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Aerobic remediation of petroleum sludge through soil supplementation: Microbial community analysis

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ABSTRACT

The effect of soil concentration on the aerobic degradation of real-field petroleum sludge was studied in slurry phase reactor. Total petroleum hydrocarbons (TPH) and polycyclic aromatic hydrocarbons (PAHs) showed effective removal but found to depend on the soil concentration. Aromatic fraction (48.12%) documented effective degradation compared to aliphatics (47.31%), NSO (28.69%) and asphaltenes (26.66%). PAHs profile showed efficient degradation of twelve individual aromatic compounds where lower ring compounds showed relatively higher degradation efficiency compared to the higher ring compounds. The redox behaviour and dehydrogenase activity showed a linear increment with the degradation pattern. Microbial community composition and changes during bioremediation were studied using denaturing gradient gel electrophoresis (DGGE). Among the 12 organisms identified, *Proteobacteria* was found to be dominant representing 50% of the total population (25% of γ-*proteobacteria*; 16.6% of β-*proteobacteria*; 8.3% of α-*proteobacteria*), while 33.3% were of *uncultured bacteria* and 16.6% were of *firmicutes*.

1. Introduction

Petroleum industry unavoidably generates large quantities of oily and viscous residue, i.e., the petroleum based oily sludge formed during various productions, transportation and refining processes. The safe disposal of oily waste generated during the processing of crude oil is one of the major problems faced by the oil industry as, improper disposal pollutes environment which includes freshwater, marine and terrestrial habitats. Crude petroleum oil is a complex mixture of hydrocarbons and other organic compounds generally classified into four fractions: aliphatics, aromatics, nitrogen sulphur oxygen containing compounds (NSO) and asphaltenes. Aromatic and polar constituents are less biodegradable than aliphatics, while asphaltenes are regarded as non biodegradable that can bring up serious environmental problems when spills occur [1]. There are many techniques available to remediate hydrocarbon polluted soil including in situ methods, such as soil vapour extraction and ex-situ methods such as soil removal and disposal, incineration and chemical treatment [2]. One *in situ* technique for the remediation of hydrocarbon contaminated soil is bioremediation, which is the application of biological remediation for the treatment of hazardous materials [2,3]. However bioremediation is recognized as an efficient, economic and versatile alternative to physicochemical treatment of oil contaminants. Aerobic microbial degradation is the main process, where microorganisms play an important role in the degradation of PAHs. As crude oil contains various compounds, a single strain might not be able to degrade all components. In general, the combined bacterial consortium show better results due to their synergistic effects [4]. Soil slurry bioremediation is a technique used to optimize abiotic conditions for biodegradation. It consists of a mixture of soil and water in various ratios and greatly enhances degradation rates over solid treatment systems by maximizing the contact between microorganisms, hydrocarbons, nutrients and oxygen [2,5]. In slurry bioreactors, the increase in soil moisture results in a larger amount of soluble contaminant, therefore increasing the bioavailability [6]. A better understanding of the microbial communities diversity inhabiting PAHs contaminated soils and their response to biostimulation strategies could provide clues about the type of bacteria that are able to adapt to exploit such habitats [7]. It is well known that majority of microbes in environmental samples cannot be cultured at present in laboratory media, which are biased for the growth of specific microorganisms [8]. In light of this, molecular biological techniques viz., denaturing gradient gel electrophoresis (DGGE) offers new opportunities and allows us to directly determine the presence and relative levels of different 16S rRNA gene amplicons both qualitatively and semi quantitatively to perform a community analysis [9]. The present investigation provides an insight in to the aerobic remediation of real field petroleum sludge and the functional role of soil supplementation on the bioremediation efficiency. The study was performed in slurry phase system. Degradation pattern of TPH

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fractions (aliphatics, aromatics, NSO and asphaltenes) and PAHs profile was analysed during the study. The community analysis and its changes during degradation was also evaluated.

2. Materials and methods

2.1. Petroleum sludge

Petroleum based oil sludge sample acquired from an oil refinery, India was used in the aerobic bioremediation studies. After procurement, the sample was packed tightly in polythene bags and stored in a dark place at 4 °C prior to experimentation.

2.2. Soil

Soil used for horticulture activities was used in bioremediation studies. The selected soil belongs to silt-loam category as per US department of agriculture triangular soil classification chart and was composed of 24% clay, 28% sand, 1.2 g density, 5% silt and moisture content [field/airdry (%)] 20.1/1.9. The soil has a distribution coefficient (k_{sd}) of 26.649×10^{-7} m^3/g, and a colony forming unit (CFU) of 2.46×10^4 CFU/g soil. Prior to use, the soil sample was sieved using 2 mm sieve to remove debris, unwanted matter and then air-dried to eliminate the moisture content. After drying, the sample was stored in closed containers at 4 °C in order to sustain the biological activity.

2.3. Inoculum

Domestic sewage (DS) (1.78×10^7 CFU/ml) and soil (2.46×10^4 CFU/g) provided as the microbial base for remediation. DS (chemical oxygen demand (COD), 420 mg/l; biochemical oxygen demand (BOD5), 320 mg/l; carbohydrates, 235 mg/l; nitrates, 115 mg/l; chlorides, 350 mg/l; phosphates, 48 mg/l; alkalinity 280 mg/l and pH 7.2) was used to feed the reactors. As it provides adequate amount of pH, buffer and minerals required to optimize the pH condition and nutrient balance.

2.4. Experimental methodology

Batch experiments were performed in slurry reactors to evaluate the degradation of petroleum based oil sludge under aerobic conditions. Based on varying soil concentrations the reactors were labelled as reactor A (0% soil, control), reactor B (5% soil), reactor C (10% soil) and reactor D (20% soil). Except variations in soil concentration rest all other operating conditions were maintained uniform. The reactors used in the experiments were with a total and a working volume of 1 l and 0.25 l respectively. To 2 g of petroleum sludge, 2 ml of ether was added to facilitate proper dissolution and then fed to reactor along with DS (100 ml) followed by 1 ml of triton 10x surfactant solution for the purpose of homogenation. Prior to inoculation, the pH of the feed was adjusted to 7 using orthophosphoric acid (88%) or 3 N NaOH. Prior to start up, the sludge was mixed with equal amount of sodium sulphate to promote dryness in the samples and to prevent losses caused due to its sticky nature. After adding all the required elements the reactors were closed with sterile cotton plugs to ensure exchange of gases and to prevail aerobic conditions throughout the experiment. Later the reactors were mounted on a horizontal shaker at 100 rpm for 10 days.

2.5. Analyses

2.5.1. Total petroleum hydrocarbons (TPH)

Soxhlet extraction was used for extracting TPH from all the reactors at different time intervals (0th day, 5th day and 10th day). The extraction was carried out in 100 ml soxhlet apparatus using dichloromethane and acetone in the ratio of 1:1 as solvent mixture according to the procedure outlined in standard AOCS official methods [10].

2.5.2. TPH fractional analysis

After extraction, TPH comprised of different components like soluble (aliphatics, aromatics, NSO) and insoluble fractions (asphaltenes). Insoluble fraction was separated by filtration method by adding 50 ml of n-pentane to 100 mg of TPH mixture. Column chromatography was performed to separate the soluble fractions. Soluble fractions were loaded onto the pre activated silica gel columns (45 × 2 cm, mesh size 60–120, activation period 16 h at 160 °C) and separated according to the method described by Mishra et al. [11]. Each fraction was analysed gravimetrically. Aromatic fractions separated were detected using HPLC (Shimadzu LC10A VP) with a UV-VIS detector at 254 nm with ODS (octadecasilicosane) column. Peaks derived were compared with the standard PAH mixture (M/S Dr. Ehrenstofer, Supelco).

2.5.3. Bioprocess evaluation

Along with TPH analysis, daily process performance was monitored in terms of pH, dissolved oxygen (DO) and dehydrogenase activity according to the procedure outlined in the standard methods APHA, 1998 [12]. pH was measured using pH analyzer (Elico, India). DO was measured using probe (YSI 5100, USA). Dehydrogenase enzyme activity of the sample was estimated by a method based on the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) [13]. All the analytical estimates were carried out in duplicate and the average values were presented.

2.5.4. Microbial diversity

Microbial diversity analysis was done for reactor D, which showed a higher biodegradability. Diversity analysis was done for the identification of potential organisms present in the soil and to evaluate their role in degradation of TPH and PAHs. The samples for diversity analysis were collected from the reactor at 0th day, 5th day and 10th day respectively.

2.5.4.1. DNA extraction and PCR amplification. The culture collected from the reactor was centrifuged at 8000 rpm for 10 min, and the cell pellets were washed thrice with sterile distilled water. Genomic DNA was extracted and purified using phenol–chloroform method [14]. The concentration of DNA obtained was measured at a wavelength of 260 nm using UV/Vis-spectrophotometer (Thermo Scientific, USA). The variable V3 region of 16S rDNA was amplified by PCR with primers to conserved regions of the 16S rRNA genes. The nucleotide sequences of the primers were as follows: primer 341F, 5′-CCT ACG GGA GGC AGC AG-3′; primer 517R, 5′-ATT ACC GCG GCT GCT GG-3′; GC clamp was added to primer 63FGC, 5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAG GCC TAA CAC ATG CAA GTC-3′ [9]. The GC-rich sequence attached to the 5′-end of forward primer prevents the PCR products from complete melting during separation via DGGE. Both primers have been observed to anneal to the majority of bacterial sequences in the ribosomal database. All the PCR amplifications were conducted in 50 μl of PCR reaction mixture containing 2 μl of total DNA (100 ng/μl concentration), 200 μM each of the four deoxynucleotide triphosphates, 15 mM $MgCl_2$, 0.1 μM of individual primers and 1 unit of Taq polymerase. An automated thermal cycler (Eppendorf) was used for PCR amplification with program for an initial denaturation at 96 °C for 5 min, 35 cycles of denaturation (40 s at 94 °C), annealing (50 s at 52.6 °C) and extension (1 min at 72 °C) and a final extension at 72 °C for 8 min. Finally the amplified PCR product was stored at 4 °C. The samples were checked by using 1% agarose gel.

2.5.4.2. Denaturing gradient gel electrophoresis (DGGE). DGGE was performed using the DCode™ Universal Mutation Detection System (Bio-Rad). Samples containing approximately equal amounts of PCR amplicons (35 μl) were loaded into 1 mm thick vertical gels containing 6% (w/v) polyacrylamide with a linear gradient of denaturants (urea and formamide). A denaturing gradient of 30–70% was applied to separate 16S rDNA fragments. Gels were prepared in 1× TAE buffer which was also used as the electrophoresis buffer. Electrophoresis was run at 60 °C, initially for 15 min and then at a constant voltage of 100 V for 12 h. After electrophoresis, the gels were stained with ethidium bromide (0.5 mg/l) for 15 min followed by destaining in distilled water for 20 min. Gel images obtained were captured using Molecular Imager G:BOX EF System (Syngene).

2.5.4.3. Sequencing and phylogenetic analysis. The middle portions of the predominant selected DGGE bands were excised with a sterile pipette tip. Bands from 0th day were not excised due to their less intensity, but they were assigned to the comigrating bands of 5th and 10th days, which exhibited higher intensity. The excised gel was incubated overnight at 4 °C in 50 μl of sterile distilled water. Three microlitres of eluted DNA was used as the template for PCR amplification performed under the conditions described above, except that the forward primer lacked GC clamp. A 5 μl sample of each PCR amplified product was subjected to agarose gel electrophoresis to confirm product recovery and to estimate product concentration. Five microlitres of each reaction mixture was subjected to DGGE analysis to check the purity and to confirm the melting behaviour of the recovered band. Some DNA samples still contained the mixed products of multiple DGGE bands. In each of these cases, the target band was excised from the recovered pattern. For sequencing analysis, amplified PCR products after purification (Fermentas purification kit) were sent to MWG Biotech. All the 16S rDNA partial sequences were aligned with the closest relative strains available in the GenBank database by using the BLASTN facility and were also tested for possible chimera formation with the CHECK CHIMERA program (http://www.35.8.164.52/cgis/chimera.cgi?su=SSU). These sequences were further aligned with the closest matches found in the GenBank database with the CLUSTALW function of Molecular Evolutionary Genetics Analysis package (MEGA). Neighbor-joining phylogenetic tree was constructed with the MEGA version 2.1 [15]. A bootstrap analysis with 500 replicates was carried out to check the robustness of the tree. Bootstrap re-sampling analysis for the replicates was performed to estimate the confidence of tree topologies.

2.5.4.4. Nucleotide sequence accession numbers. The 12 nucleotide sequences identified in this study have been deposited in the GenBank database under accession numbers FR728425, FR728428 to FR728430 and FR734174 to FR734181.

3. Results and discussion

3.1. TPH

During the experimental run the reaction mixture comprised of both soil and sludge. To avoid soil interferences during TPH extraction and fractionation, a soil portion of 110 mg was separated from 1 g of sludge. The data presented here represent only petroleum sludge portion. In order to differentiate biological removal of TPH from physico-chemical sorption removal onto soil, adsorption studies were performed separately with uniform composition of reaction mixture without bacteria. The sorption profile showed rapid removal of TPH initially (up to 24th h) and approached a maximum of 2 ± 0.42% removal prior to levelling off at 40th h to 3 ± 0.31% (data not shown). For each of the experimental set physico-chemical based sorptive removal was eliminated and the degradation data represented herewith are confined to the biological process only. Initial concentration of TPH in all the four reactors was almost same (758 mg/g of sludge). All the reactors showed higher TPH removal on 10th day (D, 371 mg/g of sludge; C, 442 mg/g of sludge; B, 482 mg/g of sludge; A, 594 mg/g of sludge) compared to 5th day (D, 554 mg/g of sludge; C, 594 mg/g of sludge; B, 638 mg/g of sludge; A, 669 mg/g of sludge) which might be attributed to the adaptation and survivability of potential TPH/PAH degraders in the reactor environment (Fig. 1a). Higher removal of TPH was observed in reactor D (51.05%) followed by reactor C (41.68%), reactor B (36.41%) and reactor A (21.63%). Control operation (reactor A) showed least TPH removal which might be due to the absence of soil microflora. Higher the soil fraction, higher will be the TPH degradability. The oil sludge degradability was found to depend on the soil concentration in the reaction mixture which might be due to the concentration of microbial community in the soil [7].

Degradation of the fractions was found to depend on the soil concentrations employed. All the TPH fractions showed maximum degradation at higher soil concentrations. Among which, aromatics showed higher degradation followed by aliphatics, NSO and asphaltenes. Aromatics and aliphatics showed almost similar and higher degradation efficiency, while the other components showed less degradation efficiency. All the fractions showed higher removal at 10th day in reactor D (aromatics, 48.1%; aliphatics, 47.3%; NSO, 28.6%; asphaltenes, 26.6%) followed by reactor C (aromatics, 43.4%; aliphatics, 39.2%; NSO, 19.1%; asphaltenes, 15.8%), reactor B (aromatics, 38.1%; aliphatics, 34.2%; NSO, 16.5%; asphaltenes, 14.8%) and reactor A (aromatics, 16.5%; aliphatics, 11.4%; NSO, 6.9%; asphaltenes, 6.1%). Degradation of aromatics in the present study was observed to be higher when compared to the previous remediation works reported using mixed culture as biocatalyst. There observed a 35% degradation in the aromatic fraction from the crude oil [16]. Reports earlier showed 90% degradation of the aliphatic fraction using a two strain defined culture (*Pseudomonas* and *Bacillus*) [17]. Compared to aromatics, aliphatic compounds require less energy for degradation. However, the noticed higher degradation of aromatics over aliphatics might be due to the breakdown of aromatic rings to straight chain compounds (aliphatics). This might also be due to the capability of the organisms to degrade aromatics. Least degradation of asphaltenes might be due to their insoluble nature which makes them unavailable to the biocatalyst.

3.2. PAH compounds

Inspite of complex nature, aromatic fraction showed higher degradation among other constituents of TPH. Twelve individual PAHs with different ring structures were studied in detail to evaluate the degradation pattern of aromatics. Maximum PAHs degradation was observed in reactor D during 10th day of operation (Fig. 1b, Table 1). Higher ring compounds showed lower degradation due to their complexity, higher hydrophobicity, high molecular weight and ring angularity, while, lower ring compounds with simple structures showed higher degradation. As in the case with TPH and individual fractions, reactor D showed higher degradation with individual PAH compounds later followed by reactor C and reactor B. Reactor A showed negligible removal pattern compared to other reactors. Among the twelve individual PAH compounds, naphthalene showed higher degradation (81%) due to its simple structure and volatility. Naphthalene degradation was later followed by phenanthrene (77%), fluorene (75%), anthracene (74%) and acenaphthylene (73%). Among the three ring compounds, acenaphthylene showed marginally less degradation due to its higher hydrophobic nature. In the case of four ring compounds, chrysene showed higher degradation (74%) compared to pyrene (71%), benzo(A)anthracene (63%) and fluoranthene

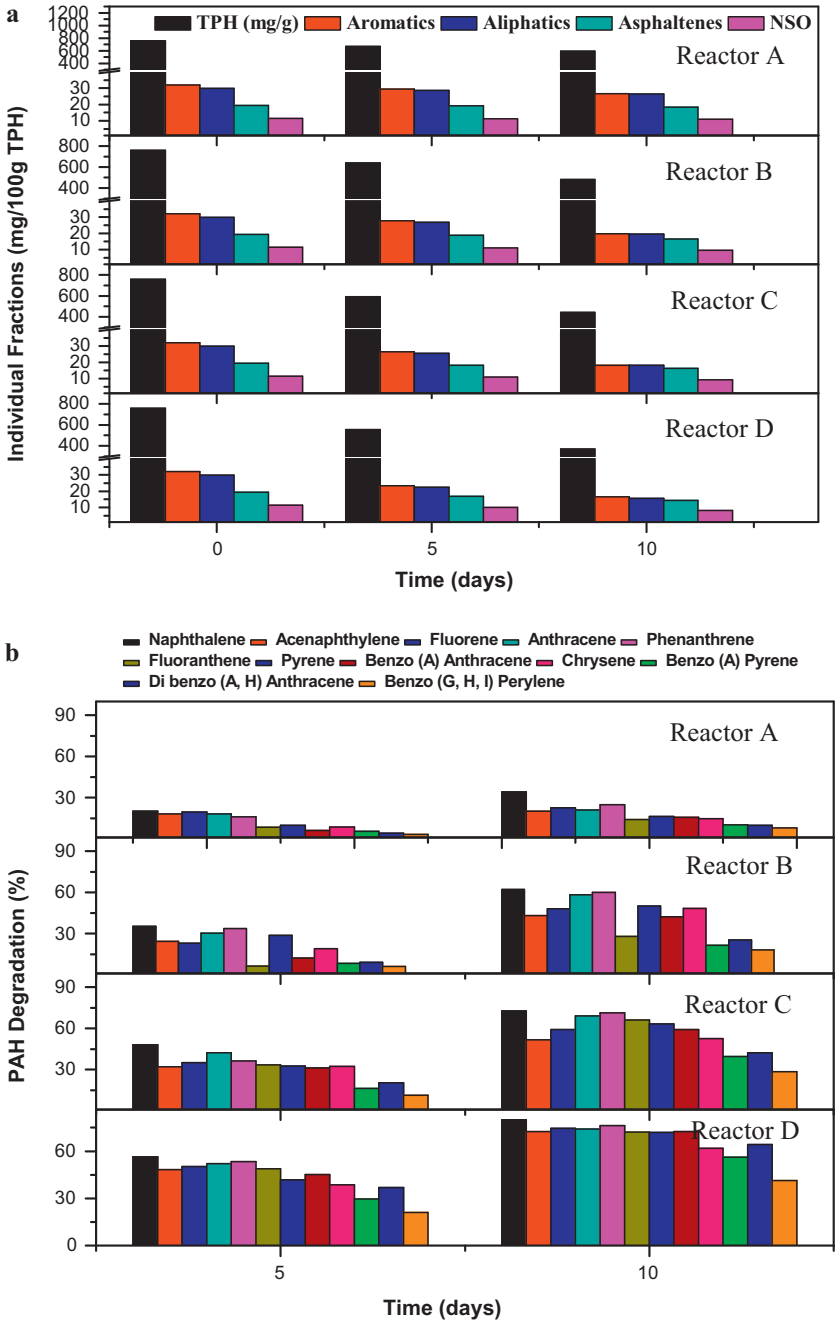

Fig. 1. (a) Degradation profile of individual fractions in reactor A, reactor B, reactor C and reactor D under aerobic remediation during the period of 10 days. (b) Degradation percentage of individual PAH compounds in reactor A, reactor B, reactor C and reactor D evaluated using HPLC during 5th day and 10th day.

(59%). Among the five ring compounds dibenzo(A,H)anthracene showed higher degradation (65%) followed by benzo(A)pyrene (57%). Benzo(G,H,I)perylene a six ring compound showed maximum degradation of 42%.

3.3. Dehydrogenase activity

Dehydrogenase (DH) enzyme is involved in the oxidation reduction reactions and releases protons and electrons which are used for the cleavage of compounds. Generally, bacteria metabolize PAH compounds via initial ring oxidation by dioxygenases to form *cis*-dihydrodiols, which are transformed to catechol compounds by dehydrogenases and further get oxidized to simpler compounds [18]. In the present study, DH concentration was observed to increase from 1st day to 10th day for all the reactors which may be due to the active and effective functioning of the existing bacteria (Fig. 2). During day one, the organisms present in the soil were in inactive stage, after supplementing with domestic sewage the organisms utilised the native nutrients, became active and documented higher DH activity. All the reactors showed higher activity on 10th day. Among the reactors, reactor D showed higher enzyme activity (12.01 μg/ml of toluene), followed by the reactors C (11.87 μg/ml of toluene), B (6.94 μg/ml of toluene) and A (5.12 μg/ml of toluene). The DH activity correlated well with the degradation of PAH compounds.

Table 1
Type of PAH compounds, number of aromatic rings, concentration studied at different time intervals.

PAH compound	No. of rings	Concentration of PAHs (μg/ml)								
		Reactor A			Reactor B		Reactor C		Reactor D	
		0thday	5th day	10th day	5th day	10th day	5th day	10th day	5th day	10th day
Naphthalene	2	276.5	220.9	182.2	179.5	104.5	143.5	75.5	120.6	55.0
Acenaphthylene	3	58.6	48.1	46.8	44.2	33.3	39.8	28.3	30.3	16.1
Fluorene	3	84.2	67.7	65.1	64.7	43.6	54.6	34.4	41.9	21.4
Anthracene	3	49.2	40.2	38.8	34.3	20.5	28.3	15.2	23.5	12.7
Phenanthrene	3	101.5	85.1	76.2	67.4	40.5	64.6	29.0	47.3	24.1
Fluoranthene	4	140.3	128.4	120.6	131.2	100.7	93.4	47.4	71.5	39.1
Pyrene	4	171.6	154.6	143.5	122.4	85.6	115.7	63.0	100.0	48.0
Benzo(A)anthracene	4	295.3	276.8	249.4	259.2	170.2	203.1	120.3	161.9	81.1
Chrysene	4	37.1	33.8	31.6	30.1	19.1	25.0	17.5	22.7	14.1
Benzo(A)pyrene	5	158.9	150.2	142.6	145.6	124.6	132.8	95.9	111.8	69.5
Dibenzo(A,H)anthracene	5	124.81	119.6	112.4	113.4	93.1	99.4	72.0	78.7	44.6
Benzo(G,H,I)perylene	6	72.9	70.6	67.2	68.4	59.6	64.7	52.1	57.5	42.7

3.4. Bioprocess monitoring

Aqueous phase pH prior to startup was adjusted initially to 7. After the experimental run, the final pH in all the reactors showed a shift towards basic condition (Fig. 3). The increment in pH towards basic condition might be due to the release of basic metabolites. Both reactor D and reactor C showed higher pH values, where reactor B showed slight increment. However, the pH in control condition did not show any significant variations throughout the operation. DO is crucial for effective functioning of an aerobic bioreactor and also an index to understand the biochemical activity occurring in the reactor microenvironment. During the startup period of the cycle, DO in all the reactors was around 4 mg/l. During the experimental run DO levels decreased with time in all the reactors indicating the presence of microbial activity (Fig. 3). Control operation (reactor A) showed negligible decrement in DO due to lower microbial activity (3.5 mg/l). Reactor D showed comparatively higher surge in DO levels (from 4.0 mg/l to 1.4 mg/l) which might be due to higher metabolic activity resulting in oxygen consumption. Next to reactor D, reactor C showed higher DO surge (2.2 mg/l) followed by reactor B (3 mg/l).

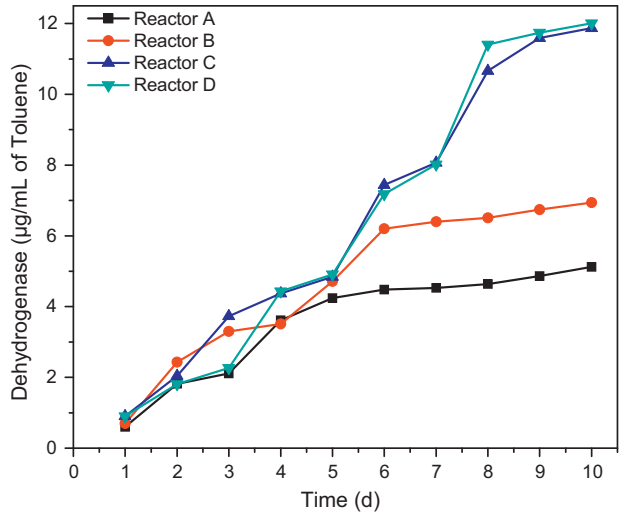

Fig. 2. Variation in dehydrogenase activity with time in four reactors during aerobic remediation.

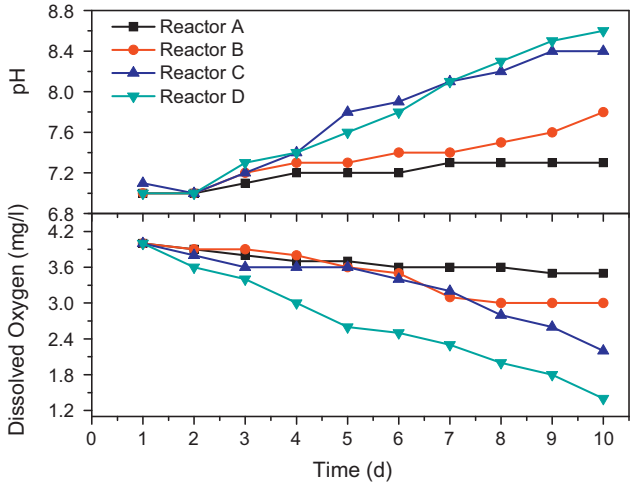

Fig. 3. Bioprocess monitoring in terms of pH and DO against time in four reactors during aerobic remediation.

3.5. Structure of microbial community

DGGE profiles generated using the universal bacterial primers based on V3 region of 16S rRNA gene demonstrated structural composition of communities in the reactor D during 0, 5 and 10 day of operation. Each of the distinguishable bands in the separation pattern represents an individual bacterial genus (Fig. 4a). The phylogenetic distribution was established with a neighbor-joining method (Fig. 4b). Some bands migrated to similar position suggesting the existence of common bacterial population. Bands that show different migration behaviour were sequenced and phylogenetically classified. Multiple bands may have possibly resulted from the microheterogenecity of 16S rRNA nucleotide sequences within a strain. In total 12 dominant true operational taxonomic units (OTUs) were identified which can be divided into five classes viz., *α-proteobacteria*, *β-proteobacteria*, *γ-proteobacteria*, *Uncultured bacteria* and *Firmicutes* (Table 2). Major bands were phylogenetically related to class *γ-proteobacteria* (25%) followed by *β-proteobacteria* (16.6%), *α-proteobacteria* (8.3%), *Uncultured bacteria* (33.3%) and *Firmicutes* (16.6%).

Among all the classes *γ-proteobacteria* and its members *Pseudomonas* were found to be dominant on 0th and 5th day and then the population declined on 10th day. These are gram negative bacteria and are both aerobic and facultative anaerobes known for degrading petroleum compounds [19,20]. Previous results have reported complete degradation of phenanthrene, naphthalene and anthracene by a *Pseudomonas* isolate and *P. aeruginosa* [21]. Some of the species of *Pseudomonas* viz., *P. citronellolis* and *P. aeruginosa* are reported to have the ability to degrade phenanthrene and pyrene in soil. Hwang and Cutright reported that bioaugmentation with *P. aeruginosa* achieved an extensive increase in the total

Table 2
Phylogenetic sequence affiliation and similarity to the closet relative of amplified 16 rDNA sequence excised from DGGE.

Band number	Blast similarity (%)	Organism	Accession number	Phylogenetic affiliation
SVM-1	88 88	*Uncultured bacterium* *Uncultured bacterium*	HM328532.1 HM325973.1	Bacteroidia
SVM-2	89 90	*Firmicutes* *Uncultured bacterium*	GU958683.1 HM831087.1	Firmicutes
SVM-3	86 86	*γ-Proteobacterium* *γ-Proteobacterium*	GU594670.1 DQ117539.1	*γ-Proteobacteria*
SVM-4	97 97	*Pseudomonas* sp. *Uncultured bacterium*	FJ013297.1 EF149071.1	*γ-Proteobacteria*
SVM-5	90 90	*α-Proteobacterium* *α-Proteobacterium*	GU230227.1 GQ350382.1	α-*Proteobacteria*
SVM-6	98	*Uncultured bacterium* *Uncultured bacterium*	AB286561.1 AB286560.1	Bacteroidia
SVM-7	95 95	*Comamonas* sp. *Comamonas* sp.	GU255474.1 GU255473.1	*β-Proteobacteria*
SVM-8	97 97	*Uncultured bacterium* *Uncultured bacterium*	AB286481.1 AB286474.1	Bacteroidia
SVM-9	88 87	*β-Proteobacterium* *β-Proteobacterium*	FR682708.1 GU213315.1	*β-Proteobacteria*
SVM-10	92 92	*Jeotgalibacillus* sp. *Marinibacillus* sp.	FR693626.1 GQ169103.1	Firmicutes
SVM-11	93 93	*Uncultured bacterium* *Uncultured bacterium*	EU790065.1 EU316208.1	Bacteroidia
SVM-12	88 88	*Lysobacter defluvii* *Lysobacter* sp.	AM283465.1 DQ490982.1	*γ-Proteobacteria*

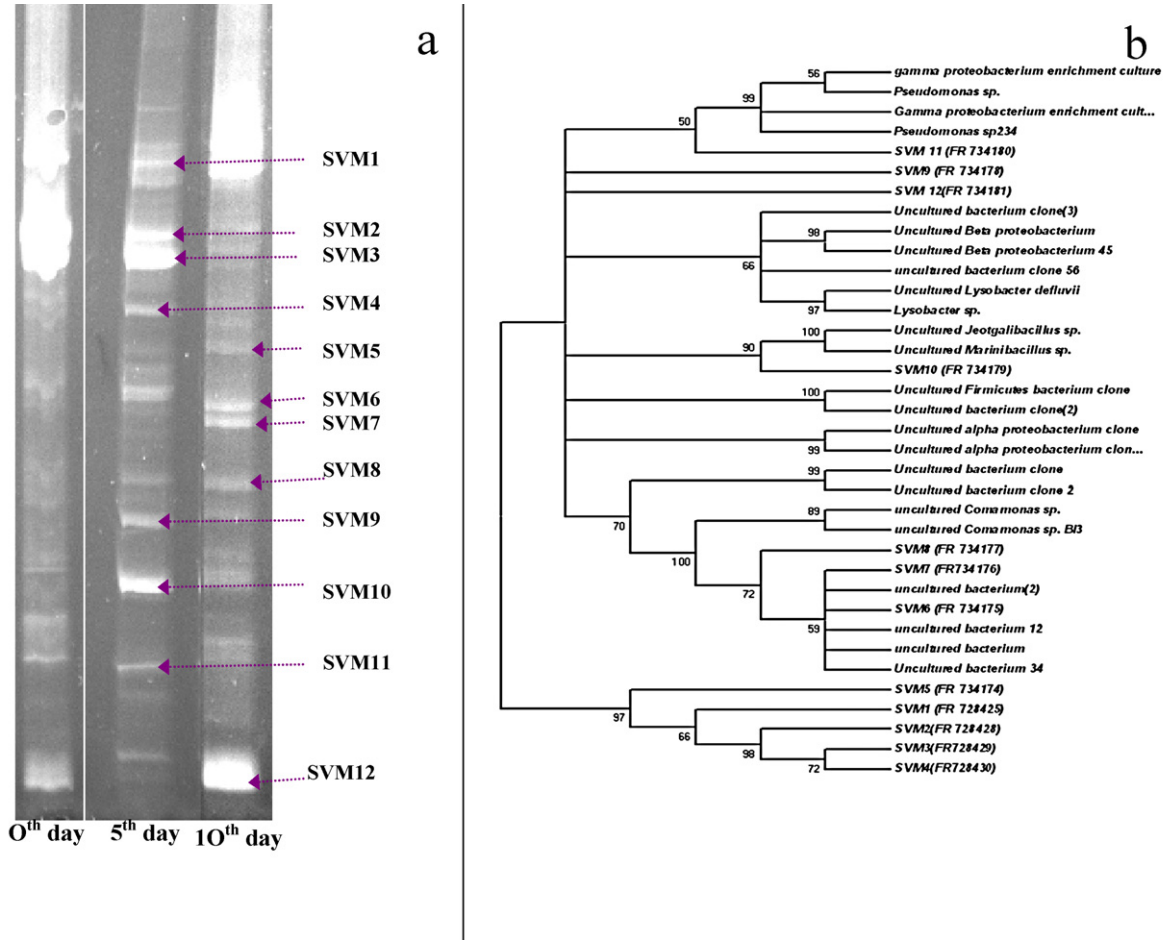

Fig. 4. Microbial diversity analysis of reactor D (a) Denaturing gradient gel electrophoresis profile of PCR amplified V3 region of 16S rDNA in the microbial communities; (b) neighbor-joining trees constructed using Mega 4.0 showing phylogenetic relationships of 16S rDNA sequences from closely related sequences from GenBank.

biodegradation of phenanthrene and pyrene in soil [22]. *Pseudomonas cepacia*, have the higher capacity to degrade benzo(A)pyrene, dibenzo(A,H)anthracene as sole carbon and energy sources, there was a 20–30% decrease in the concentration of benzo(A)pyrene and dibenzo(A,H)anthracene [23]. Improved degradation and phenanthrene solubility was also observed when surfactants produced by *P. aeruginosa* were added [24]. *Lysobacter defluvii* one of the *γ-proteobacteria*, which have the ability to degrade naphthalene, phenanthrene and carbazole [25]. This organism has high resistance against PAHs compounds which was evidenced by appearance of bright band during 0th, 5th and 10th day. After *γ-proteobacteria*, *β-proteobacteria* and *Comamonas* sp., member of *β-proteobacteria* were found to be dominant. These are aerobic, facultative and efficiently used in the degradation of phenanthrene and PAHs. *Comamonas* sp. have the capacity to degrade organic compounds by utilizing some of the NSO compounds viz., quinoline and carbazole as a sole carbon and energy source [26]. It was also reported that it can be utilised p-chloroaniline as a sole carbon, energy and nitrogen source [27]. *Comamonas* sp. showed high band intensity on 10th day while less on 5th day and absence on 0th day. Where as *β-proteobacteria* showed dominance on 5th day and 10th day and absence on 0th day. Next to *β-proteobacteria* the band pattern detected the dominance of *α-proteobacteria*. These are gram negative aerobes which enhance the bioremediation of hydrocarbons, TPH and PAHs [28]. Bands showed high intensity on 10th day, low on 5th day and absence on 0th day. Along with the above mentioned classes the dominance of *Uncultured bacteria* was also observed. Four bands of *Uncultured bacteria* displayed little similarity, but were evolutionarily close. We assume these four bands may represent some of the new genera. Among these SVM1 showed bright bands on all the three intervals viz., 0th, 5th and 10th days. Where as SVM6, SVM8 showed bright band on 10th day, light bands on both 5th and 0th day. SVM11 showed bright bands on 0th day and 5th day where as light bands on 10th day. *Firmicutes* and its members *Marinibacillus* are gram positive, motile, aerobic and facultative. These are the dominant hydrocarbon degraders and play a major role in degrading PAHs from crude oil. During the diversity studies the population was observed to be dominant on 0th and 5th day but low on 10th day. Studies evidenced, greater ability of *Bacillus* sp. to degrade hydrocarbons and PAH compounds (pyrene) [29]. Some of the species of *Bacillus* are known for the degradation of lower ring compounds viz., naphthalene [30]. *Bacillus subtilis*, a member of *Firmicutes* produces a lipopeptide surfactant named surfactin which can reduce the surface tension of an aqueous solution. Along with *Bacillus*, *Pseudomonas* is also reported as hydrocarbon degrader [31]. Presence of these potential species of *Bacillus* and *Psuedomonas* in the reactor supported the efficient degradation of the four ring compounds.

Both the presence and intensity of the organisms were varied in accordance to the time intervals. 0th day showed bright bands of *γ-proteobacteria*, *Firmicutes* and *Uncultured bacterium* (SVM1, SVM11), light bands of *Uncultured bacterium* (SVM6, SVM8) and absence of *α-proteobacteria* and *β-proteobacteria*. 5th day showed dominance of *γ-proteobacteria*, *Firmicutes*, *β-proteobacteria* and *Uncultured bacterium* (SVM1, SVM11), low band intensity of *Uncultured bacterium* (SVM6, SVM8) and *α-proteobacteria*. 10th day showed high intense bands of *Lysobacter defluvii*, *Uncultured bacterium* (SVM1, SVM6, SVM8), *α-proteobacteria* and *β-proteobacteria*, low intensity of *γ-proteobacteria*, *Uncultured bacterium* (SVM11) and *Firmicutes*. During time intervals 5th day and 10th day showed sharp increase in the number of PAHs degrading microbial populations with increase in time, which was well correlated with the observed TPH/PAHs degradation. Literature described both increase and decrease in the microbial diversity during the experimental run. Our results matched with previous reports [32], whereas Vinas et al. reported a decrease in diversity in their experiments [7]. *α-Proteobacteria* and *β-proteobacteria* are absent on 0th day and showed their presence on 5th day and 10th day due to the utilization of domestic sewage. *Firmicutes*, *uncultured bacterium* and *γ-proteobacteria* showed high intensity at 0th day, low intensity on 5th day and 10th day. This is due to the accumulation of metabolites resulting from the oxidation of PAHs which can reduce the viability of several PAHs degraders [33]. The study performed at various soil concentrations demonstrated efficient and rapid degradation of petroleum based oil sludge under aerobic conditions. Enhanced degradation of aromatic compounds was significantly noticed. Diversity studies detected the presence of specific strains surviving in PAHs microenvironment.

4. Conclusion

Aerobic bioremediation of real-field petroleum sludge was studied using various soil concentrations was found to be effective and dependent on the soil supplementation. The results demonstrated maximum degradation of aromatic compounds with high soil concentration and significant variation amidst experimental and control conditions. Lower ring compounds showed higher degradation indicating dependency of degradation on ring complexity. Microbial community analysis was found to vary in accordance with time and detected the presence of efficient aromatic degrading organisms. The redox behaviour pattern and dehydrogenase activity showed an increasing trend with increasing degradation while dissolved oxygen showed decreasing trend with increasing degradation time.

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