



ORIGINAL ARTICLE

Elucidation of Pyrene Degradation Pathway in Bacteria

Babita Kumari^a, S.N.Singh^{*a}, Farah Deeba^b, Marisha Sharma^a, Vivek Pandey^b and D.P.Singh^c

^a Environmental science Division, CSIR-National Botanical Research institute, Lucknow

^b Plant Physiology Lab., CSIR-National Botanical Research institute, Lucknow

^c School of Environmental Science, Babasaheb Bhimrao Ambedkar University, Lucknow

*corresponding author Email: drsn06@gmail.com

ABSTRACT

In the present investigation, degradation of a highly persistent PAH, pyrene (100 mg Kg⁻¹) spiked in soil, was studied separately by three PAH degrading bacteria. The results revealed that BP10 could degrade a maximum of 96% of pyrene, followed by P2 (80%) and the least was recorded in PSM11 after 14 days of incubation period. These bacterial strains were also found to produce different types of biosurfactants. Glycolipid was produced by BP10 and PSM11, while P2 produced proteolipid. Although the biosurfactant produced by BP10 and PSM11 was the same, but its emulsification index in benzene and desorption efficiency for pyrene from soil were found higher in BP10 than PSM11. Based on 2D analysis of proteins in BP10, several proteins were found upregulated indicating their direct or indirect role in pyrene degradation. Besides, two new proteins i.e. aldehyde dehydrogenase family protein and isocitrate lyase were also synthesized during pyrene degradation. BP10 followed a single pathway of pyrene degradation mediated through formation of 2-carboxybenzaldehyde, ortho phthalic acid and catechol as intermediates.

Keywords: Pyrene; Bacteria; Catechol dioxygenases; Biosurfactant; Degradation pathway; Proteomics

Received: 14/04/2013 Accepted: 05/05/2013

© 2013 Society of Education, India

INTRODUCTION

Polycyclic Aromatic Hydrocarbons (PAHs) are found in considerable amounts in crude oil and oily effluents of petroleum refineries [1]. Low molecular weight (LMW) PAHs are relatively volatile, high soluble in water and more degradable than high molecular weight (HMW) PAHs [2]. Since HMW PAHs are adsorbed strongly to soils [3], they are highly resistant to microbial degradation due to their low solubility, higher toxicity and toxic metabolite formation, like quinone, during pyrene degradation [4]. Among the HMW PAHs, pyrene is one which is found abundantly in the environment emanating from various sources [5]. Pyrene is a tetracyclic aromatic hydrocarbon with a symmetrical structure and ranked as one of the top 129 hazardous pollutants by the U.S Environmental Protection Agency (USEPA) [6].

In recent years, there has been an increasing interest in the use of microbes to metabolize PAH compounds in soil, as bioremediation technology is considered as more efficient, economical, self-sustainable and versatile alternative to physico-chemical treatments [7]. Several species of bacterial genera *Pseudomonas*, *Alcaligenes*, *Mycobacterium*, *Rhodococcus*, *Corynebacterium*, *Diaphorobacter*, *Pseudoxanthomonas*, *Bacillus vallismortis* etc. have been found highly capable of degrading pyrene [6, 8, 9]. Zhang et al. [10] even reported 97.7% degradation of pyrene with initial concentration of 500 µg g⁻¹ in liquid culture within 5 days of incubation without adding any co-metabolism substrates or surfactants.

In order to enhance desorption of PAHs to facilitate the bioavailability of PAH compounds, many workers used synthetic surfactants [11, 12]. However, in view of increasing reports of toxicity of synthetic surfactants, Liu et al. [13] advocated in favour of using biosurfactant producing bacteria to enhance the availability of oil hydrocarbons and also to facilitate their degradation process.

There are many degradative enzymes like dioxygenase, aldehyde dehydrogenase, putative monooxygenase, hydratase, aldolase, catalase-peroxidase, flavoprotein, phthalate dihydrodiol dehydrogenase etc. which have been reported to be involved in the pyrene degradation [14, 15]. However, the proteomics has been used in recent years for the elucidation of pyrene degradation pathway by *Mycobacterium* sp. based on the identification of induced enzymes in the presence of pyrene as a sole source of carbon and energy [15, 16]. Kim et al. [17] identified a total of 1028 different proteins species,

including 142 proteins that showed upregulation upon exposure of the bacterium to pyrene. The proposed pathway in *Mycobacterium* is initiated by the oxidation of pyrene in the K region by a dioxygenase to form cis-4, 5 pyrene-dihydrodiol. Once 3, 4- dihydroxyphenanthrene is formed, it enters the phenanthrene degradation pathway [15]. During mineralization of pyrene, 4-phenanthrenecarboxylic acid, 4, 5 pyrene dihydrodiol [18] and 4, 5 phenanthrene dicarboxylic [19] were formed as stable intermediates.

In this investigation, pyrene degradation was studied in isolation by three bacterial strains (BP10, PSM11 and P2) in soil. Since BP10 was found to be the highest degrader of pyrene, proteomics was used to trace the pathway of pyrene degradation based on the upregulation of degradative enzymes and also compared to that reported in *Mycobacterium* sp. [15, 16, 17].

MATERIAL AND METHODS

Isolation, screening and identification of hydrocarbon degrading bacterial strains

2 g of petroleum oil, collected from Barauni Oil Refinery, India was added to 100 ml minimal salt medium (MSM) (composition: 7 g dipotassium phosphate, 2 g mono potassium phosphate, 0.5 g sodium citrate, 1 g ammonium sulphate, 0.1 g magnesium sulphate in 1 litre medium pH= 7.0 ± 0.2) and incubated for 7 days in an orbital shaker set at 35°C and 150 rpm. After 1 week of incubation, 1 ml inoculum of bacterial culture was transferred to the flasks having fresh sterilized MSM supplemented with petroleum oil (2% w/v) and incubated in an orbit shaker set at 37°C and 150 rpm for the enrichment of petroleum hydrocarbon degrading bacterial strains. After repeating this procedure for 3 times, the active inoculum was used for the isolation of TPH (Total petroleum hydrocarbon) degrading bacteria, following serial dilution method and spreading over the nutrient agar (composition: 10 g peptic digest of animal tissue, 5 g beef extract, 5 g sodium chloride and 15 g agar in 1 litre medium pH 7.4 ± 0.2) plates.

Ten bacterial strains isolated from petroleum oil along with *Acinetobacter* PSM11 (a hydrocarbon degrading bacterial strain isolated from crude oil contaminated soil of Mathura oil refinery, Mathura, India) were first screened on the basis of their growth in the MSM with pyrene in the range of 25- 200 mg l⁻¹ to serve as a sole source of carbon and energy, separately. Two strains i.e. coded as BP10 and P2 isolated from petroleum oil of Barauni oil refinery and *Acinetobacter* PSM11 were selected based on their highest growth for higher dose of pyrene (200 mg l⁻¹).

Selected strains were identified by M/S Chromous Biotech, Bengaluru (India) on the basis of 16S rDNA technology using ABI 3130 Genetic Analyzer and their homology (>99%) with DNA sequence of NCBI databases of bacteria. 5' AGAGTRTGATCMTYGCTWAC -3' and 5'-CGYTAMCTTWTACGRCT-3' were used as 16s forward primer and 16s reverse primer, respectively. The sequencing reaction (10 µl) contained 4 µl Big Dye Terminator Ready reaction mixture, 1 µl of 100 ng µl⁻¹ Template, 2 µl of 10 pmol µl⁻¹ primer and 3 µl Milli Q water and PCR conditions (25 cycles) were: Initial Denaturation : 96°C for 1min, Denaturation : 96°C for 10 sec, Hybridization : 50°C for 5 sec and Elongation : 60°C for 4 min.

Biosurfactant production and their biochemical characterization

All three bacterial strains were grown separately in the nutrient medium (1 l) in 2 l conical flasks for 5 days in an orbital shaker set at 150 rpm and 35°C. Medium was centrifuged at 10,000 x g for 20 min to remove the bacterial cells. Cell free medium was acidified up to pH 2.0 with 6M HCl. Acidified medium was kept at 4°C to precipitate out the crude biosurfactant. Precipitate was collected as pellet by centrifugation at 10,000 rpm for 20 min. The pellet was resuspended in 0.1 M NaHCO₃ solution. Pure biosurfactant was extracted by a mixture of chloroform and ethanol (2:1 v/v, CHCl₃-C₂H₅OH) at room temperature [20].

To determine the protein and/ or carbohydrate moiety of extracted biosurfactants, biosurfactants were qualitatively analyzed by TLC method. The biosurfactants extracted in mixture of organic solvents were spotted on TLC paper (Merk: Silica gel 60 F254, 20 x 20 cm). A mobile phase of 500ml was prepared with chloroform, methanol, acetic acid and water in the ratio of 25:15:4:2 v/v. After air drying, the spots were stained by spraying ninhydrin solution (0.2% w/v) to confirm the presence of free amino groups, while carbohydrate moiety was detected as black spot by dipping TLC plate in AgNO₃ for 5 min, followed by an alkaline MeOH solution for 30 min [21].

Emulsification index, surface tension and desorption capacity of biosurfactants

To evaluate the efficiency of biosurfactants produced by different bacterial strains, their emulsification index and desorption capacity of pyrene (100 µg g⁻¹ spiked in soil) were determined. For this purpose, 5 ml of benzene or toluene was added to 5 ml of aqueous phase containing 500 mg l⁻¹ biosurfactant and agitated vigorously for 5 min on a vortex. After 24 h, total height of both organic and aqueous phase (H_T) and height of emulsified layer (H_E) were recorded. Emulsification index was calculated as following:

$$E_{24} (\%) = (H_E / H_T) \times 100$$

Amount of pyrene desorbed from soil (spiked 100 $\mu\text{g g}^{-1}$ of soil) in the presence of isolated biosurfactants (500 $\mu\text{g ml}^{-1}$) in 20 mM phosphate buffer (pH 7.0) was determined by HPLC.

Tagging of bacterial strains against antibiotics

As soil was not to be autoclaved for sterilization in experiment of degradation study of pyrene in soil because of two reasons; (i) heat decreases the soil nutrition value, hence doesn't support bacterial growth and (ii) autoclaving is not possible in the field application. Hence, bacterial strains were tagged with antibiotics to know the survivability of inoculated bacterial strains in the soil (unsterilized) during experiment. BP10 and P2 were tagged with vancomycin (200 $\mu\text{g ml}^{-1}$), while PSM11 was tagged with ampicillin (100 $\mu\text{g ml}^{-1}$). Tagging of bacteria was performed by growing them separately on NA plate supplemented with different antibiotics with increasing concentration of vancomycin and ampicillin. Final concentration of antibiotics for tagging for each bacterial was decided when the native bacteria of soil could not grow NA plate for that dose of selected antibiotics.

Experimental setup

Garden soil, collected from National Botanical Research Institute, Lucknow, U.P. (India) was air dried, sieved through 2 mm sieve. The pH, TOC, available phosphorus and bacterial biomass of soil were found 7.39, 0.57 %, 0.5 g kg^{-1} and 1.4×10^5 bacterial count g^{-1} of soil, respectively. Soil was spiked with pyrene (100 $\mu\text{g g}^{-1}$ of soil) by adding pyrene with acetone and mixed by being vigorously stirred and left in dark place for removal of acetone for 24 h. Sixty gram of spiked garden soil was uniformly spread over each petri dish (n=12). Three selected bacterial strains, BP10, P2 and PSM11, were enriched in 100 ml NB (composition: 5g peptic digest of animal tissue, 5 g sodium chloride, 1.5 g beef extract and 1.5 g yeast extract in 1 l medium pH 7.4) enriched with 100 mg l^{-1} pyrene in 250 ml Erlenmeyer flask in an orbital shaker set for 15 h at 37°C and 150 rpm. Cells were harvested by centrifugation at 5000 x g for 10 min to get pellets of intact bacterial cells. Subsequently, cell pellets were washed with sterilized double distilled water (DDW) to remove nutrients and pyrene of the media. Cell pellets were resuspended in sterilized DDW and vortexed properly before they were inoculated in spiked soil in petri dishes. The bacterial inoculum was maintained in the range of $6-7 \times 10^5$ CFU g^{-1} of soil. Three petri plates were kept without any bacterial inoculum to study natural degradation of pyrene in soil to serve as control.

All the petri plates were kept in culture room at 37°C for 28 days. 5 g soil samples from each set were initially taken out at zero day and then at the interval of 7 days till 28 days of incubation period to study bacterial degradation of pyrene. Simultaneously, growth of bacteria in terms of CFU, protein content and soil pH were also monitored. Induction of catabolic enzymes particularly catechol 1, 2 dioxygenase and catechol 2, 3 dioxygenase was also investigated in relation to pyrene degradation.

Extraction and analysis of pyrene

One gram of spiked soil with 10 ml benzene was kept for shaking in an orbital shaker at 180 rpm for 24 h. Benzene extracted sample was collected after centrifugation at 10,000 x g for 10 min. Solvent was evaporated under a gentle nitrogen hood and residue was dissolved in acetonitrile. Prepared sample was analyzed for remaining pyrene and its degradation products using HPLC (Ultimate 3000), having C18 column (25 cm x 4.6 mm x 5 μm) and by applying linear gradient of acetonitrile (50-85% in 35 min) in HPLC grade water at constant flow of 1.5 ml min^{-1} . PAH peaks were detected at 230 nm using an UV detector.

Bacterial growth

Bacterial growth was assessed by CFU counting. CFU g^{-1} of BP10 and P2 in soil was determined on NA plates supplemented with vancomycin (250 mg l^{-1}) and of PSM11 with ampicillin (100 mg l^{-1}) after each 7 days interval. Colonies were counted on NA plates after incubation for 48 h at 37°C in the culture cabinet.

Protein extraction and estimation

Soil protein was extracted in 1N NaOH solution by following the procedure of Rahman *et al.* [22]. 1 g of soil was suspended in 10 ml of distilled water and mixed vigorously on vortex. After settling the soil particles, 1 ml of supernatant was centrifuged at 13000 rpm for 10 min. Then after, supernatant was discarded and the pellet was dissolved in 1 ml of 1N NaOH. It was kept for 3 min in water bath at boiling temperature for cell lysis. 1 ml of 1M H_3PO_4 was added after samples were cooled to room temperature (25°C). Sample, thus prepared, was used for the protein estimation and enzyme assay. Protein was estimated by following the method of Lowry *et al.* [23].

Enzyme assay

Catechol 1, 2 dioxygenase activity

Catechol 1, 2 dioxygenase (C120, EC 1.13.11.1) was assayed by the method of Hegeman [24] using the colorimetric procedure to detect the product *cis-cis* muconate (pH = 7.0, $\lambda = 260 \text{ nm}$; $\epsilon = 25600 \text{ mol}^{-1}\text{cm}^{-1}$). Reaction mixture contained 1 μmol of EDTA, 0.1 μM of catechol, 8.7 μM of sodium phosphate buffer (pH=7.0) and protein sample (0.02-0.06 mg of protein) in a final volume of 1 ml. The increase in OD_{260} was used as a measure for accumulation of *cis, cis* muconic acid.

Catechol 2, 3 dioxygenase activity

Catechol 2, 3 dioxygenase (C230, EC 1.13.11.2) was determined with an increase in OD₃₇₅ concomitant with the formation of 2-hydroxymuconic semialdehyde (pH = 7.5, $\lambda = 375$ nm; $\epsilon = 33400$ mol⁻¹cm⁻¹) using UV-VIS Spectrophotometer. Reaction mixture contained 48 μ M of sodium phosphate buffer (pH = 7.5), 0.1 μ M catechol and protein extract (0.02-0.06 mg of protein) in a final volume of 1 ml. Protein samples were heated for 10 min at 60°C before the enzyme activity was assayed following the method of Klecka and Gibson [25].

Elucidation of degradation pathway of pyrene by BP10 through proteomics

BP10 was grown in 150 ml of sterilized MSM in two conical flasks of 250 ml for one week in an orbital shaker set at 150 rpm and 35°C. One flask was supplemented with 100 mg l⁻¹ of pyrene to be termed as treated, while as another one without pyrene serve as control. After 1 week of incubation period, cell pellets were harvested through centrifugation at 10,000 x g for 10 min and washed with saline water (0.85% NaCl) thrice and then resuspended in extracting buffer pH 8.0 (10mM Tris HCl, 1mM EDTA and 1mM PMSF) and finally sonicated at 50% amplitude with pulse interval of 0.5 s for 15 min.

Two-DE and Gel staining

Two-dimensional electrophoresis (2-DE) was carried out as described by Lehesranta et al. [26] but with some modifications. Iso electric focusing (IEF) was conducted at 20°C with an Ettan IPGphor-3 (GE Healthcare) on 7 cm IPG strip (pH 4-7, GE Healthcare). The focused strips were equilibrated twice for 15 min in 10 ml of equilibration solution. The first equilibration was performed in a solution containing 6M urea, 30% w/v glycerol, 2% w/v sodium dodecyl sulphate (SDS), 1% w/v DTT and 50mM Tris HCl buffer, pH 8.8, while the second equilibration was performed in a solution with 2.5% w/v idoacetamide. The operating conditions were: 250 V for 30 min, 450 V for 15 min, 750 V for 15 min, and 2000 V for 30 min and 8000 V for 2 h for a total of 15 kWh. The second dimension was run in Hoefer mini-gel apparatus in 7-8 cm homogeneous 12% SDS-PAGE gels. Electrophoresis was performed in a standard Tris Glycine running buffer at a constant voltage of 200 V. Gels were stained in Sypro-Ruby and gel images were acquired with the Typhoon 9200 scanner (GE Healthcare) The data was analyzed using Image Master 2D Platinum 6.0 software (Amersham Bioscience) [27].

Protein identification

Tryptic digestion of the protein spots excised from the gels and samples were prepared according to Koistinen et al. [28].

MS and MS/MS

A 4800 Proteomics Analyzer (Applied Biosystems) with TOF/ TOF optics was used for all MALDI-MS and MS/MS applications. Samples were prepared by mixing 0.5 ml of sample with 0.5 ml of matrix solution (5 mg ml⁻¹ of α -Cyano-4-hydroxycinnamic acid in 50% ACN containing 0.1% TFA) and spotted on stainless steel 384 well target plate [27]. For MS/MS experiments, the instrument was externally calibrated with fragment of Glufibrino- peptide B. The mono isotopic peptide masses obtained from MALDI-TOF were analyzed by the 4000 Series Explorer software version 3.5 (ABI). On the basis of mass signals, protein identification was performed through Mascot software (<http://www.matrixscience.com>) against Swiss Prot, NCBI nr and MSDB protein databases.

RESULTS**Isolation and identification of pyrene utilization bacterial strains**

Ten bacterial strains were isolated from petroleum oil of Barauni Oil Refinery, Barauni, Bihar (India). Among these isolates, two bacterial strains coded as BP10 and P2 and *Acinetobacter* PSM11 showed faster growth with the increasing pyrene concentration (25-200 mg l⁻¹). Therefore, these three strains were selected for study of pyrene degradation in soil.

Bacterial strains P2 and BP10 were found as *Ochrobactrum* sp.P2 Accession No. KC493414 and *Pseudomonas* sp. BP10, KC493413, respectively, based on 16S rDNA technology (Chromous Biotech, India).

Nature of biosurfactants produced by selected bacterial strains

Analysis of biosurfactants produced by three bacterial strains based on TLC indicated that the biosurfactants produced by BP10 and PSM11 were glycolipid, while that synthesized by P2 was proteolipid in nature.

Emulsification index of biosurfactants produced by BP10, P2 and PSM11 was found 75%, 53% and 35% with benzene and 60%, 50% and 55% with toluene, respectively. Further, desorption of pyrene from soil in presence of biosurfactants produced by BP10, P2 and PSM11 was found to be 35%, 22% and 15%, respectively.

Degradation of Pyrene in soil

Degradation of pyrene ($100 \mu\text{g g}^{-1}$) spiked in soil was studied separately by three bacterial strains isolated from the crude oil contaminated soil. It was noted that the bacterial strains P2, BP10 and PSM11 could degrade pyrene between 33-42% after incubation of 7 days, showing no significant difference in their ability for pyrene degradation (Fig. 1). However, as the incubation period increased, rate of pyrene degradation was enhanced exponentially and significantly varied among the bacterial strains. After 14 days of incubation, BP10 metabolized pyrene by 96%, followed by P2 (80%) and the least (75%) was recorded in PSM11 after deducting the contribution made by the bacteria in the control. At the end of 28 days of incubation period, although the degradation rate was slowed down, but pyrene degradation reached to 98% in both BP10 and P2, but only 94% degradation was recorded in PSM11. On the other hand, natural degradation of pyrene by native microbes in control was recorded 3%, 17%, 30% and 44% for the incubation period of 7, 14, 21 and 28 days, respectively, which was found significantly lower than the bacterial strains added.

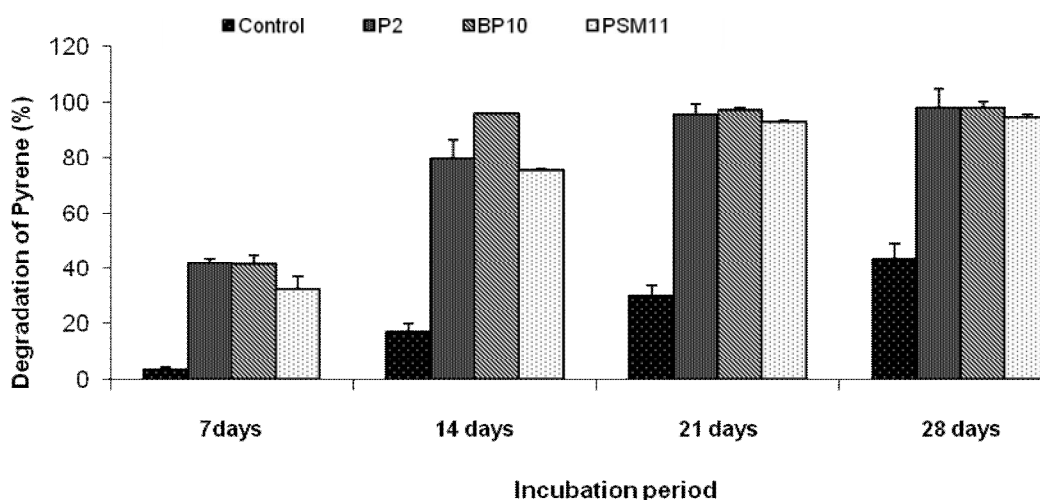


Fig. 1. Degradation (%) of pyrene in spiked soil by bioaugmented bacterial strains and native microbes (control).

Growth of inoculated bacterial strains

At the time of inoculation, CFU of bacterial cell feed was about $6-7.1 \times 10^5 \text{ g}^{-1}$ of soil. Subsequently, it was observed that individual bacterial strains P2, BP10 and PSM11 continued to multiply till 14 days of incubation (Fig. 2a) and then declined, indicating utilization of pyrene as a sole source of carbon and energy. The maximum growth ($5.26 \times 10^7 \text{ CFU g}^{-1}$ of soil) was recorded for BP10, followed by P2 ($1.97 \times 10^7 \text{ CFU g}^{-1}$ of soil) and minimum ($1.72 \times 10^7 \text{ CFU g}^{-1}$ of soil) was noted for PSM11 after 14 days of incubation.

Bacterial growth was also evidenced by increased protein content of the bacterial cells. The cell protein content continued to increase in all bacterial strains for 14 days of incubation and then declined progressively with increasing incubation period (Fig. 2b). The protein content was recorded maximum ($4.87 \pm 0.16 \text{ mg g}^{-1}$) in BP10, followed by P2 ($3.65 \pm 0.57 \text{ mg g}^{-1}$) and minimum ($1.9 \pm 0.04 \text{ mg g}^{-1}$) was found in PSM11 after 14 days of incubation. Increasing and decreasing trend of cell protein corresponded to the CFU values of individual bacterial strains through out the incubation periods.

Activity of catechol dioxygenases

When the activity was assayed for two enzymes catechol 1, 2 dioxygenase (C120) and catechol 2, 3 dioxygenase (C230) usually involved in PAH degradation, it was observed that the maximum activity of ($444.12 \pm 18.8 \mu\text{mol mg}^{-1} \text{ min}^{-1}$) C120 was found in a bacterial strain BP10, followed by P2 ($313.95 \pm 15.35 \mu\text{mol mg}^{-1} \text{ min}^{-1}$) and the least was observed in PSM11 ($232.25 \pm 14.18 \mu\text{mol mg}^{-1} \text{ min}^{-1}$) after 14 days of incubation period. However, the minimum activity ($55.86 \pm 37.7 \mu\text{mol mg}^{-1} \text{ min}^{-1}$) of C120 was noted in the control during the same incubation period (Fig. 3a). On the contrary, maximum activity of catechol 2, 3 dioxygenase (C230) was recorded in PSM11 ($77.19 \pm 0.08 \mu\text{mol mg}^{-1} \text{ min}^{-1}$), followed by P2 ($39.85 \pm 2.5 \mu\text{mol mg}^{-1} \text{ min}^{-1}$) and minimum ($26.28 \pm 2.34 \mu\text{mol mg}^{-1} \text{ min}^{-1}$) was noted in BP10 (Fig. 3b).

This indicated that with the use of pyrene as a substrate, the catechol 1, 2 dioxygenase activity was enhanced many folds as compared to control. However, activity of catechol 2, 3 dioxygenase was very low as compared to catechol 1, 2 dioxygenase in all three bacterial strains. This evidences that in the

degradation process of pyrene, catechol 1, 2 dioxygenase played a major role in pyrene degradation in comparison to catechol 2, 3 dioxygenase.

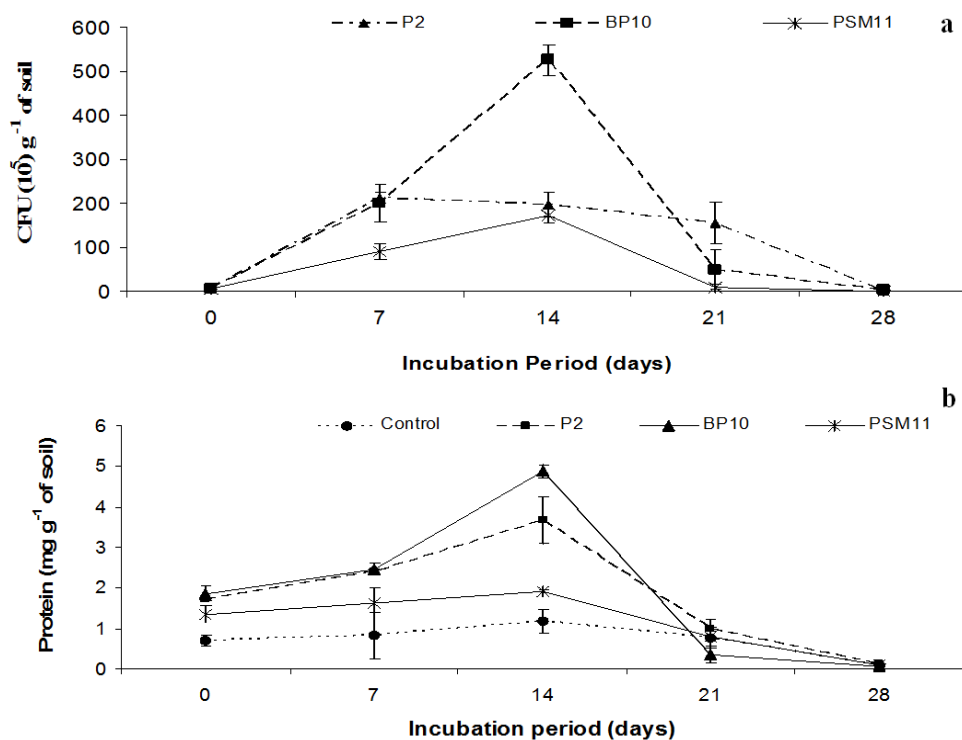


Fig. 2. Growth (CFU 10^5 g $^{-1}$ of soil) of bioaugmented bacterial strains (a) and soil protein (mg g $^{-1}$ of soil) at different incubation periods (b).

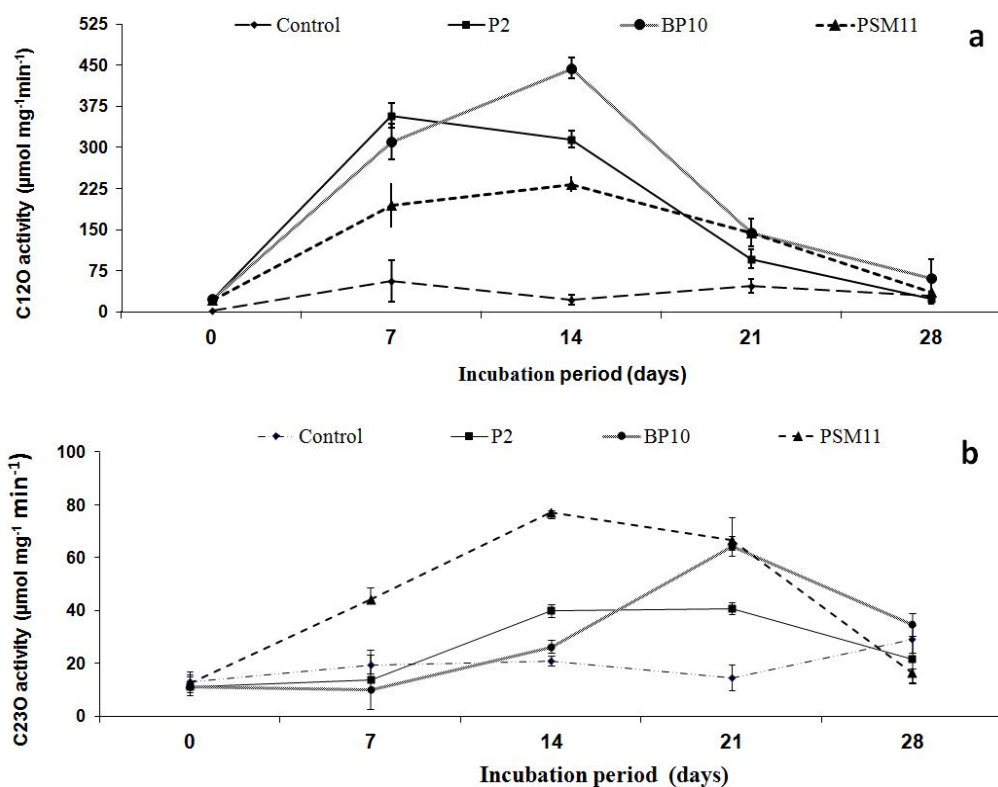


Fig. 3. Activity of Catechol 1, 2 dioxygenase (a) and catechol 2, 3 dioxygenase (b) in μ mol mg $^{-1}$ of soil protein in presence of bacterial strains during pyrene degradation.

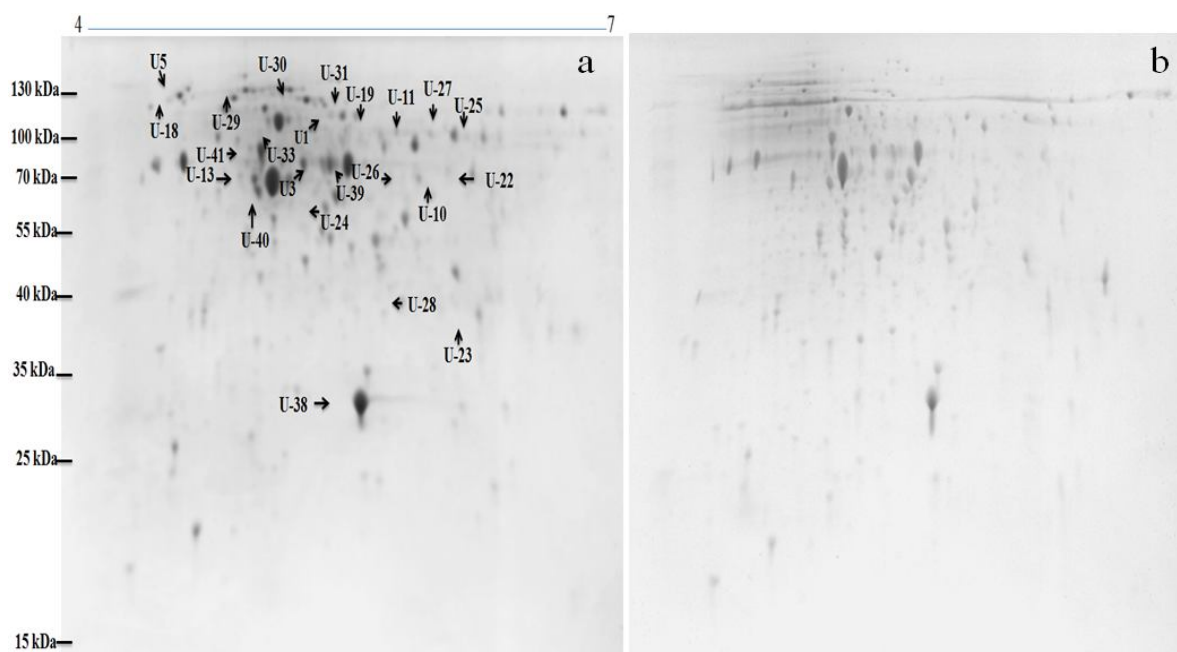


Fig. 4. Two-dimensional gel of proteins induced by BP10 in presence (a) and absence (B) of pyrene. The arrows indicate upregulated and downregulated proteins identified by mass spectrometry.

Study of proteomics in pyrene degradation

BP10 produced various proteins when incubated in presence of pyrene (100 mg l^{-1}) in MSM. Among them, a few were upregulated) as compared to control (without pyrene), while others were either downregulated or remained unchanged (Fig 4). The upregulated proteins are elongation factor Tu (spot no: U3), surface antigen family outer membrane protein (spot no: U5), serine hydroxymethyl transferase (spot no: U10), FOF1 ATP synthase subunit alpha (spot no: U11), porin (spot no: U13), TonB-dependent ferric siderophore receptor (spot no: U18), adenylosuccinate synthetase (spot no: U19), serine hydroxymethyl transferase (spot no: U22), alkyl hydroperoxide reductase subunit C (spot no: U23), succinyl-CoA synthetase subunit beta (spot no: U24), acetyl-CoA hydrolase (spot no: U25), 3-oxoacyl-(acyl carrier protein) synthase I (spot no: U26), dihydrolipoamide dehydrogenase (spot no: U27), succinate dehydrogenase iron-sulfur subunit (spot no: U28), TonB-dependent siderophore receptor (spot no: U29), TonB-dependent receptor plug (spot no: U30), chaperonin GroEL (spot no: U33), outer membrane protein H1 (spot no: U38), porin (spot no: U40) and FOF1 ATP synthase subunit beta (spot no: U41). Besides, two new proteins were also found i. e. aldehyde dehydrogenase family protein (spot no: U1) and isocitrate lyase (spot no: U31). Aldehyde dehydrogenase family proteins are 1-hydroxy-2-naphthaldehyde dehydrogenase and 2-carboxybenzaldehyde dehydrogenase which catalyse the formation of 1-hydroxy-2-naphthoate from 1-hydroxy-2-naphthaldehyde and O-phthalate from 2-carboxy benzaldehyde. In addition, catechol was also formed from protocatechuate following the vanillin pathway. In the presence of both catechol 1, 2 dioxygenase and catechol 2, 3 dioxygenase, pyruvate, acetaldehyde and *cis,cis* muconate finally entered the TCA cycle. Upregulated enzymes like succinyl coA synthetase, succinate dehydrogenase, acetyl coA hydrolase and dihydro lipoamide dehydrogenase (part of two enzyme complex i. e. pyruvate dehydrogenase and α - keto glutarate dehydrogenase) are also associated with TCA cycle which can enhance metabolic activity. Another new enzyme isocitrate lyase involved in glyoxylate cycle forms glyoxylate from isocitrate. Similarly, upregulated proteins like 3-oxoacyl sythetase 1, serine hydrooxy methyl transferase, adenylosuccinate synthetase and FOF1 ATP synthase are involved in fatty acid metabolism, amino acid biosynthesis, purine biosynthesis and ATP synthesis, respectively. Upregulation of porin protein could boost the uptake of substrate by bacterial cell through enhanced diffusion process. Elongation factor Tu and chaperonin GroEL may be correlated with growth of bacteria as they are involved in translation and post translational protein folding, respectively. Based on these identified proteins, it may be concluded that bacterial strain BP10 followed the pathway of pyrene degradation as illustrated in Fig 5. Pyrene degradation followed the phenanthrene pathway as it was converted in to 3, 4 dihydroxy phenanthrene and then 1 hydroxy-2 naphthaldehyde which was further oxidized to 2 carboxy benzaldehyde. Like *Mycobacterium*, BP10 also followed O-phthalate pathway for further mineralization of pyrene.

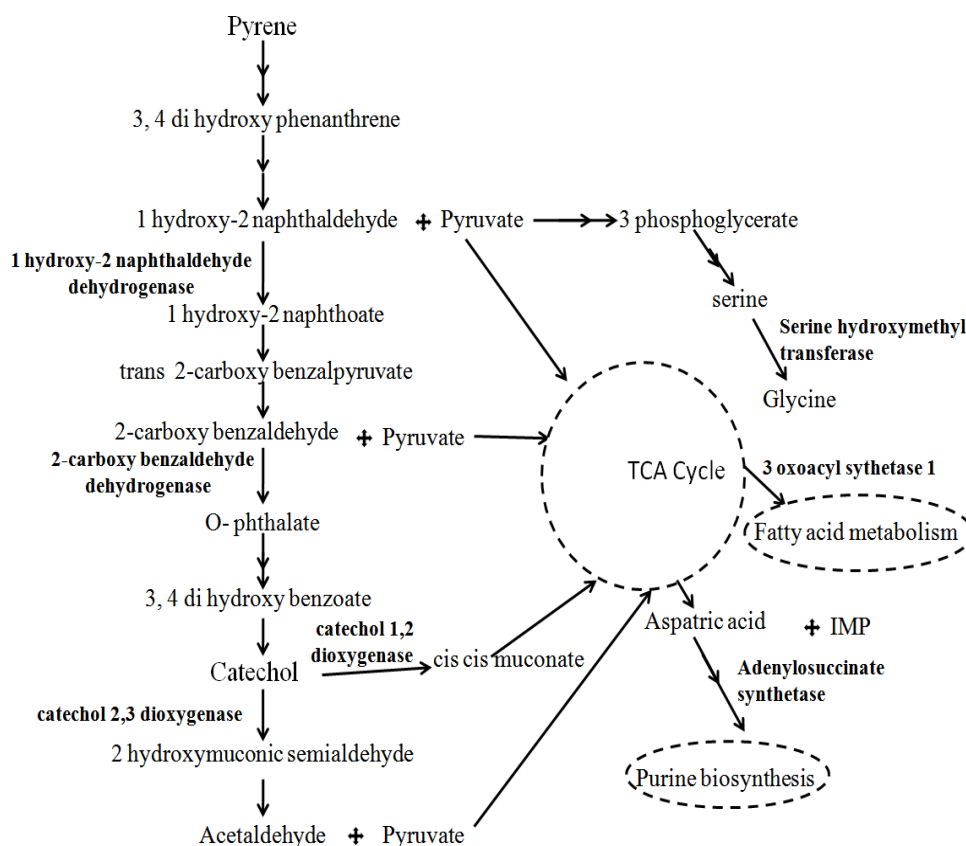


Fig. 5. Elucidation of pyrene degradation pathway in *Pseudomonas* sp. BP10 showing the involvement of degradative enzymes based on our proteomics study.

DISCUSSION

Kafilzadeh et al. [6] isolated different bacterial species like *Mycobacterium* sp., *Corynebacterium* sp., *Nocardia* sp., *Pseudomonas* sp., *Rhodococcus* sp. and *Micrococcus* sp. which were capable of degrading pyrene by 89.1%, 79.4%, 75.3%, 68.2%, 62.3% and 56.8%, respectively, after 10 days of incubation. Sarma et al. [5] also observed 61.5% degradation of pyrene by an enteric bacterium, *Leclercia adecarboxylata* PS4040 within 20 days when used as a sole source of carbon and energy. Cheung and Kinkle [29] observed the higher initial rates of pyrene degradation with shortest acclimation periods when soils were pre-exposed with phenanthrene or a mixture of phenanthrene and pyrene for 14 weeks. However, in our case, all three strains were able to degrade pyrene between 75-96 % in 14 days incubation without any pre-exposure with pyrene or phenanthrene. In naturally contaminated soil, a large amount of pyrene ($54.14 \pm 7.43\%$) was mineralized after 2 months incubation which was significantly higher ($P < 0.05$) than that of autoclaved control i.e. $19.07 \pm 0.20\%$ [30]. This clearly indicates the role of native bacteria in PAH degradation at the contaminated sites.

Degradative enzymes catechol 1, 2 dioxygenase and catechol 2, 3 dioxygenase are actively involved in PAH degradation pathway. Cenci and Caldini [31] also observed about 100 times higher activity of C120 than C230 during degradation of various PAH compounds by *Pseudomonas fluorescens*. However, Dhote et al. [32] and Kumar et al. [33] reported that meta cleavage by C230 was a predominant process of degradation of chrysene and fluoranthene, respectively.

Biosurfactants produced by microbes have a diverse group of surface active chemical compounds which include lipopeptides, synthesized by many species of *Bacillus*, glycolipids synthesized by *Pseudomonas* sp., and polysaccharide-lipid complexes, synthesized by *Acinetobacter* sp. [34]. Shavandi et al. [35] observed that the biosurfactant concentration became sufficient for micelle formation after 36 h when *Rhodococcus* sp. strain TA6 was inoculated in basal salt medium supplemented with 1% kerosene oil. Abouseoud et al. [36] observed that the biosurfactant produced by *Pseudomonas fluorescens* was stable even at 120°C and in a range of pH 4-9 with no significant effect on the surface activity of the culture.

Detailed investigation on pyrene degradation based on proteomics has been only reported in *Mycobacterium* sp. [16, 37]. Hussain [38] reported pyrene mineralization in *Pseudomonas fluorescens* 29L via formation of salicylate and catechol. According to him, the commonly identified pathways in PAH biodegradation include the ortho- and meta- cleavage pathways, gentisate pathways, o-phthalate

pathway, β ketoadipate pathway, and the upper and lower pathways. The o-phthalate pathway involves the formation of o-phthalate and its transformation to other intermediates, usually 4,5-dihydroxyphthalate which is further degraded to 3,4-dihydroxybenzoic acid by the enzyme 4,5-dihydroxyphthalate decarboxylase [39]. In the proposed metabolic pathway for the degradation of pyrene by *Mycobacterium* sp., Krivobok et al. [15] reported involvement of 15 enzymes in pyrene degradation. Some of the enzymes are common for the degradation of other PAHs also.

Pyrene degradation by *Mycobacterium* sp. was also extensively studied by Heitkamp et al. [18]. This bacterium could start the degradation of pyrene either via initial dioxygenation at C1 and C2 position or C4 and C5 position and formed pyrene 1-2 diol [14] and pyrene cis-4, 5-dihydrodiol [18] as initial degradation products. A variety of substituted benzene ring compounds were also formed as intermediates and end products from pyrene degradation in bench studies. These include the formation of 2-carboxybenzaldehyde and β ketoadipyl CoA as intermediates and 1, 2-dimethoxypyrene as end product in *Mycobacterium vanbaleeni* PYR-1 [17]. However, Sarma et al. [5] reported formation of different metabolites like hydroxypyrene, 1-2 phenanthrene dicarboxylic acid, 2 carboxybenzaldehyde and ortho phthalic acid during the pyrene degradation by an enteric bacterium *Leclercia adecarboxylata* PS4040.

Based on this study, it may be concluded that all three bacterial strains BP10, P2 and PSM11 were found to be potential degraders of pyrene spiked in soil. Further, higher activity of Catechol 1, 2 dioxygenase than Catechol 2, 3 dioxygenase indicates that ortho cleavage is a major route for pyrene initial degradation. However, the proteomics analysis revealed upregulation of many other enzymes in BP10 which played important role at different stages of pyrene degradation.

ACKNOWLEDGEMENT

Dr. S. N. Singh and Mrs. Babita Kumari are thankful to Director, CSIR National Botanical Research Institute, Lucknow for his active support and CSIR for financial support to the project NWP019.

REFERENCES

1. Seo, J.S., Keum, Y.S. & Li, Q.X. (2009). Bacterial degradation of aromatic compounds. *Int. J. Environ. Res. Public Health*. 6:278-230.
2. Cerniglia, C.E. (1990). The use of *Mycobacterium* sp. in the remediation of polycyclic aromatic hydrocarbon wastes. In: Biological remediation of contaminated sediments, with special emphasis on the Great Lakes. Jafvert, C.T. and Rogers, J.E., Office of Research and Development U.S. Environmental Protection Agency, Washington, D.C. P. 91-107.
3. Means, J.C., Wood, S.G., Hassett, J.J. & Banwart, W.L. (1980). Sorption of polynuclear aromatic hydrocarbons by sediments and soils. *Environ. Sci. Technol.* 14: 1524-1528.
4. Bolton, J.L., Trush, M.A., Penning, T.M., Dryhurst, G. & Monks, T.J (2000). Role of quinones in toxicology. *Chem. Res. Toxicol.* 13:135-160.
5. Sarma, P.M., Duraja, P., Deshpande, S. & Lal, B. (2010). Degradation of pyrene by an enteric bacterium, *Laclercia adecarboxylata* PS4040. *Biodegrad.* 21(1): 59-69.
6. Kafilzadeh, F., Hoshyaripour, F., Tahery, Y. & Azad, H.N. (2012) Bioremediation of pyrene by isolated bacterial strains from the soil of the landfills in Shiraz (Iran) *Ann Biol Res* 3 (1): 486-494.
7. Margesin, R. & Schinner, F. (1998). Low-temperature bioremediation of a waste water contaminated with anionic surfactants and fuel oil. *Appl. Microbiol. Biotechnol.* 49:482-486.
8. Klankeo, P., Nopcharoenkul, W. & Pinyakong, O. (2009). Two novel pyrene-degrading *Diaphorobacter* sp. and *Pseudoxanthomonas* sp. isolated from soil. *J. Biosci. Bioeng.* 108(6): 488-495.
9. Ling, J., Zhang, G., Sun, H., Fan, Y., Ju, J. & Zhang, C. (2011). Isolation and characterization of a novel pyrene-degrading *Bacillus vallismortis* strain JY3A. *Sci. Total Environ.* 409: 1994-2000.
10. Zhang, G.Y., Ling, J.Y., Sun, H.B., Luo, J., Fan, Y.Y. & Cui, Z.J. (2009). Isolation and characterization of a newly isolated polycyclic aromatic hydrocarbons-degrading *Janibacter anophelis* strain JY11. *J. Hazard. Mater.* 172:580-586.
11. Doong, R. & Lei, W. (2003). Solubilization and mineralization of polycyclic aromatic hydrocarbons by *Pseudomonas putida* in the presence of surfactant. *J. Hazard. Mater.* 96(1):15-27.
12. Li, J.L. & Chen, B.H. (2009). Effect of non-ionic surfactants on biodegradation of phenanthrene by a marine bacteria of *Neptunomonas naphthovorans*. *J. Hazard. Mater.* 162(1):66-73.
13. Liu, Y.C., Li, L.Z., Wu, Y., Tian, W., Zhang, L.P., Xu, L., Shen, Q.R. & Shen, B. (2010). Isolation of alkane-degrading *Alcanivorax* sp. strain 2B5 and cloning of alkB gene; *Bioresour. Technol.* 101: 310-316.
14. Kim, Y.H., Moody, J.D., Freeman, J.P., Engesser, K.H. & Cerniglia, C.E. (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.
15. Krivobok, S., Kuony, S., Meyer, C., Louwagie, M., Willison, J.C. & Jouanneau, Y. (2003). Identification of Pyrene-induced proteins in *Mycobacterium* sp. Strain 6PY1: evidence for two ring hydroxylating dioxygenases. *J. Bacteriol.* 185(13): 3828-3841.

16. Liang, Y., Gardner, D.R., Miller, C.D., Chen, D., Anderson, A.J., Weimer, B.C. & Sims, R.C. (2006) Study of biochemical pathways and enzymes involved in pyrene degradation by *Mycobacterium* sp. strain KMS. *Appl. Environ. Microbiol.* 72(12): 7821-7828.
17. Kim, S.J., Kweon, O., Jones, R.C., Freeman, J.M., Edmondson, R.D. & Cerniglia, C.E. (2007). Completed and integrated pyrene degradation pathway in *Mycobacterium vanbaalenii* PYR-1 based on systems biology. *J. Bacteriol.* 189(2):464-472.
18. Heitkamp, M.A., Freeman, J.P., Miller, D.W. & Cerniglia, C.E. (1988). Pyrene degradation by a *Mycobacterium* sp.: identification of ring oxidation and ring fission products. *Appl. Environ. Microbiol.* 54: 2556-2565.
19. Cerniglia, C.E. (1992). Biodegradation of polycyclic aromatic hydrocarbons. *Biodegrad.* 3: 351-368.
20. Pansiripat, S., Pornsunthorntawe, O., Rujiravanit, R., Kitiyanan, B., Somboonthanate, P. & Chavadej, S. (2010). Biosurfactant production by *Pseudomonas aeruginosa* SP4 using sequencing batch reactor: Effect of oil- to glucose ratio. *Biochem. Engineer. J.* 49: 185-191.
21. Han, N.S. & Robyt, J.F. (1998). Separation and detection of sugars and alditols on thin layer chromatograms. *Carbohydrate Res.* 313(2): 135-7.
22. Rahman, K.S.M., Rahman, T.J., Banat, I.M., Lord, R. & Street, G. (2007). Bioremediation of petroleum sludge using bacterial consortium with biosurfactant. In: Singh SN, Tripathi RD (Eds), *Environmental Bioremediation Technologies*, Springer, Germany, p. 391-408.
23. Lowry, O.H., Roserbrough, N.T., Farr, A.L. & Randall, R.J. (1951). Protein measurement with folin phenol reagent. *J. Biol. Chem.* 193: 265-75.
24. Hegeman, G.D. (1966). Synthesis of the enzymes of the mandelate pathway by *Pseudomonas putida*. I. Synthesis of the enzymes in the wild type. *J. Bacteriol.* 91: 1140-1154.
25. Klecka, G.M., Gibson, D.T. (1981). Inhibition of catechol 2, 3-dioxygenase from *Pseudomonas putida* by 3-Chlorocatechol. *Appl. Environ. Microbiol.* 41(5): 1159-1165.
26. Lehesranta, S.J., Davies, H.V., Shepherd, L.V.T., Nunan, N., McNicol, J.W., Auriola, S., Koistinen, K.M., Suomalainen, S., Kokko, H.I., Kärenlampi, S.O. (2005). Comparison of tuber proteomes of potato varieties, landraces, and genetically modified lines. *Plant Physiol.* 38: 1690 - 1699.
27. Deeba, F., Pandey, A.K., Ranjan, S., Mishra, A., Singh, R., Sharma, Y.K., Shirke, P.A. & Pandey, V. (2012). Physiological and proteomic response of cotton (*Gossypium herbaceum* L.) to drought stress. *Plant Physiol. Biochem.* 53: 6-18.
28. Koistinen, K.M., Hassinen, V.H., Gynther, P.A.M., Lehesranta, S.J., Keinänen, S.I., Kokko, H.I., Oksanen, E.J., Tervahauta, A.I., Auriola, S. & Kärenlampi, S.O. (2002). Birch PR- 10c is induced by factors causing oxidative stress but appears not to confer tolerance to these agents. *New Phytol* 155: 381-391.
29. Cheung, P.Y. & Kinkle, B.K. (2005) Changes in *Mycobacterium* spp. population structure and pyrene mineralization in polycyclic aromatic hydrocarbon-amended soils. *Soil Biol. Biochem.* 37: 1929-1937.
30. Zeng, J., Lin, X., Zhang, J. & Li, X. (2010). Isolation of polycyclic aromatic hydrocarbon (PAHs)-degrading *Mycobacterium* spp. and the degradation in soil. *J. Hazard. Mater.* 183(1-3): 718-723.
31. Cenci, G. & Caldini, G. (1997). Catechol dioxygenase expression in a *Pseudomonas fluorescens* strain exposed to different aromatic compounds. *Appl. Microbiol. Biotechnol.* 47:306-308.
32. Dhote, M., Juwarkar, A., Kumar, A., Kanade, G.S. & Chakarbarti, T. (2010). Biodegradation of chrysene by the bacterial strains isolated from oily sludge. *World J Microbiol. Biotechnol.* 26(2): 329-335.
33. Kumar, S., Upadhyay, S.K., Kumari, B., Tiwari, S., Singh, S.N. & Singh, P.K. (2011). *In vitro* degradation of fluoranthene by bacteria isolated from petroleum sludge. *Bioresour. Technol.* 102: 3709-3715.
34. Mousa, T.A.A., Ahmed, A.M. & Abdel-hamid, S.M.S. (2006). Optimization of cultural conditions for biosurfactant production from *Nocardia amarae*. *J. Appl. Sci. Res.* 2: 844-850.
35. Shavandi, M., Mohebbi, G., Haddadi, A., Shakarami, H. & Nuhi, A. (2011). Emulsified potential of a newly isolated biosurfactant-producing bacterium, *Rhodococcus* sp. strain TA6. *Colloids and Surfaces B: Biointerfaces* 82: 477-482.
36. Abouseoud, M., Yataghene, A., Amrane, A. & Maachi, R. (2008). Biosurfactant production by free and alginate entrapped cells and *Pseudomonas fluorescens*. *J. Ind. Microbiol. Biotechnol.* 35: 1303-1308.
37. Kim, Y.H., Engesser, K.H. & Cerniglia, C.E. (2005). Numerical and genetic analysis of polycyclic aromatic hydrocarbon degrading *Mycobacteria*. *Microbiol. Ecol.* 50: 110-119.
38. Husain, S. (2008). Identification of pyrene degradation pathways: bench-scale studies using *Pseudomonas fluorescens* 29L. *Remediat. Summer* 1-24.
39. Pujar, B.G. & Ribbons, D.W. (1985). Phthalate metabolism in *Pseudomonas fluorescens* PHK: Purification and properties of 4, 5-dihydroxyphthalate decarboxylase. *Appl. Environ. Microbiol.* 49: 374-376.

How to Cite This Article

Babita K., S.N.Singh, F. Deeba, M. Sharma, V. Pandey and D.P.Singh. (2013). Elucidation of Pyrene Degradation Pathway in Bacteria. *Adv. Biores.* 4 (2): 151-160.