009). However, owing to its physico-chemical properties, PAH-polluted sites frequently resist a fast and complete cleanup (Seo et al. 2009; Yutse et al. 2000). Among the reasons suggested for the attenuation in the biodegradation rates are the accumulation of toxic metabolites and the fact that the residual concentrations comprise the components more resistant to degradation (Watanabe 2001; Sarma et al. 2004b; Yutse et al. 2000). Strategies to improve bioremediation technologies for PAH-contaminated soils require a broader understanding of the biochemical pathways involved in degradation and in the eventual formation of partially oxidized products.

Pyrene is one of the most abundant high-molecular-weight PAHs in environmental samples. The pathways for the biodegradation of pyrene are well documented for actinomycetes and fungal strains (Kim et al. 2005, 2008; Liang et al. 2006; Anastasi et al. 2008). However, in the recent years, few bacterial strains capable of metabolizing pyrene have also been reported (Demaneche et al. 2004; Seo et al. 2007; Lafortune et al. 2009; Sheng et al. 2009). Pathway of pyrene degradation has been proposed for *Mycobacterium* PYR-1 growing on pyrene which involves ring oxidation and ring cleavage metabolites (Heitkamp et al. 1998; Vila et al. 2001; Brezna et al. 2006). This pathway was later confirmed and studies by many investigators and also in other microorganisms that use pyrene as a sole source of carbon and energy (Demaneche et al. 2004; Kim et al. 2007, 2008).

However, considering the complexity and ubiquity of the PAH compounds in the environment, it necessitates the further understanding of different environmental routes for their degradation and identification of metabolites by new strains. It will assist in designing bioremediation strategies for remediation and reclamation of sites contaminated with such hazardous compounds.

We have recently reported the capability of a novel enteric bacterial strain to utilize PAH compounds (Sarma et al. 2004a). The groups of enteric bacteria under the family enterobacteriaceae are mainly regarded as inhabitants of animal gut. The ability of this group to degrade high molecular weight PAH compounds appears to be an unusual feature as this phenomenon has been associated with typical soil bacteria (Diaz et al. 2001). Although there are several reports on bioremediation of PAHs, researches pertaining to biodegradation of the same by enteric bacteria are relatively less (Diaz et al. 2001; Watanabe 2001).

In this study we report the metabolic profile of *Leclercia adecarboxylata* PS4040 (Sarma et al. 2004a), when grown in pyrene as the sole source of carbon and energy. We also report identification of intermediate metabolic products arise during degradation of pyrene when used as sole carbon source by *L. adecarboxylata* PS4040. Based on the intermediates identified in this study, we also attempt to propose a metabolic pathway for pyrene biodegradation.

## Materials and methods

### Source of bacteria

The novel enteric bacterial strain *L. adecarboxylata* PS4040 used in this study was isolated from oily sludge contaminated soil samples from Digboi oil refinery as reported in our previous study (Diaz et al. 2001).

### Degradation analysis

Growth of the strain *L. adecarboxylata* PS4040 was verified by demonstrating an increase in bacterial cell protein concentration concomitant with a decrease in the pyrene concentration as mentioned in our previous study (Sarma et al. 2004a). Batch culture experiments were conducted in triplicate in 500-ml Erlenmeyer flasks with 200 ml minimal salts medium (MSM) (Lal and Khanna 1996) containing pyrene (300 mg $l^{-1}$) as sole carbon source for detection of intermediates. Pyrene was dissolved in acetone and added to the flasks and acetone was then allowed to evaporate. The flasks were incubated on a rotary shaker (200 rpm) at 30°C in dark. The bacterial strain

was grown previously on MSM containing pyrene (200 mg $l^{-1}$) as carbon source to an exponential phase and a cell density of $10^8$ cells $ml^{-1}$, was used as inoculum (Sarma et al. 2004a). Uninoculated and killed controls were kept with each set of experiments to monitor natural and abiotic weathering of pyrene. Residual pyrene and the intermediates in the culture broth was extracted just after inoculation and there after at 5th, 10th, 15th and 20th day of incubation. The culture was extracted five times with equal volume of ethyl acetate as per the protocols described elsewhere (Sarma et al. 2004a). The intermediates from one set of cultures were extracted under acidic conditions. The culture was acidified with 5 N HCl and then extracted again in the same manner as described above. For detection and characterization of metabolites aliquots of neutral and acidic extracts were evaporated under a gentle nitrogen stream in fume hood. The residue was dissolved in 500 µl of acetone was used for gas chromatography (GC) analysis and later for GC-mass spectroscopy (GC-MS) analysis.

## Identification of the intermediates

A volume of 1 µl acetone-residue mixture was analyzed by gas chromatography (Hewlett Packard 5890 series II) fitted with flame ionization detectors and a 30-m long DB 5.625 column (ID 0.25 µm and film thickness 0.25 µm) and the analysis was done as per the methods described previously (Sarma et al. 2004b). Another volume of 1 µl acetone-residue mixture from the culture extract was also subjected to GC-MS (Perkin Elmer Clarus 500, USA) analysis. A PE-Elite-5 capillary column (ID 0.32 mm, length 15 m and 0.25 µm film thickness) was used with helium as the carrier gas. The oven temperature was initially kept at 80°C and was increased to 300°C at a rate of 5°C/min. The mass spectrometer was operated at 70 eV of electron ionization energy. The injector and detector temperatures were set at 200 and 300°C, respectively. The selective ion monitoring (SIM) mode of the GC-MS (Perkin Elmer Clarus 500, USA) was used with specific *m/z* values for confirming the identification of the metabolites. Derivatization prior to GC/EI-MS analysis was performed by silylation with N,O-bis(trimethylsilyl)-trifluoroacetamide with 1% trimethylchlorosilane (Regis Technologies, Morton Grove, IL). The samples were dissolved in 400 µl of acetonitrile. Two hundred microliters of dissolved sample and 100 µl of silylation reagent were mixed in an Xpertek high-recovery 1.5-ml sample vial (P. J. Cobert, St. Louis, MO) sealed with a septum cap and allowed to react for 1 h at 68°C. The injection volumes were 0.5 µl. All mass spectrometric measurements were at low resolution, and no tandem mass spectrometry methods were employed. Therefore, all fragmentation losses are reported as assumptions based on the proposed structures and available moieties.

## Analysis of metabolites by HPLC and NMR studies

Purification of the metabolites was done by preparative high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) spectroscopy. For HPLC analysis, the bacterial strain was grown in 2,000-ml Erlenmeyer flasks with 1,000 ml MSM containing pyrene (300 mg/l) as sole carbon source for 20 days under similar conditions as mentioned above. Residual metabolites were extracted from the culture by the similar methods described above and the solvent was evaporated under a gentle nitrogen flow. The residue was dissolved in 100 µl of acetone.

HPLC analysis were done on an Agilent 1,100 series HPLC (Agilent, USA) system equipped with a variable wavelength detector. Injections (50 µl) were made on a Supelcosil LC-PAH (Supelco, USA) column (2 m by 4.6 mm [internal diameter], particle size 5 µm) and the samples were analysed at 254 nm with a linear gradient of methanol (50–100% [vol/vol] in 20 min) in water. Flow was kept at 1 ml/min.

Fractions of Metabolite 1 were collected by the fraction collector fitted with the HPLC (Agilent, USA) with concordance to its retention time. The fractions were pooled together as one collective sample and the sample was evaporated in a flash evaporator (Retovac, Heidolph, Germany). The purity of the evaporated collective fraction sample was confirmed by re-injection in HPLC and GC-MS as per the methods described earlier. The HPLC purified samples was then analysed for $^{1}H$ NMR and $^{13}C$ NMR spectra on a Bruker (300 htz, UK) spectrometer. For both, the central signal of the solvent (methanol and $CdCl_3$) was used as an internal standard.

### Experiments with metabolite 1

Growth of strain PS4040 on metabolite 1 as a sole source of carbon was determined. The bacterial strain was grown previously on MSM containing pyrene (200 mg/l) as carbon source to an exponential phase and a cell density of $10^8$ cells $ml^{-1}$, was used as inoculum. Metabolite 1 (1-hydrozypyrene, Sigma USA) was added to the flasks containing 100 ml MSM at concentrations equivalent to 500 μM and cultures were incubated for 4 weeks. Protein concentration and residual metabolite in the culture flasks were monitored as describe above and as per methods described in an earlier study (Sarma et al. 2004a).

### Chemicals

The standards of the parent compound and the metabolites were purchased from Aldrich Chemical Co., USA. Ethyl acetate used for extraction purposes were of HPLC grade and were purchased from Merk, USA. The standards in were analysed GC-MS after extraction in culture broth under similar conditions mentioned above. The GCMS spectras so obtained were then analysed with NIST library database and were used later to identify the metabolites.

## Results

### Degradation of pyrene as sole source of carbon and energy

The studies on degradation of PAH compounds showed that the bacterial strain *L. adecarboxylata* PS4040 could degrade pyrene when used as sole source of carbon (Sarma et al. 2004a). As discussed in our earlier study (Sarma et al. 2004a), we reconfirmed the results of pyrene degradation. The concentration of pyrene in culture decreased from 200 to 77 mg $l^{-1}$, exhibiting 61.5% degradation of pyrene by the strain PS4040 in 20 days.

### Detection and identification of metabolites during degradation of pyrene

The intermediate metabolic products of pyrene degradation were initially detected by GC analysis of the residual pyrene of the culture through the time course degradation experiment The GC analysis of neutral extracts and the acidic extracts did not show much variability. The same residual samples was then analysed by GC-MS and metabolites were identified on the basis of molecular ion peak and fragment ion peaks. GC and GC-MS analysis of neutral extracts revealed a total of four metabolites. Acidic extracts revealed another metabolite along with the five metabolites detected in neutral extracts. The retention time and fragmentation pattern depicting the mass spectrum for all the five metabolites are shown in Table 1. Further, detection and identification was also confirmed by comparing the retention times of GC and mass spectra in GC-MS with that of respective authentic standards procured from Sigma Chemicals (USA). The standard metabolites were also analysed under similar conditions (without inoculation and with heat killed cells) to rule out any chemical modifications during incubation or extraction procedures. The results ruled out formation of any artifacts that could be mistaken for intermediates of pyrene degradation.

The metabolites were detected after derivitization and the first metabolite was detected on the 5th day in the culture extract and was observed till the end of exponential phase (Fig. 1). This metabolite was identified as 1-hydroxypyrene (Table 1). The second metabolite was detected on the 5th day culture extracts (Fig. 1). The mass spectrum and fragmentation pattern of Metabolite II was similar, but not identical to a metabolite formed from pyrene biodegradation by a *Mycobacterium* strain (32) (Table 1). The mass spectrum of Metabolite II showed a molecular ion (*m/z* 270) and a fragmentation pattern (Table 1), which is compatible with an aromatic structure having two carboxylic groups. On the basis of mass spectrum, Metabolite II was identified as, 2-phenanthrenedicarboxylic acid (Table 1) and was detected in the culture extracts from 10th to 15th day (Fig. 1).

Similarly based on the fragmentation pattern, Metabolite III was identified as 2-carboxybenzaldehyde and Metabolite IV as *ortho*-phthalic acid (Table 1). These two metabolites were also detected in 10th day extracts and were observed till 15th day extracts (Fig. 1). The fragmentation pattern of Metabolite V is shown in Table 1. Based on the fragmentation pattern, Metabolite V was identified as 1,2-benzene diol (catechol) and was detected in 15th

**Table 1** GC $R_t$s and electron impact mass spectral properties of metabolites formed from pyrene utilization by *L. adecarboxylata* TERIPS4040

| Metabolite | $R_t$ (min) | *m/z* of fragment ions (% relative intensity) | Identification |
|---|---|---|---|
| Metabolite I | 20.93 | **218** (100), 189 (24.6),109 (20.33), 94 (19.63) | 1-Hydroxypyrene |
| Metabolite II | 30.21 | 270 (10.15), 252 (24.21), 226 (14.54), **180** (100), 179 (63.2), 165 (24.32) | 1,2-Phenanthrene dicarboxylic acid |
| Metabolite III | 27.18 | 150 (20.5), 122 (23.5), **105** (100), 76 (33.07), 65 (21.53), 50 (16.24) | 2-Carboxy benzaldehyde |
| Metabolite IV | 28.34 | 166 (23.34), 148 (34.76), **105** (100), 77 (82.26), 50 (22.43), 38 (19.8) | *ortho*-Phthalic acid |
| Metabolite V | 4.74 | **110** (100), 108 (15.55), 82 (18.37), 64 (28.13), 55 (18.64), 54 (28.27), 53 (26.17) | 1,2-Benzene diol (catechol) |

Figures in bold indicate highest *m/z* ion fragment of the compound

day extracts, which substantially reduced on day 20 (Table 1; Fig. 1). The results of the GC-MS analysis of the samples by selective ionization mode respective *m/z* ratios with also inferred identical results, confirming the identification of all these metabolite.

The mass spectrum obtained for 1-hydroxypyrene was different from the pyrene metabolites as commonly described in most literature. The mass spectrum of metabolite 1, indicate a molecular ion peak at *m/z* 218 ($M^+$) (100% base peak), with fragment ion peaks at *m/z* 189 ($M^+$ −29, C–OH), *m/z* 163 ($M^+$ −55, O–C–CH=CH). This indicated presence of only one hydroxyl group in this compound and was identified as 1-hydroxypyrene as the first ring oxidation product of pyrene metabolism by *L. adecarboxylata* PS4040. Metabolite 1-hydroxypyrene was reported earlier from fungal and mammalian metabolism of pyrene, but was never reported as a bacterial metabolic product. Therefore, the identity of Metabolite I was further confirmed by NMR analysis.

### Analysis of metabolite I

The Metabolite I was purified by preparative HPLC and its purity was confirmed by GC and HPLC analysis. The fraction of Metabolite I when subjected to GC-MS, showed a fragmentation pattern that is identical to the fragmentation pattern of 1-hydroxypyrene. The structure was further confirmed by $^1$H NMR spectroscopy. The resonance assignments ($\delta$) for the metabolites are as follows: 7.73 (d, 1H, $J = 8.4$ Hz), 7.94(d, 1H), 7.99 (t, 1H), 8.03(d, 1H), 8.10(d, 1H), 8.15 (d, 1H), 8.18 (d, 1H), 8.23(d, 1H), 8.43(d, 1H) (Fig. 2a). The $^{13}$C-NMR spectrum (Fig. 2b) showed a multiplet of absorption signals at $\delta$ 109–133 indicating the aromatic carbon atoms. The singlet at $\delta$ 151.68 corresponds to a carbon atom bearing a hydroxyl group(C–OH) (Fig. 2b). Upon analysis of the spectra, it was observed that there were no singlet protons and it indicates that the hydroxyl group is attached to carbon 1. This was in concordance with the mass spectra obtained by GC-MS analysis. Thus the NMR chromatogram further confirms the hypothesis of incorporating a single hydroxyl group to the parent pyrene compound to produce 1-hydroxypyrene as the oxidation product.

### Utilization of 1-hydroxypyrene as sole source of carbon

Strain PS 4040 could utilize 1-hydroxypyrene as sole source of carbon and energy as determined by significant increases in optical density and protein concentration relative to inoculated flasks with no carbon source (Fig. 3).

## Discussion

The ability of an enteric bacterium to degrade high molecular weight PAH compound pyrene, appears to be an uncommon finding. However, as discussed by Diaz et al. (2001) upon analyzing the ecology of enteric bacteria it can be seen that an enteric bacterium may easily encounter aromatic compounds in both the intestinal and extra-intestinal habitats. We

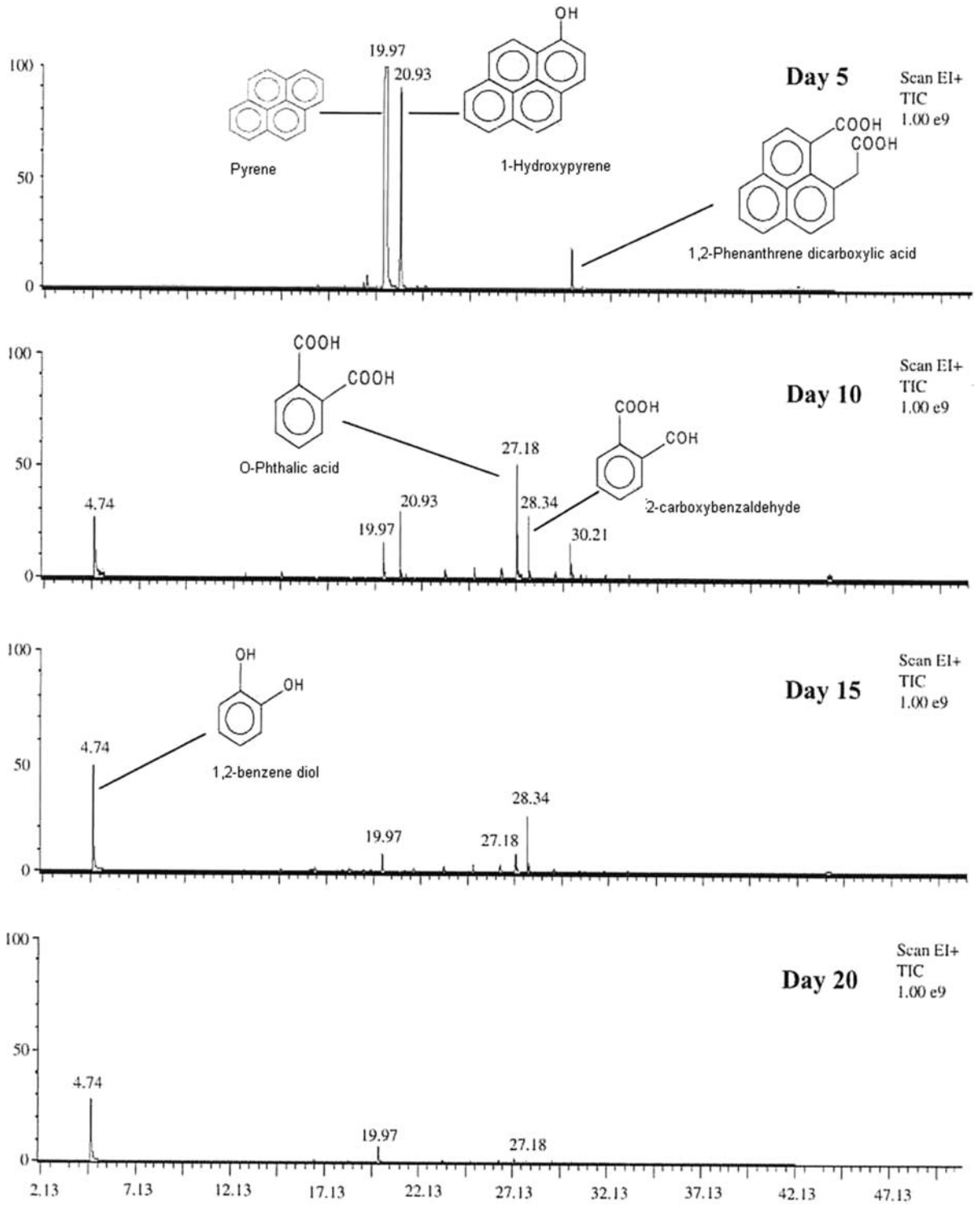


**Fig. 1** Intermediate metabolic products of pyrene degradation by *L. adecarboxylata* PS4040 analyzed by gas chromatography (*GC*) mass spectrometry (*MS*). GC MS analysis was done at 5, 10, 15 and 20 days of incubation of *L. adecarboxylata* PS404 in MSM containing pyrene (300 mg/l)

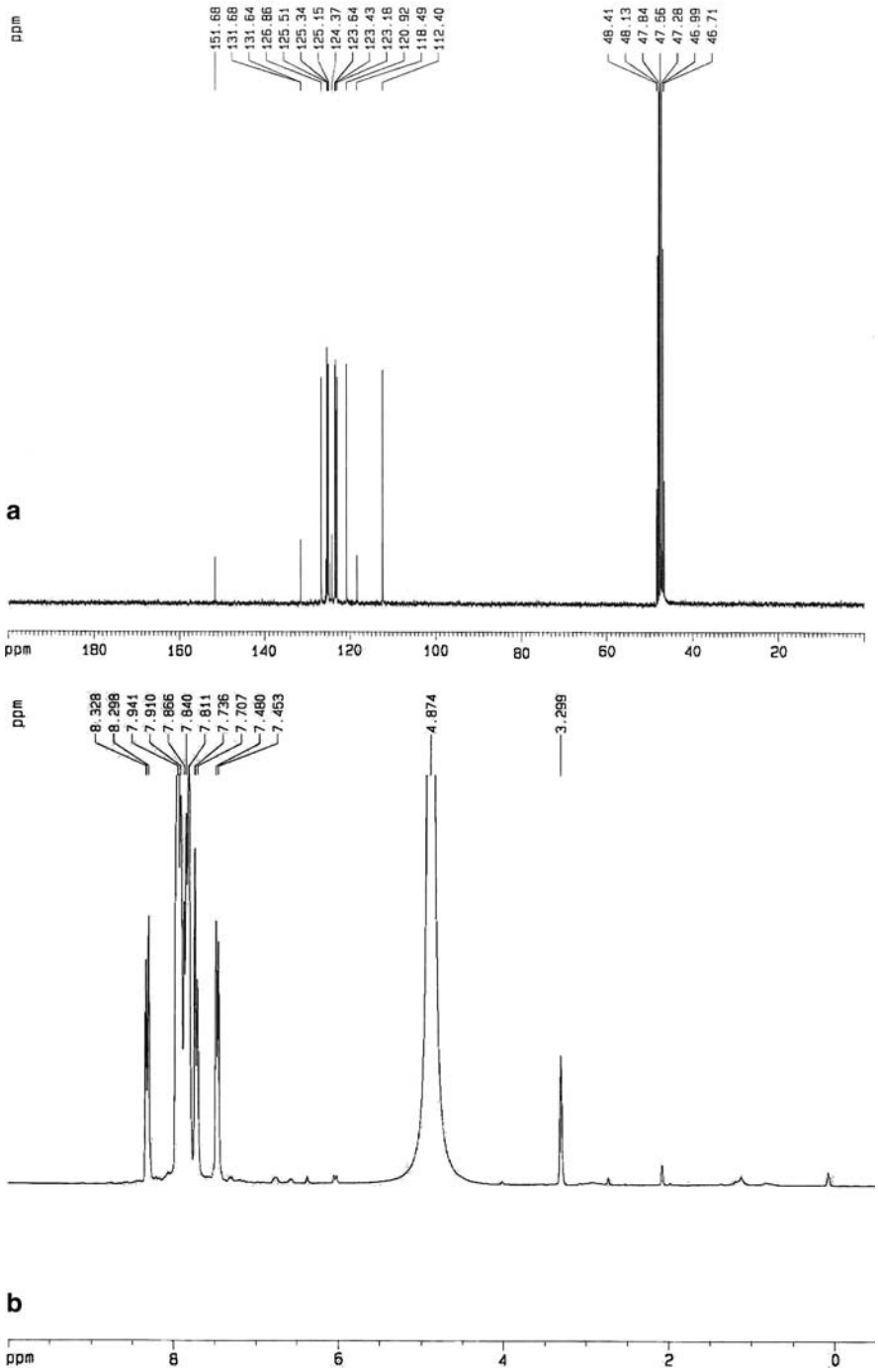


**Fig. 2** **a** $^{1}$H NMR spectroscopy and **b** $^{13}$C-NMR spectrum data of Metabolite I

have discussed the PAH degrading capability of this enteric bacterial strain in our last publication (Sarma et al. 2004a). The strain *L. adecarboxylata* PS4040 was isolated from soil samples collected from an oily sludge contaminated site that had a contamination history of over 100 years (Sarma et al. 2004a).

In most of the reports, the first step in the microbial degradation of PAH compounds is the action of dioxygenases, which incorporates atoms of oxygen at two carbon atoms of a PAH compound. However, in this study, identification of metabolites accumulated during growth indicates that *L.*

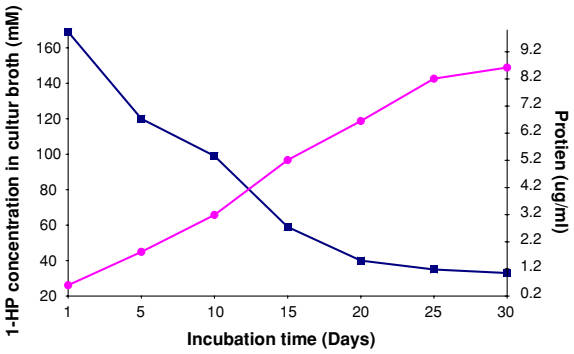


**Fig. 3** Degradation of 1-hydroxypyrene (Metabolite I) by *L adecarboxylata* PS4040 as sole source of carbon and energy

*adecarboxylata* PS4040 initiates its attack on pyrene by mono-oxygenation of C-1 position. We detected 1-hydroxypyrene as an initial ring oxidation product, while most reports on pyrene metabolism suggest formation of a didhydrodiol by dioxygenase catalyzed reaction (Kazunga and Aitken 2000; Liang et al. 2006; Kim et al 2007). In this study, there was no detection of *cis* or *trans* pyrene hydrodiol. Thus the possibility of monooxygenase activity as the initial ring oxidation product is 1-hydroxypyrene is suspected, though this aspect warrants further investigation. Further we attempted to detect dioxygenase genes with the help of reported primers (Demaneche et al. 2004; Baek et al. 2009), but we failed to detect any positive amplicons (data not shown). Though this study does not establish the fact of presence or absence of dioxygenase genes, we are in the process of analyzing the genomic library of the strain *L. adecarboxylata* PS4040 to characterize the role of degradative enzymes.

Kanaly and Harayama (2000) reviewed the biodegradation of high molecular weight PAH compounds and discussed that was unclear if the small amounts of pyrenol detected had occurred by nonenzymatic dehydration of pyrene dihydrodiols or by oxidative metabolism of pyrene by the mycobacterium strains. In this study, measures were taken to rule out detection of any artifacts that might form due to any chemical modifications during incubation or extraction procedures. All the standard metabolites were procured and analysed under similar conditions as it was done for the test samples. Further, the reports on detection of both *cis*- and *trans*-4,5-dihydrodiols suggested multiple pathways for the initial oxidative attack on pyrene (Vila et al. 2001). In few studies where the investigators have used $^{18}O_2$, or otherwise, confirmed that these products were catalyzed by both dioxygenase and monooxygenase enzymes, respectively (Heitkamp et al. 1998; Brezna et al. 2006). Bezalel et al. (1996), reported metabolism of pyrene at 4,5 bond (K- region) to form an epoxide and then further transformed to a didydrodiol and suggested the role of a cytochrome P-450 monooxygenase. Furthermore, in this study detection of 1-hydroxypyrene till 10th day of incubation further reiterates the possibility of an initial monooxygenase activity and 1-hydroxypyrene as initial ring oxidation product for *L. adecarboxylata* PS4040. There are reports on existence of several cytochromes and monooxygenase enzyme systems for PAH degradation (Brezna et al. 2006).

Vila et al. (2001) mentioned the possibility of monooxygenation of C-4 and C-5 positions and subsequent hydrolysis to *trans*-4,5-dihydroxy-4,5-dihydropyrene in case of pyrene degradation by a Mycobacterium strain. They have reported formation of phenanthrene 4,5-dicarbolylic acid through an *ortho* cleavage of a diol formed from *cis* dihydroxypyrene. In this study, we have detected 1,2 phenanthrene carboxylic acid as a ring fission product. It might be suggestive of possible rearomatization of the initial dihydrodiol and subsequent cleavage of the ring as reported by Rehmann et al. (1998). However, as mentioned by Vila et al. (2001), the mechanisms to the *ortho* cleavage of the central ring of pyrene and the on carbon excision from the K region of phenanthrene dicarboxylic acid remain to be elucidated, thus requires further investigation.

There are reports of monoozygenation of fluorene at C-9 position to give 9-fluorenol by bacterial strains (Kasuga et al. 2001). However, this monooxygenation activity was due to complementation of the non-specific ferrodoxin and ferrodoxin reductase component of *E. coli* cells to which the PAH degrading genes (*dbf*A1 and *dbf*A2) were cloned (Kasuga et al. 2001; Demaneche et al. 2004). Though we could not detect either of these genes in PS 4040 (data not shown), it may be speculated that initial ring fission occurs due to a monooxygenase activity. Further, Wiele et al. (2005), have reported 1-hydroxypyrene as an intermediate product of pyrene metabolism by gut microflora. It is also note worthy that 1-hydroxypyrene has been reported as a major intermediate of

pyrene occurring in urine of animals exposed orally to pyrene and the strain of *L. adecarboxylata* PS4040 is from the family enterobacteriaceae. There are reports detecting 1-hydroxypyrene as an intermediate for mammalian pyrene degradation (Buratti et al. 2000; Costera et al. 2009). This metabolite is also very extensively used as a bio-monitoring of pyrene exposure to mammals including humans (Buratti et al. 2000; Wiele et al. 2005; Costera et al. 2009).

There are also reports of detecting 1-hydroxypyrene in biodegradation of pyrene by fungal isolates (Capotorti et al. 2004; Anastasi et al. 2008) However, there are no reports of detecting this metabolite as an intermediate for pyrene degradation by bacterial strains except for the report of Wiele et al. (2005) that used a gastrointestinal stimulator to establish colon microflora to transform pyrene and benzo(a)-pyrene to 1-hydroxypyrene and 1-hydroxy-benzo(a)pyrene compounds to esterogenic metabolites. In this study, extensive analysis of the Metabolite 1, encompassing the analytical methods of GC, GC-MS, preparative HPLC and finally NMR, verified the identity of Metabolite I to be 1-hydroxypyrene. We thus report for the first time 1-hydroxypyrene as an initial ring oxidation product of pyrene degradation by an enteric bacterial strain PS4040. With the detection of other four metabolites, we also propose a pathway for pyrene degradation by enteric bacterial strain *L. adecarboxylata* (Fig. 4).

The late metabolites identified (Table 1; Fig. 2) suggests that, further breakdown follows the phthalic acid pathway as discussed earlier (Rehmann et al. 1998). This hypothesis is further supported by the finding that strain *L. adecarboxylata* PS4040 could utilize catechol (Sarma et al. 2004a), the final aromatic intermediate of this pathway as the sole source of carbon and energy. The elucidation of the intermediates and the order of reaction suggest that there are no dead end products.

This study also suggests that there is production of no dead end metabolites. The initial reactions suggest production of energy for growth of the enteric bacterial strain *L. adecarboxylata* PS4040. This is consistent with the growth observed in logarithmic phase when the strain was grown in pyrene as some source of carbon in our previous study (Sarma et al. 2004a). The metabolite I (1-hydroxypyrene) was utilized by the strain *L. adecarboxylata* PS4040 as sole source of carbon and energy. Though all the individual metabolites were not analysed as sole source of carbon and energy, growth pattern of the strain suggest that the late metabolites do not produce energy for growth. The growth rate indicated low yields in total protein and turbidity as observed in the time course pyrene degradation experiment of *L. adecarboxylata* PS4040 in our previous study (Sarma et al. 2004a).

In summary the metabolites were described for pyrene metabolism and transformation will give a new insight into the understanding of the biochemical process that determine the environmental fate on PAHs by enteric bacteria and this demands further biochemical and genetic studies. As describes in the preceding discussion, this study does not establish

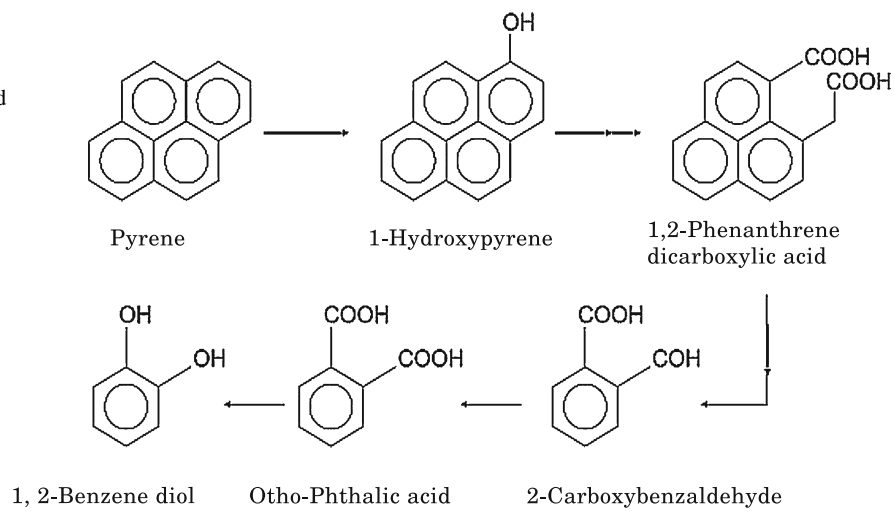


**Fig. 4** Proposed degradation pathway for pyrene by *Leclercia adecarboxylata* PS4040. Simple *arrows* indicate single reaction and *double arrows* indicate two/more step transformation

presence or absence of dioxygenase genes and we are in the process of analyzing the genomic library of the strain *L. adecarboxylata* PS4040 to identify and characterize the degradative enzymes.

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