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Biodegradation of phenanthrene using adapted microbial consortium isolated from petrochemical contaminated environment

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ABSTRACT

In developing countries like India, there are many industrial areas discharging effluent containing large amount of polyaromatic hydrocarbon (PAH) which causes hazardous effect on the soil-water environment. The objective of this study was to isolate and characterize high-efficiency PAH-degrading microbial consortium from 3 decade old petrochemical refinery field located in Nagpur, Maharashtra with history of PAH disposal. Based on biochemical tests and 16S rDNA gene sequence analysis the consortium was identified as *Sphingobacterium* sp., *Bacillus cereus* and a novel bacterium *Achromobacter insolitus* MHF ENV IV with effective phenanthrene-degrading ability. The biodegradation data of phenanthrene indicates about 100%, 56.9% and 25.8% degradation at the concentration of 100 mg/l, 250 mg/l and 500 mg/l respectively within 14 days. The consortium and its monoculture isolates also utilized variety of other hydrocarbons for growth. To best of our knowledge this is the first time that *Achromobacter insolitus* has been reported to mineralize phenanthrene effectively. GC–MS analysis of phenanthrene degradation confirmed biodegradation by detection of intermediates like salicylaldehyde, salicylic acid and catechol. All the results indicated that the microbial consortium have a promising application in bioremediation of petrochemical contaminated environments and could be potentially useful for the study of PAH degradation and for bioremediation purposes.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) constitute a group of priority environmental pollutants in soils and sediments which are of concern because of their toxic, mutagenic and carcinogenic effects [1]. Some of these PAHs are considered to be possible or probable human carcinogens, and their distributions in the environment causes hazard to human beings [2]. Exposure to PAHs constitutes a significant health risk for people living in industrialized areas of the world [3]. For pollution abatement, these organic compounds are generally removed through physico-chemical methods. These Conventional chemical or physical technologies have inherent drawbacks due to high operating cost, difficulty in operation and production of secondary pollutants [4]. Biodegradation involving the use of naturally occurring microorganisms presents an alternative, yet better approach for the degradation of PAH [5].

Bacteria of the genera *Sphingomonas*, *Burkholderia*, *Pseudomonas*, *Acinetobacter*, *Rhodococcus* and *Mycobacterium* are well-known PAH-degraders. They successfully mineralized both low molecular weight PAHs and high molecular weight PAHs [6,7]. Although pure cultures of several PAH-degrading bacteria can readily utilize PAHs as a carbon source [8,9], degradation results may improve if a mixed bacterial culture or bacterial–fungal consortium was used [10–12]. Generally, biodegradation using a pure strain does not represent the actual behavior of environmental microorganisms during bioremediation in natural PAH-contaminated soils, because in nature, bioremediation depends on cooperative metabolic activities of mixed microbial populations. One important advantage of using microbial consortia is they possess multiple metabolic capacities that increase the efficiency of the bioremediation process [13].

Phenanthrene is among the 16 PAH listed as primary pollutants by US Environmental Protection Agency (EPA) [14] and is known to be a human skin photosensitizer and mild allergen [15]. In the present study we have evaluated the capacity of microbial consortium (*Sphingobacterium* sp., *Bacillus cereus* and a novel bacterium *Achromobacter insolitus* MHF ENV IV) enriched from a petrochemical refinery field to degrade and mineralize different concentrations of solid phenanthrene as a model PAH in liquid medium. The potential phenanthrene degraders were isolated and identified by different morphological, physiological, biochemical assays and 16S rDNA technology. Further the phenanthrene biodegrading capability of the consortium was assessed and the potential pathway inducers, which are produced as intermediates during PAH degradation such as salicylaldehyde, salicylic acid and cat-

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echol was detected by GC–MS to determine biodegradation. The increase in the microbial count and the changes in Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD) were monitored and quantified as an indicator for growth and proliferation of microorganisms along the degradation of the phenanthrene in the mineral medium. The growth of the isolates on other hydrocarbon sources was also evaluated to determine the cooperative metabolic activity of mixed microbial population and the adaptability of microorganisms to survive in hydrocarbon contaminated area.

2. Materials and methods

2.1. Site description and soil analysis

The soil was collected from area around petrochemical industries and oil refineries at Wardha road, Khapri, located on the outskirts of Nagpur city. The petrochemical industry located at Khapri have been disposing petrochemical waste effluent which contains a mixture of various PAH, motor oils and grease dumped at this site through underground waste effluent channels since past 3 decades. Soil samples were collected aseptically from a layer 0–30 cm deep site a few meters away from the petrochemical plant. The soil samples were collected in sterilized seal pack polythene bags which was later ground and sieved through a 2 mm pore size sieve and stored at 4 °C for further physico-chemical and microbial assay for isolation of potential PAH degrading microorganisms. The soil sample was used for microbial enumeration immediately after collection. For isolation of bacteria from the petrochemical contaminated soil/sediments, 1 g of the mixed soil was added to 9 ml of deionized water and 0.1 ml of this diluted sample was spread plated on Nutrient Agar medium from the appropriate dilution tubes and incubated at room temperature for 24 h. The plates showing isolated colonies were tallied, and the results were determined for each soil samples. The fungal colonies were counted after 48–72 h of incubation [16]. Isolated colonies were plated on specific agars which were used for identifying specific microorganisms and fungi in the contaminated soil.

Tanner's Mineral Medium (MM) medium was used as a culture medium for enrichment and isolation of phenanthrene degrading strains. The composition of Tanner's Mineral Medium (MM) medium was as follows (g/l): 0.04 $CaCl_2{\cdot}H_2O$; 0.1 KH_2PO_4; 0.8 NaCl; 1.0 NH_4Cl; 0.2 $MgSO_4{\cdot}7H_2O$; 0.1 KCl. Micronutrients used were (mg/l) 0.1 $CoCl_2{\cdot}6H_2O$; 0.425 $MnCl_2{\cdot}4H_2O$; 0.05 $ZnCl_2$; 0.015 $CuSO_4{\cdot}5H_2O$; 0.01 $NiCl_2{\cdot}6H_2O$; 0.01 $Na_2MoO_4{\cdot}2H_2O$; 0.01 $Na_2SeO_4{\cdot}2H_2O$. The pH of the medium was adjusted to 7.0.

2.2. Enrichment and isolation of the phenanthrene degrading microbial consortium

The petrochemical refinery oil was selected because it was expected to consist of microorganisms adapted and survived at a higher contaminant concentration and also has potential to degrade phenanthrene. The isolates were exposed to increasing concentration of phenanthrene to isolate the microorganisms that could utilize phenanthrene as sole carbon source in enrichment study by the method given by Siddique et al. [17] using nutrient medium. The present bioremediation study was carried out by shake flask method. A quantity of fresh petrochemical contaminated soil was added into 100 ml sterilized MSM containing 250 mg/l solid Phenanthrene in conical flask. Phenanthrene was added directly into MSM in solid state. Although this concentration was much higher than the real contamination level in environment, it was common to use an elevated concentration for enrichment to ensure that the PAH degraders were selected. The flask was shaken in an orbital shaker (120 rpm) at 37 °C for one week for microbial enrichment, then an aliquot of 5 ml enriched culture was inoculated into another 250-ml conical flask containing 100 ml fresh MSM with the same amount of solid Phenanthrene for the second enrichment. After four consecutive enrichments, the cultures with a series of concentration gradient were inoculated on the MSM agar plates containing a thin layer of Phenanthrene respectively to get the enriched consortium and the separated Phenanthrene degrading microorganism with a clearing zone around the inoculated region. The isolation and purification of the bacterial consortium were carried out on nutrient agar plates by conventional spread plate techniques. Plates were incubated at 37 °C for 48 h after which isolated colonies were selected for further identification. All isolates were stored at −20 °C as liquid cultures containing 20% glycerol (v/v) [18].

2.3. Identification of microbial consortium

The microbial isolates were first identified based on the morphological, cultural characteristics of individual colonies, then by traditional biochemical tests. Individual isolated colony was re-streaked on mineral agar plates for identification. The isolated colony was gram stained and different standard morphological, physiological and biochemical tests were performed using KB003 kit (KB003 Hi25, Himedia, India). For further identification of the microbial consortium the 16S rRNA gene fragment was amplified by PCR from genomic DNA using 16S gene universal primers: 8F: 5′ AGA GTT TGA TCC TGG CTC AG 3′, 1492R: 5′ ACG GCT ACC TTG TTA CGA CTT 3′. PCR was carried out in a final reaction volume of 25 μl in 200 μl capacity thin wall PCR tube in Eppendorf Thermal Cycler. PCR tubes containing the mixture were tapped gently and spin briefly at 10,000 rpm. The PCR tubes with all the components were transferred to thermal cycler. The PCR reaction mixture (final volume = 25 μl) was prepared in a single tube as follows: 7.50 μl of DNase-RNase free water, 12.50 μl 1× PCR master mix (MBI Fermentas), 1.00 μl Forward Primer (10 pmole/μl), 1.00 μl Reverse Primer (10 pmole/μl), 3.0 μl Diluted DNA (30 ng/μl). PCR was carried out in a final reaction volume of 25 μl in 200 μl capacity thin wall PCR tube in Eppendorf Thermal Cycler. PCR tubes containing the mixture were tapped gently and spin briefly at 10,000 rpm. The PCR tubes with all the components were transferred to thermal cycler. Thermal cycling for PCR was done with a profile consisting of 30 cycles at 95 °C for 2 min, 52 °C 30 s, 72 °C 90 s. To confirm the targeted PCR amplification, 5 μl of PCR product from each tube was mixed with 1 μl of 6× gel loading dye and electrophoresed on 1.2% agarose gel containing ethidium bromide. The amplified product was visualized as a single compact band of expected size under UV light and documented by gel documentation system (Bio-Rad).

Further the 16S rDNA gene sequence was used to carry out BLAST (Basic local Alignment Search tool) with 'nr' database of NCBI GenBank using MEGABLAST algorithm. The BLAST data was arranged in maximum percentage identity and first ten sequences was selected and exported in FASTA format. Based on maximum identity score and query coverage the best highly identical 10 sequences were selected and aligned using multiple alignment software program ClustalW (MEGA tool). The evolutionary history was inferred using the Neighbor-joining method. The bootstrap consensus tree inferred from 500 replicates is taken to represent evolutionary history of the taxa analyzed [19,20,21].

2.4. Bioremediation experiment

2.4.1. Spiking of organic compound

Erlenmeyer flasks (250 ml) and Tanner's Mineral Media were autoclaved for 20 min at 121 °C. 1 ml acetone containing required phenanthrene concentration was aseptically added to autoclaved

Erlenmeyer flasks allowing the acetone to evaporate. After complete evaporation of acetone from the Erlenmeyer flasks, 100 ml sterile culture media was added under laminar flow hood so as to reach the desired final concentration of organic compound. The inocula containing 5% of the total volume were sampled in the logarithmic phase culture in MM broth and incubated for 24 h with orbital shaking (120 rpm). After incubation for 24 h, 1 ml medium was diluted and 0.1 ml of 10^7 dilutions were plated on nutrient agar and incubated for 24–30 °C in dark and colonies were directly counted and expressed as CFU ml^{-1}. The pH was monitored throughout the bioremediation experiment.

2.4.2. Scale-up technique

1 ml sub-cultured consortium (nutrient broth) was inoculated into Erlenmeyer flasks (250 ml) containing nutrient culture media with a phenanthrene concentration of 10 mg/l. The inoculated flasks were kept in orbital shaker incubator at 120 rpm, 37 °C. for 14 days. After 14 days, 5 ml of this culture media was transferred to flasks containing Tanner's Mineral Medium with phenanthrene concentration of 50 mg/l. The flasks were again kept on orbital shaker incubator at 120 rpm, 37 °C for 14 days. Likewise, the microbial culture was sub-cultured into Tanner's Mineral Medium with different phenanthrene concentrations of 100 mg/l, 250 mg/l and 500 mg/l and kept on orbital shaker incubator at 120 rpm, 30 °C at each concentration over a period of 14 days. At this stage, the consortium was found adapted and was able to utilize phenanthrene as sole source of carbon for growth and maintenance.

2.4.3. Phenanthrene biodegradation

Samples were centrifuged (10 min, 10,000 rpm) to separate cell mass and the supernatant. The samples were extracted in organic solvent (n-hexane) for analysis. Chromatographic separation was performed using HPLC system [Jasco, Model UV-2075 Plus], equipped with the UV–VIS diode array detector (Varian) and with Borwin software. 20 μl of extracts was injected into the isocratic mobile phase of acetonitrile and water (80:20 ratios), run at 1 ml/min, isocratic run for 10 min with Varian C-18 column (250 mm × 4.6 mm). Phenanthrene was further identified by comparing UV spectra and retention times with the standards. Detections were performed at 254 nm.

The extracts were also analyzed for identification of phenanthrene and its metabolites by Gas Chromatography–Mass Spectrometry and samples were quantified according to USEPA SW-846 Method 8270D (Gas Chromatography/Mass Spectrometry for semivolatile Organic compounds). The Shimadzu gas chromatograph (Model QT 2010) equipped with electron ionization detector and mass selective detector was used. The injector temperature was programmed from ambient to 360 °C where the oven temperature was fixed at 350 °C. Nitrogen was used as the carrier gas, and the temperature program was set as follows: 80 °C for 1 min, and then 5 °C/min up to 240 °C for 5 min. The degradation rate was estimated by calculating the decrease of the peak of the substrate PAH [22]. Qualitative analysis was based on retention indexes, mass spectra comparison with data in the literature and mass spectral libraries, comparison of the mass spectra with those of commercially available compounds.

3. Results and discussion

The waste disposal site of petrochemical industries located at Khapri, near Nagpur, Maharashtra has been studied to evaluate the potential of microbial consortium for the degradation of phenanthrene at various concentrations and enhancement of the biodegradation process at higher concentration of phenanthrene using temperature controlled shake flask bioreactor. The varying concentrations of phenanthrene were taken individually to assess bioremediation by microbial consortium. The physico-chemical characteristics of the soil are presented in Table 1, which shows the presence of macro- and micronutrient such as carbon, nitrogen, phosphate and sulfate in the petrochemical contaminated soil. The microbial characterization of the consortium shows the presence of bacteria, fungi, and actinomycetes, etc. in the soil (Table 2).

Table 1
Physico-chemical characteristics of soil.

Parameters	Value[a]
pH	7.60
Moisture content (%)	6.38
Acidity (mg/l)	60.59
Alkalinity (mg/l)	620.83
Biological oxygen demand (mg/l)	3.12
Chemical oxygen demand (mg/l)	211.65
Organic carbon (%)	0.42
Phosphate (mg/l)	20.55
Sulfate (mg/l)	196.25
Total organic carbon (%)	3.45
Total organic matter (%)	5.86
Nitrogen (%)	0.44

[a] The values are average of three replicates.

The consortium was isolated based on the formation of clear zones on solid LB plates with sprayed phenanthrene as the sole carbon source. In contrast to other strains isolated in this experiment which lost their degradation abilities after more than 2 months preservation, the consortium showed high phenanthrene degradation ability. In order to identify the isolated microorganisms, the different physiological and biochemical tests were conducted (Table 3).

For further identification of the microbial consortium the genomic DNA of bacteria was isolated, and 16S rDNA was amplified using universal primers. A 1.5-kb PCR product was obtained from the 16S rDNA amplification was sequenced and aligned using Clustal W software. The 16S rDNA sequence obtained for A2, A3 and A4 was compared with the other 16S rDNA bacterial sequences available in GenBank database. The BLAST search of available data in the EMBL/GenBank database showed a high similarity (99%) with *Sphingobacterium* sp., *Bacillus* sp. and *Achromobacter* sp. respectively (Fig. 1). A-2, A-3 and A-4 were identified as *Sphingobacterium* sp., *Bacillus cereus* and *Achromobacter insolitus* MHF ENV IV (GenBank entry: GQ334452.1) respectively. Phylogenetic tree was drawn to determine the evolutionary relationship between the isolates (Fig. 1).

The essential environmental parameters responsible for bioremediation were monitored throughout the experiment. The data show an increase in temperature from 26 °C to 28 °C, supporting the bioremediation process. The pH was monitored thought the experiment. It was found to be decreasing from 7 to the acidic range during the bioremediation process. The OD (600 nm) was measured every 24 h. Analysis of pH changes with growth of the

Table 2
Microbial characteristics of soil.

Microorganisms	Species
Bacteria	*Pseudomonas* sp.
	Bacillus sp.
	Streptococci sp.
	Salmonella sp.
	E. coli sp.
Fungi	*Aspergillus* sp.
	Mucor sp.
Actinomycetes	*Penicillium* sp.
	Rhizopus sp.
	Nocardia sp.
	Micromonospora sp.
	Rhodococcus sp.

Table 3
Physiological and biochemical characteristics of consortium.

Characteristics	Principal	A-2[a]	A-3[a]	A-4[a]
ONPG	Detects β-galactosidase activity	–	–	–
Lysine decarboxylase	Detects lysine decarboxylation	V	+	+
Ornithine decarboxylase	Detects ornithine decarboxylation	V	+	+
Urease activity	Detects urease activity	–	–	–
Phenylalanine deaminase	Detects phenylalanine deamination	–	–	–
Nitrate reduction	Detects nitrate reduction	V	–	–
Hydrogen sulfide production	Detects H_2S production	–	–	–
Citrate utilization	Detects capability of organism to utilize citrate as a sole carbon source	+	+	+
Voges-proskauer's	Detects acetoin production	–	–	–
Indole	Detects deamination of tryptophan	–	–	–
Malonate	Detects capability of organism to utilize sodium malonate as a sole carbon source	+	–	–
Esculine	Esculin hydrolysis	+	–	–
Arabinose	Arabinose utilization	–	–	–
Xylose	Xylose utilization	+	–	–
Adonitol	Adonitol utilization	–	–	–
Rhamnose	Rhamnose utilization	–	–	–
Cellobiose	Cellobiose utilization	–	–	–
Melibiose	Melibiose utilization	+	–	–
Saccharose	Saccharose utilization	+	–	–
Raffinose	Raffinose utilization	–	–	–
Trehalose	Trehalose utilization	+	+	+
Glucose	Glucose utilization	+	+	+
Lactose	Lactose utilization	–	–	–
Oxidase	Detects cytochrome oxidase production	+	–	–
Catalase	Detects catalase production	+	–	–

[a] Results were interpreted after 24 h of incubation at room temperature.

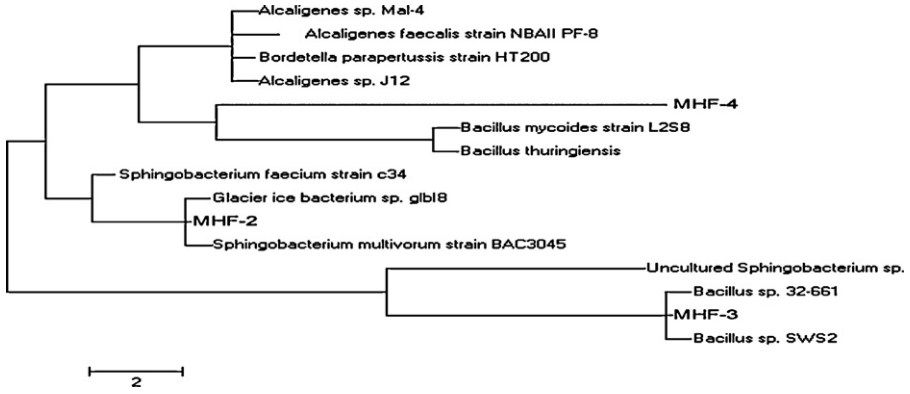

Fig. 1. Phylogenetic tree showing the genetic relatedness of bacterial consortium A-2, A-3 and A-4 isolated from petrochemical contaminated soil.

consortium revealed significant reduction in pH due to acidification (Fig. 2). Growth of the microbial consortia in the mineral medium caused a rapid decrease in pH reaching 5.6, 5.3 and 5.2 for 100 mg/l, 250 mg/l and 500 mg/l respectively up to the 14th day probably due to the accumulation of H^+ or other acidic metabolites in the mineral medium. After the 12th day of incubation, no further drastic reduction in pH was observed. The pH change of the experimental media may signify metabolic activity leading to production of acidic or alkaline metabolites during breakdown of PAHs. Kim et al.

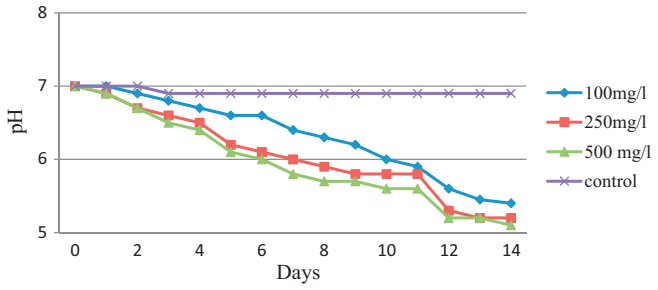

Fig. 2. Variation in pH during phenanthrene bioremediation by microbial consortium.

[23] observed that acidic pH conditions promote uptake of PAHs for degradation in *Mycobacterium vanbaalenii*. It can therefore be suggested that monitoring pH of media may be used to check the progress of PAHs degradation. This corroborates previous research where the dynamics of pH changes in cultures were consistent with that of PAH concentration change [24].

The OD (600 nm) was measured every 24 h. The results indicated the growth in the number of degrading microorganisms. Number of CFU/ml was monitored and was found to be increasing along the concentration of phenanthrene within a period of 14 days experiment and remained constant as the incubation progressed towards the end of the experiment. These research findings indicate increased degradation of phenanthrene by isolated microbial consortium. The CFU of the selected isolates (Fig. 3) are characteristically from bacteria growing under shaking using phenanthrene crystals as the sole carbon source in amounts exceeding the aqueous solubility in the MM at selected concentrations of 100 mg/l, 250 mg/l and 500 mg/l.

In the initial phase, exponential growth was based on phenanthrene dissolved in the aqueous medium, close to or at the maximum concentration. In this phase, population growth was controlled only by metabolic activity, and not by phenanthrene availability, with a high biomass increasing as verified by Wick

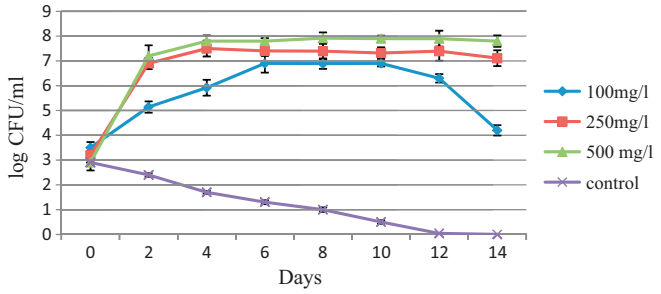

Fig. 3. Growth of consortium in MM containing phenanthrene as sole carbon source. Data are mean of three replicates; error bars represent SD.

et al. [25]. Exponential growth ceased when phenanthrene consumption by the isolates exceeded the phenanthrene dissolution rate. Isolates reached a pseudo-linear growth phase when limited physically by the maximum dissolution of phenanthrene which is converted in cells. In the pseudo-stationary phase, the phenanthrene consumption of individual cells reached the maintenance level and consequently the growth ceased [26]. The increase in cell count of the isolates during degradation was an indication that the Phenanthrene supported microbial biomass product even as a sole source of carbon and energy. Researchers have shown that population density increase in media was a reflection of degradation process and proliferation of cell mass [27]. As such the increase in population density of the isolates in the experimental media compared to the control as degradation proceeded was an indication of metabolic activity reflected in the increased cell count.

In the bioremediation experiment, initial concentrations 100 mg/l, 250 mg/l, 500 mg/l of phenanthrene were taken. The experimental findings indicated that in case of 100 mg/l phenanthrene, degradation started within 3 h, i.e. lag period observed at this concentration was very short. This phenanthrene concentration was degraded below the limit of detection within 14 days. Similarly for 250 mg/l of phenanthrene, 56.9% degradation was observed over a period of 14 days. Here degradation started after 7 h, i.e. lag phase observed for this concentration was 7 h. Biodegradation of higher concentration of phenanthrene like 500 mg/l was found relatively slow for consortium as very little decrease in concentration of about 25.8% was observed in 14 days span. However there was no decrease in the CFU during the degradation even at higher concentration of phenanthrene indicating that the consortium supported growth even at higher concentration. The degradation pattern of phenanthrene by the consortium is presented in Fig. 4.

The total abiotic loss of Phenanthrene during the degradation experiment and extraction process was about 1.5%. Evaluation of the effect of phenanthrene concentration revealed that cell counts increased with concentration up to 500 mg phenanthrene/l. The efficiency of the selected isolate on phenanthrene degradation was

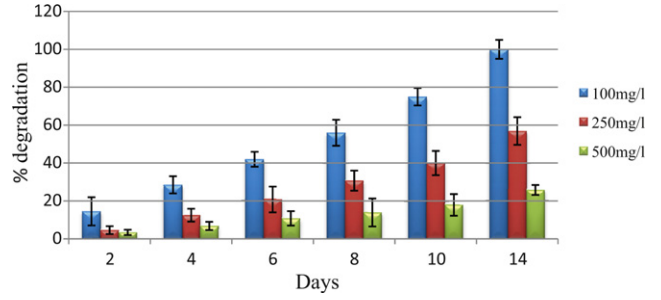

Fig. 4. Biodegradation of phenanthrene by microbial consortium. Data are mean of three replicates; error bars represent SD.

Table 4
Utilization of carbon substrates by phenanthrene-degrading consortium.

Carbon source	A-2[a]	A-3[a]	A-4[a]	Consortium[a]
Naphthalene	+[b]	+[b]	+[b]	++[b]
Phenanthrene	+	+	+	++
Anthracene	+	+	+	+
Benzene	–	+	–	+
Toluene	–	+	–	+
Xylene	+	+	+	+
Phenol	+	+	+	+
Methanol	–	+	–	+
Ethanol	–	+	–	+
Salicylic acid	+	+	+	+
Tween 80	+	+	+	++
Tween 20	+	+	+	++

[a] Growth was followed by measuring the increase of OD (600 nm) of the culture for 10 days.
[b] ++, Good growth: $OD_{600\,nm} > 0.2$; +, growth: $OD_{600\,nm} > 0.1$; –, no growth:$OD_{600\,nm} < 0.02$.

individually evaluated by Gas chromatogram and HPLC [28]. The bioremediation samples were also analyzed on mass spectrometer for detection of its metabolites and its intermediates.

When the extracts were subjected to GC–MS analysis the metabolites detected were salicylic acid (*m*/*z* 93,138) and salicylaldehyde (*m*/*z* 121) respectively. Another major metabolite was identified with *m*/*z* ratio of 109 which represents the deprotonated catechol ring (Fig. 5a–d) [29]. This suggests that phenanthrene was metabolized into simpler compounds by the strain via salicylate pathway confirming the degradation. Further investigation should be aimed at the toxicity of the intermediates; whether the intermediates produced by incomplete degradation have the potential to form the basis for a purposeful biosynthesis of compounds that are important chemicals and antioxidants in biomedicine and in the cosmetics industry [30].

During the bioremediation the chemical oxygen demand (COD) and biological oxygen demand (BOD) were also monitored as indicators for bioremediation and microbial growth. The basis for the COD test is that nearly all organic compounds can be fully oxidized to carbon dioxide with a strong oxidizing agent under acidic conditions. Fig. 6 demonstrates the decrease in COD levels over a period of bioremediation which indicates the degradation of phenanthrene by microbial consortium. The decrease in BOD values indicates the growth of microorganisms in the varying concentration of phenanthrene (Fig. 7) [31].

The capacity of members of the microbial consortium to use 12 hydrocarbons as C sources is presented in Table 4. The consortium displayed a wide spectrum of substrate utilization. It grew on all of the 12 C sources evaluated. Individually, the bacterial isolate 3 showed higher metabolic versatility because it grew in 12 C sources compared to bacterial isolates 2 and 4 which grew in 9 of the hydrocarbon C sources tested. There were great differences in the substrate utilization profile of the intermediary metabolites of the PAH degradation pathway. Salicylic acid was used as C source by all of the members of the consortium, indicating that this can be the main central intermediary metabolite of the PAH degradation pathway for the isolates from the PAH-degrading consortium. For the monoaromatic hydrocarbons, the bacterial isolate 3 grew in the presence of the four compounds. In spite of the simpler structure than PAHs, benzene, and toluene were not degraded by isolates 2 and 4. The consortium was also found capable of utilizing Tween 20 and Tween 80. However, the resistance of the isolates to the toxics effects of these compounds is also a characteristic to be considered in the selection of the degraders to soil bioremediation.

Remediation investigations and feasibility studies usually emphasize geology, hydrogeology, contaminant distribution and risk assessment, while the microbiology of the site is often ignored. Instead the characterization of naturally selected microbial consor-

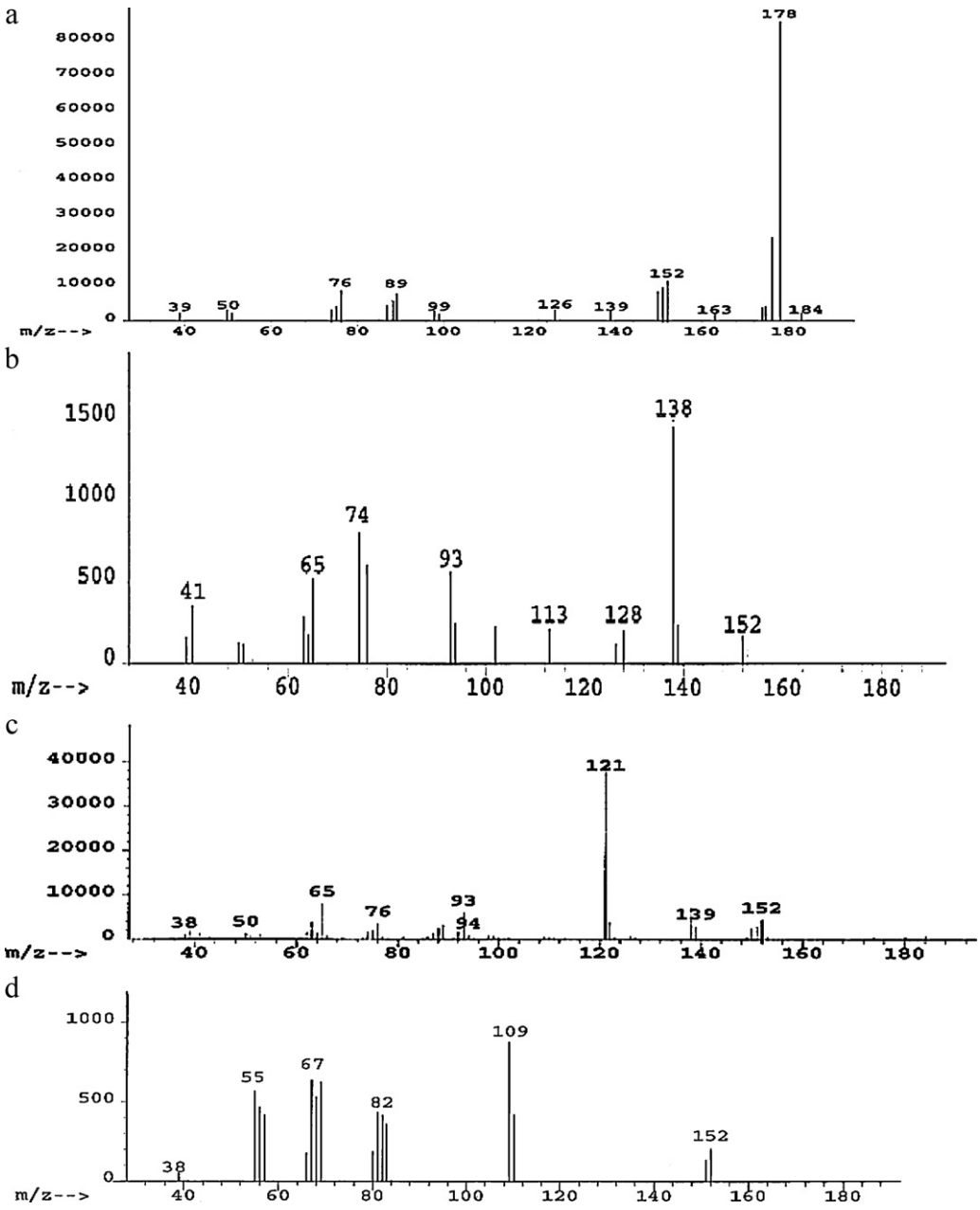

Fig. 5. (a) Mass spectrum of phenanthrene (*m*/*z* identification-178). (b) Mass spectrum of salicylic acid (*m*/*z* identification-93, 138). (c) Mass spectrum of salicylaldehyde (*m*/*z* identification-121). (d) Mass spectrum of catechol (*m*/*z* identification-109).

tia can provide important knowledge that allows evaluation of the initial biodegradative potential of the contaminated site. Moreover, molecular analysis gives the possibility of monitoring changes that takes place in the microbial community structure in response to remediation activities.

Microbial degradation of PAHs in soil is limited by the low bioavailability of high molecular weight compounds. Subsurface contamination by these hydrocarbons is a complex process, and their remediation, mainly initiated by microbes, is limited by

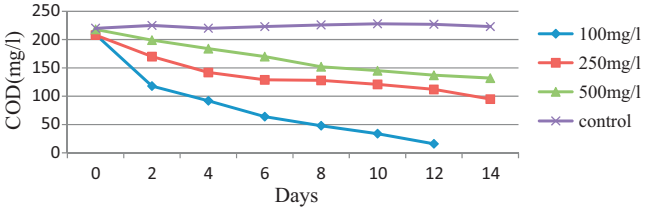

Fig. 6. Chemical oxygen demand (COD) variation during phenanthrene bioremediation by microbial consortia A4.

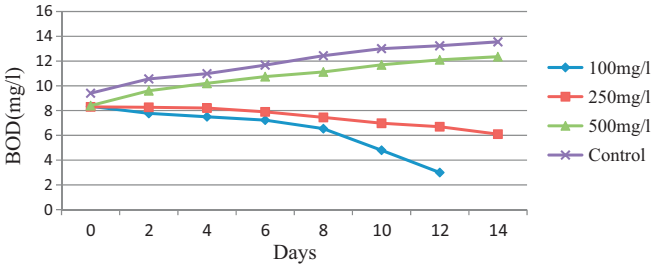

Fig. 7. Biological oxygen demand (BOD) variation during phenanthrene bioremediation by microbial consortia.

low water solubility [32] adsorption of contaminants onto soil matrix, and limited rate of mass transfer for the biodegradation. Biosurfactants serve to decrease tension at the hydrocarbon-water interface and can result in hydrocarbon pseudosolubilization via micelle or vesicle formation, leading to increased mobility, bioavailability, and subsequent biodegradation [33,34]. Nazina et al. [35] reported that biosurfactants were mostly produced by strains of *Pseudomonas* sp., *Bacillus cereus*, *Rhodococcus ruber*, and *Bacillus licheniformis* in media with hydrocarbons which could enhance the dissolved concentration of phenanthrene to above 1 mg/l, concentration higher than the saturated solubility of phenanthrene under standard condition. Carlos et al. [36] has reported biosurfactant production by *Bacillus cereus*. Moreover combining biosurfactant producing bacterium with other bacteria can enhance biodegradation [37]. Emphasis should be given on the use of such biosurfactant producing microorganisms as a practical solution of the bioremediation of hydrophobic contaminants. Wang et al. [38] and Curtright et al. [39] who reported biodegradation of phenanthrene by *Achromobacter* sp. Doddamani and Ninnekar [40] has reported phenanthrene biodegradation by *Bacillus* sp. Okparanma et al. [41] reported biodegradation of phenanthrene by *Bacillus* sp. It was found that naturally selected microbial populations are enriched with microorganisms carrying catabolic gene that degrades pollutants, and such adapted population have the advantage of being adapted to polluted sites. The isolated consortium proved to be efficient in removing phenanthrene at a higher concentration due to its surfactant production activity. Although *Sphingobacterium* sp., *Bacillus cereus* has been reported in many biodegradation studies, to our knowledge, this is the first time that *Achromobacter insolitus* is reported to possess the capability for phenanthrene degradation at such a high concentration along with other hydrocarbons. These results indicate that the consortium might possess a high potential to be applied in bioremediation of environments polluted by phenanthrene. These potential isolated can be highly recommended for bioremediation of petrochemical contaminants due to its biosurfactant and rhamnolipid production activity and versatility to utilize other related hydrocarbons present in petrochemical waste.

4. Conclusions

The soil sample from petrochemical refinery field displayed the highest microbial PAH mineralization activity in soil culture. A total of three bacterial monocultures *Sphingobacterium* sp., *Bacillus cereus* and a novel bacterium *Achromobacter insolitus* MHF ENV IV were identified as capable of degrading phenanthrene and 12 other hydrocarbons as C source and energy. The microbial consortium degraded significant amount of phenanthrene present in the mineral medium. The metabolites detected were salicylaldehyde, salicylic acid and catechol indicating that phenanthrene was metabolized into simpler compounds by the consortium via salicylate pathway confirming the degradation. The microbial consortium produced emulsification of the medium and degraded a variety of hydrocarbon substrates and its monoculture isolates displayed a wide substrate spectrum of activity and utilized a variety of toxic aromatic and aliphatic hydrocarbons that are likely to be found in sites contaminated with complex mixtures of PAHs, indicating the possibility of using the consortium for bioremediation of sites contaminated with mixtures of polynuclear aromatic and aliphatic hydrocarbons.

GenBank accession number

The 16s rDNA sequence data of *Achromobacter insolitus* MHF ENV IV reported in this paper have been submitted to NCBI GenBank under accession number GQ334452.1.

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