Bioresource Technology 242 (2017) 15–27

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Enrichment and characterization of hydrocarbon-degrading bacteria from petroleum refinery waste as potent bioaugmentation agent for *in situ* bioremediation

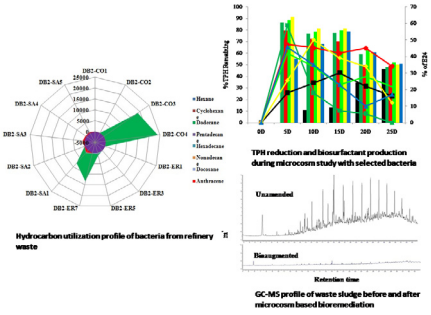
Poulomi Sarkar [a], Ajoy Roy [b], Siddhartha Pal [b], Balaram Mohapatra [a], Sufia K. Kazy [b], Mrinal K. Maiti [a], Pinaki Sar [a,*]

[a] Department of Biotechnology, Indian Institute of Technology Kharagpur, Kharagpur 721302, India
[b] Department of Biotechnology, National Institute of Technology Durgapur, Durgapur 713209, India

HIGHLIGHTS

- Petroleum refinery waste of Digboi refinery harbours hydrocarbonclastic bacteria.
- Isolation of superior hydrocarbon degrading bacteria achieved only by enrichment.
- Metabolic versatility of *Burkholderia, Pandoraea* and *Enterobacter* spp. explored.
- Microcosm study identified selected strains as potent bioaugmentation agent.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 31 January 2017
Received in revised form 30 April 2017
Accepted 2 May 2017
Available online 4 May 2017

Keywords:
Petroleum refinery waste
Bioaugmentation
In situ bioremediation
Burkholderiaceae
Enrichment
TPH reduction

ABSTRACT

Intrinsic biodegradation potential of bacteria from petroleum refinery waste was investigated through isolation of cultivable strains and their characterization. *Pseudomonas* and *Bacillus* spp. populated the normal cultivable taxa while prolonged enrichment with hydrocarbons and crude oil yielded hydrocarbonoclastic bacteria of genera *Burkholderia*, *Enterobacter*, *Kocuria*, *Pandoraea*, etc. Strains isolated through enrichment showed assemblages of superior metabolic properties: utilization of aliphatic (C6-C22) and polyaromatic compounds, anaerobic growth with multiple terminal electron acceptors and higher biosurfactant production. Biodegradation of dodecane was studied thoroughly by GC–MS along with detection of gene encoding alkane hydroxylase (*alk*B). Microcosms bioaugmented with *Enterobacter*, *Pandoraea* and *Burkholderia* strains showed efficient biodegradation (98% TPH removal) well fitted in first order kinetic model with low rate constants and decreased half-life. This study proves that catabolically efficient bacteria resides naturally in complex petroleum refinery wastes and those can be useful for bioaugmentation based bioremediation.

1. Introduction

The rapid expansion of petroleum and allied industries has added to the economic prosperity of India (and many other countries as well), but led to the generation of large volume of hazardous oily sludge (>28000 tons/annum in India and >60 million tons/annum globally) that warrants immediate attention (Hu et al., 2013; Solanki et al., 2015). Sustainable and affordable disposal/remediation of all petroleum hydrocarbon (HC) rich waste sludge/contaminants is a prime technological impediment and scope for microbial bioremediation has emerged as the most feasible, yet effective solution to attain complete pollutant degradation (Cerqueira et al., 2011). Hydrocarbon degrading microorganisms

* Corresponding author.
E-mail address: psar@hijli.iitkgp.ernet.in (P. Sar).

http://dx.doi.org/10.1016/j.biortech.2017.05.010
0960-8524/

are ubiquitous in natural as well as impacted environments (Bell et al., 2013; Kostka et al., 2011; references therein) and biodegradation was shown to be successful in remediating oil/HC contamination naturally and/or with engineered strategies (Brooijmans et al., 2009; Fuentes et al., 2014). Bioremediation can be accomplished by either boosting the growth of indigenous microbial community through biostimulation or by introducing organisms with superior catabolic abilities (bioaugmentation) (Agnello et al., 2016). Considering the theoretical limitation of bioremediation posed by the requirement of 'combined capacity of catabolic pathways', bioaugmentation with catabolically superior bacteria has been identified to be more advantageous to achieve improved remediation (Dueholm et al., 2015).

The ubiquity of diverse microbial groups with enzymatic capabilities necessary for the *in situ* breakdown and mineralization of broad range of toxic petroleum hydrocarbons have been widely reported from diverse petroleum HC enriched environment (Fuentes et al., 2014; Kostka et al., 2011; Rodriguez-R et al., 2015). Although isolation-characterization of microbial populations from diverse HC rich environments including oil reservoirs, oil spill sites, contaminated rivers, and other environment relatively well studied (Das and Kazy, 2014; Hilyard et al., 2008; Lamendella et al., 2014), there is a paucity of knowledge on indigenous bacterial communities that simultaneously catalyze alkane and aromatic degradation in petroleum refinery waste sites (Sarkar et al., 2016). Particularly, information on the identity of the cultivable members of the community within petroleum HC enriched waste sludge and evaluation of their abilities for actively degrading the constituent HCs have not been specifically addressed. Isolation and evaluation of strains for degradation of a broad range of HCs present in petroleum wastes are advantageous over culture independent molecular approaches (Hilyard et al., 2008). Isolated strains are useful to (i) elucidate links between phylogeny and catabolic abilities, (ii) explore sources of novel HC degrading genes, (iii) for bioaugmentation in HC contaminated sites/wastes. Introduction of allochthonous bacteria into the contaminated sites often show reduced efficacy due to lack of their adaptability (Abed et al., 2014) and therefore isolation of metabolically superior bacteria from the same habitat and their use as bioaugmentation agent is a sought after technique for developing more effective bioremediation processes (Dueholm et al., 2015; Tao et al., 2016).

With increased economic pressure for producing more oil, priority for sustainable management of HC enriched wastes remained high for the foreseeable future, and hence the development of improved biotechnological processes for their treatment gets prioritized. Isolation and characterization of petroleum HC degrading bacterial groups from refinery waste and evaluation of their candidature as bioaugmentation agent are considered a prerequisite for directing the bioremediation strategies for refinery waste/similar systems (Kostka et al., 2011). Thus, using the refinery waste sludge of Digboi oil refinery, Indian Oil Corporation Limited, Digboi, India, the present study was designed with following objectives: (i) to identify and unravel the ecophysiology of hydrocarbonoclastic bacterial that may serve as bioaugmentation agent (ii) to evaluate performance of the selected strains for *in situ* bioremediation. The entire study depicts the usefulness of bacterial strains as bioremediation candidates for eradicating perilous petroleum enriched refinery wastes and other petroleum contaminated environments.

2. Materials and methods

2.1. Sample collection and characterization

Petroleum refinery waste composed of semi-solid oily sludge (designated as DB2) were aseptically collected from ~30 cm below the top layer of a waste disposition site of Indian Oil Corporation Ltd. (IOCL) oil refinery, Digboi, Assam, India (27° 23′ 33.45″ N and 95° 36′ 55.458″ E) during June 2012. Collected samples were stored at −20 °C in air-tight (Schott-Duran) glass bottle up to the brim and transported to laboratory within 48 h. Enrichment and isolation of cultivable populations were started immediately as the samples reached the laboratory. Major physicochemical parameters (temperature, pH, etc.) were measured on site using OrionStar140™ multiparameter meter (Thermo Electron Corporation, USA). Total petroleum hydrocarbon (TPH) was measured by gravimetric method (Sarkar et al., 2016) followed by gas chromatography (GC) (Perkin Elmer Clarus 580, USA). Major anions were quantified through ion chromatography (IC) (Thermoscientific ICS Dionex 2100, USA) and metals were estimated using inductively coupled mass spectrometry (ICPMS) (Varian Palo Alto CA USA) and /or atomic absorption spectroscopy (AAS) (Perkin Elmer MA, USA). Details of the methods are same as described in Sarkar et al., (2016) and discussed in the later section.

2.2. Enrichment, enumeration and isolation of cultivable bacteria

Bacterial strain enumeration and isolation were done using (a) direct isolation through plating on to Reasoner's 2A (R2A) agar medium and (b) following enrichment with various aliphatic and aromatic hydrocarbons (HCs) and plating on multiple agar media. Heterotrophic bacterial cells from the refinery waste sample were enumerated by dislodging one gram sample in sodium pyrophosphate (0.1% w/v) for 12 h at 30 °C, 150 rpm followed by dilution plating on to Reasoner's 2A (R2A) (Himedia, India) agar plates and incubation at 30 °C. CFUs (colony forming units) were counted at periodic interval till 7 days and morphologically distinct colonies were collected. Enumeration of hydrocarbonoclastic cultivable bacteria and their isolation through enrichment was done in three different media, *viz*., Bushnell-Haas (BH) (Himedia, India) (for isolation of hydrocarbon degrading population), M9 (Ciric et al., 2010) (for assessing the effect of minimal inorganic nutrients on bacterial growth and diversity) and reduced BH (0.1% w/v cystein-HCl added and nitrogen purged, for anaerobic members). Enrichment experiments were set up with one gram waste sample as initial inoculum in Bushnell-Haas (BH), M9 and reduced BH (0.1% w/v cystein-HCl added and nitrogen purged) media with two carbon source supplements:1-crude oil (1% v/v) and mixture of each 1% v/v (hexane, dodecane, pentadecane, hexadecane, docosane and nonadecane) of aliphatic and aromatic 1% v/v (BTEX) or 1% w/v (naphthalene, anthracene) hydrocarbons and 2-crude oil (1% v/v). For the anaerobic enrichments reduced BH medium along with crude oil (1% v/v) was used as the sole carbon source and all the transfers were done inside anaerobic chamber (Coy Labs, USA). Enrichments were incubated at 30 °C in static (anaerobic) or shaking (150 rpm) (aerobic) conditions. Following growth (15 days) each enrichment broth were subcultured (thrice) in the same medium with similar HC mixture and incubated. After 45 days of growth distinct colonies were isolated from the three enrichment setups by plating 100 μl culture on to BH and M9 agars (overlaid with 0.5% v/v crude oil) incubated at 30 °C and on to the anaerobic agar plates incubated at 30 °C within anaerobic jar filled with N_2. Colonies appeared on each plates were inspected for morphological details, and unique colonies were picked up. All colonies obtained were subcultured thrice to check culture purity; all pure cultures were designated and stored (15% glycerol, −80 °C) for future analysis. Bacterial cells obtained from each of the isolation methods were designated as DB2 (direct isolation), DB2AN (enrichment – anaerobic), DB2CO (enrichment –only crude oil in BH medium), DB2ER (enrichment- only crude oil in M9 medium) and DB2SA (enrichment – HC mix in BH medium) along with their strain designations.

2.3. Molecular identification of isolated bacterial strains

Identification of bacterial strains was done by analyzing the 16S rRNA gene from respective genomes. Genomic DNA was extracted from each of the strains following standard procedure, 16S rRNA gene was PCR amplified and sequenced. Details of these methods including primers and PCR conditions are same as described elsewhere (Paul et al., 2015). Sequences were analyzed through similarity search in NCBI GenBank and RDP database followed by phylogenetic interpretation through constructing Neighbour Joining tree using Jukes Cantor algorithm with 1000 bootstrap values in MEGA 6.0 (Koichiro et al., 2011).

2.4. Metabolic characterization of bacterial strains

Metabolic ability of the representative aerobic strains was ascertained by performing different biochemical and eco-physiological tests. Tolerance of the strains towards various physiological stresses was assessed by growing them under different pH (3–10), temperature (5 °C–45 °C) or NaCl (1–5% w/v) concentrations in R2A with required modifications at 150 rpm. For rest of the experiments neutral pH, 30 °C and 150 rpm were used unless otherwise stated. For assessment of different heavy metals separate 50 mM stock solutions of arsenite (As^{3+}) cadmium (Cd^{2+}), mercury (Hg^{2+}), nickel (Ni^{2+}) and lead (Pb^{2+}) were prepared and filter sterilized. Strains were inoculated in BH medium (inorganic phosphate replaced with glycerol phosphate and 2% yeast extract as carbon source) and each heavy metals in different concentrations [As^{3+} (0.6 mM), Cd (0.4 mM), Hg (0.24 mM), Ni (0.85 mM) and Pb (0.24 mM), and a mixture of all of them (0.3 mM final concentration)] and incubated under similar conditions. The cell growth was calculated by monitoring cell turbidity measured as optical density at 600 nm for liquid broth or appearance of visible bacterial colonies up to 24–72 h of incubation (in case of solid medium. Optical density of $\geq$0.3 at 600 nm was considered as positive growth. Growth with BTEX was measured using BH agar plates, briefly each strain was inoculated on BH agar plates and a sterile filter paper soaked with 2% v/v BTEX was adhered to the inner side of the petri plate lid and the plates were incubated inside a glass chamber saturated with BTEX. Emulsification index (E24) as a measure of biosurfactant produced by the test strains with different HCs and/or crude oil following published method (Mnif et al., 2011). Swimming motility and production of siderophore were evaluated as per reported protocols (Paul et al., 2015). For determining the catalase activity of each of the bacterial strains overnight grown pure colonies were subjected to 0.3% v/v hydrogen peroxide solution. Formation of effervescence by the colonies was indicated as positive response. Oxidase test was performed using by soaking oxidase discs (HiMedia, India) with distilled water followed by swabbing of overnight grown bacterial colonies in each disc.

2.5. Growth with aliphatic and aromatic HCs and measurement of cell surface hydrophobicity

Bacterial cell growth in the presence of wide range of aliphatic (*viz.,* hexane, cyclohexane, dodecane, pentadecane, hexadecane, nonadecane, docosane), aromatic [*viz.,* benzene, toluene, ethylbenzene and xylene (BTEX), naphthalene, anthracene] and crude oil was tested using BH medium. For all the compounds, except BTEX, BH broth was amended with 2% (v/v) of the test substance, inoculated with overnight grown culture ($\sim10^5$–10^6 CFU/ml) and incubated (up to 14 days, at 30 °C, 150 rpm). Growth was estimated measuring CFU at an interval of 48 h. Emulsification index (E24) was determined for each of the strain using the method described above. Growth with BTEX was measured using BH agar plates containing 2% v/v BTEX.

Strains showing superior growth with HCs were subjected to Microbial Adhesion To Hydrocarbons (MATH) assay to determine their cell surface hydrophobicity following the method described by (Brima et al., 2010).

2.6. Alkane (dodecane) metabolism by selected bacterial strains and detection of alkane hydroxylase (alkB) gene

Hydrocarbon biodegradation by selected bacterial strains was studied in detail with dodecane as the substrate. Overnight grown cultures of the selected strains were inoculated (with final cell densities $\sim10^6$–10^7 CFU/ml) in BH medium amended with dodecane (100 mM) and incubated (at 30 °C, 150 rpm). Control sets with only yeast extract and uninoculated BH medium with 100 mM dodecane were also set up. Cell growth was monitored by determining CFUs/ml at 24 h interval using BH agar medium overlaid with dodecane (0.5% v/v). Degradation of dodecane was measured by extracting the HC with and equal volume of hexane, evaporation to reduce volume and analysis by GC-FID (details provided in the Supplementary information).

To investigate possible metabolic pathway of alkane degradation, cellular fatty acids were extracted from all the selected strains at the end of their growth with dodecane, esterified and analyzed by GC-FID (details provided in Supplementary information). Gene encoding alkane hydroxylase enzyme, the key component in bacterial alkane degradation, *alk*B was detected in selected strains by using *alk*B specific primers and following the protocol as described by Kloos et al., (2006).

2.7. Microcosm set up and analysis

Laboratory scale bioremediation experiment was performed with isolated strains as bioaugmentation agent in sludge microcosms. DB2 sludge sample (5 g) was bioaugmented with selected bacterial strains (individually, with initial cell number added $\sim10^5$–10^6 CFU/ml) in serum vials (100 ml) containing modified BH medium (50 ml) and incubated (up to 4 weeks, at 30 °C, 150 rpm). Total seven microcosms (five: with five different bacterial bioaugmentation, one: with no bioaugmentation control and one: 5% (w/w) mercuric chloride killed control) in quadruplet were set up (details of this experimental setup are provided in the Supplementary information). The level of TPH in each set up was determined at five days interval using gravimetric (Sarkar et al., 2016) and GC based method. For this, respective vials were sacrificed, and the content was extracted with hexane-dichloromethane (1:1) and analyzed. Hydrocarbon constituents and intermediates within the most biodegraded sets were ascertained through GC–MS analysis. Chemical oxygen demand (COD) of the samples with and without bioaugmentation by selected bacterial strains was analyzed (Barker et al., 1999). Production of emulsifiers within each microcosm during the bioremediation process was estimated by calculating the E24 index of the aqueous phase (Mnif et al., 2011).

2.7.1. Bioremediation kinetic study and estimation of half-life times of the process

Biodegradation of petroleum contaminated sample was explained by first order kinetics depicted by the following equation (Kuppusamy et al., 2016):

$$Ct = Cie^{-kt} \quad (1)$$

where Ct = remaining TPH concentration at time t (g Kg^{-1}), Ci = initial TPH concentration (g Kg^{-1}), k = biodegradation rate (day^{-1}) and t = time (day).

Biodegradation half life was calculated using the following equation:

$$t_{1/2} = \ln 2/k \quad (2)$$

where ln2 = 0.693 and k = biodegradation rate (day^{-1}).

2.8. Extraction and analysis of total petroleum hydrocarbons form the samples

Solvent extractable petroleum hydrocarbons were extracted from the petroleum refinery waste before and after bioaugmentation treatment using solvents, hexane-dichloromethane (1:1), centrifuged at 12,000 rpm for 30 min, passed through anhydrous sodium sulfate and glass wool and detected using both gas chromatography-flame ionization detector (GC-FID) and gas chromatography-mass spectrometer (GC–MS) (Clarus 580 series, Perkin Elmer, USA). The column program for GC-FID/MS used was initial temperature of 80 °C followed by a ramp of 6 °C/min up to final temperature of 260 °C for 3 min. MS parameters used were: EI^+ mode, Source temperature 200 °C, Electron beam energy 70 eV, mass range 30–450 amu. Nitrogen was used a carrier gas at a constant flow rate of 1 ml/min for both the systems. The data analysis was done by the Total Chrom software.

2.9. Statistical analysis

Statistical correlation between bacterial strains was estimated by comparing their metabolic profiles using unweighted pair group method with arithmetic mean (UPGMA) and principal component analysis. All statistical analyses were done using Multi-Variate Statistical Package (MVSP, version 3.2) and Palaeontological Statistics (PAST, version 3.x). MS-Excel 2007 was used for performing the 2-way ANOVA estimation.

2.10. Nucleotide sequence accession numbers

The nucleotide sequences from this study were deposited in NCBI GenBank under following accession numbers (KM357838-KM357865) and (KX219966-KX219974).

3. Results and discussions

3.1. Sample characteristics

The sludge sample was characterized to be of circumneutral pH (6.84), moderate temperature (35.6 °C), reduced (ORP-182 mV), rich in sulphate (6621 mg/kg) and paucity of nitrate (13.63 mg/kg). Elevated TPH (154 g/kg) content and presence of many toxic heavy metals such as Arsenic (2.3 mg/kg), chromium (48.4 mg/kg), cobalt (2.6 mg/kg), copper (12.9 mg/kg), iron (101.2 mg/kg), lead (2.7 mg/kg), nickel (38.8 mg/kg), zinc (68.2 mg/kg) was also prominent in the waste sample. TPH content was primarily constituted of alkanes (C7-C30) and polyaromatic hydrocarbons. Petroleum hydrocarbon rich wastes from refinery and other production related operations are often characterized with similar properties (Sarkar et al., 2016; Ze et al., 2016) Combination of high TPH, reducing state, low O_2 and insufficient nitrogen and phosphorus nutrients have been attributed to be the limiting factors for intrinsic bioremediation by indigenous microbial communities (Bell et al., 2013; Sarkar et al., 2016., and references therein).

3.2. Enumeration and isolation of bacterial strains

The abundance of heterotrophic bacterial cells was enumerated followed by isolation of pure culture strains using both specific enrichment and direct (non-enrichment) isolation procedures. Enrichment condition yielded nearly three orders of magnitude higher colony counts (2.7×10^9 CFU/g) over the same obtained through the direct count, clearly indicating the efficacy of added hydrocarbons in improving bacterial cultivability. The observation corroborates numerous reports showing a significant increase in viable bacterial counts during enrichment for biostimulation/bioremediation (Kostka et al., 2011; Leahy and Colwell, 1990; Sarkar et al., 2016, references therein). It has been established that during enrichment with HC compounds in nutrient medium bacterial abundance increased as the added compounds provide the major sources of carbon and electrons as well as other essential nutrients (N, P, K, Mg, S, etc.) (Kostka et al., 2011). A total of forty-five morphologically distinct strains were obtained from enrichment (15 isolates) as well as non-enrichment (30 isolates) conditions.

3.3. Taxonomic identification of the isolated bacterial strains

Taxonomic identity of all the isolated bacterial strains (45) was ascertained through 16S rRNA gene sequence analysis. Sequence similarity search in NCBI Genbank and RDP database revealed the presence of culturable members of different genera under the phyla: *Proteobacteria, Firmicutes* and *Actinobacteria* (Fig. 1a-b). The 16S rRNA gene sequences of the test strains showed close phylogenetic relatedness with different strains isolated from similar or other extreme habitats (Fig. 1b) as well as several validly described/type strains (Supplementary Table 2). *Proteobacteria* was composed of α-, β- and γ- subdivisions. γ *Proteobacteria* with its four genera *Pseudomonas*, *Burkholderia, Enterobacter* and *Rahnella* represented nearly 60% of the total population. Twenty-one strains, all obtained through direct isolation were affiliated to the genus *Pseudomonas*. 16S rRNA gene sequences of pseudomonads showed high identity (≥99%) among themselves, with sequences of strains *P. fluorescens*, *P. fragi*, *P. gessardii* and *P. libanensis* and well known hydrocarbon degrading strain *P. putida* KT2440. Members of the genus *Enterobacter* were the next dominant group within γ-*proteobacteria* represented by 3 strains, all isolated via enrichment only. A close phylogenetic relatedness of these three bacteria among themselves, with type strains *E. hormaechei* and *E. asburiae* and HC degrading *Enterobacteria* spp. was evident. Anaerobically isolated bacterium strain DB2-AN4 was identified to be a member of the genus *Rahnella* (*Enterobacteriaceae*) showing 100% sequence identity with drinking water isolate *R. aquatilis*. Nine strains were affiliated to β-proteobacterial genera *Burkholderia, Pandoraea* and *Microvirgula*. Strains affiliated to the genera *Burkholderia* and *Pandoraea* were all isolated from enrichment culture only. 16S rRNA gene sequences of six *Burkholderia* strains shared 100% sequence identity (Supplementary Table 1) among themselves and showed close affinity with trichloroethylene degrading *B. kururiensis* KP23^T and members of the same species isolated from nitrate reducing environment. Strain DB2-CO3 was closely related to hydrocarbon degrading *B. multivorans* DDS 15A-1 and several *Burkholderia* strains isolated from xenobiotic compound contaminated sites. Isolates DB2-7 and DB2-ER7 were the sole representatives of genera *Microvirgula* and *Pandoraea,* respectively. A close relatedness of these strains with nitrogen oxide respiring *M. aerodenitrificans* Sgly2^T and HC degrading strains *P. pnomenusa* MCB032, *Pandoraea* sp. HPC154, respectively, was noted. Strain DB2SA4 showed phylogenetic similarity with a Cu resistant *Brevundimonas* sp. LM18 whereas DB2-SA5 was more related to chlorpyrifos-degrading *Brevundimonas* sp. Dsp-7 and Fe transforming *Brevundimonas* sp. cf01. Six strains isolated from non-enrichment culture were affiliated to the genus *Bacillus* (*Firmicutes*). Detailed phylogenetic analysis of *Bacillus* clade unveiled their closeness with aliphatic polyester degrading (*B. megaterium*

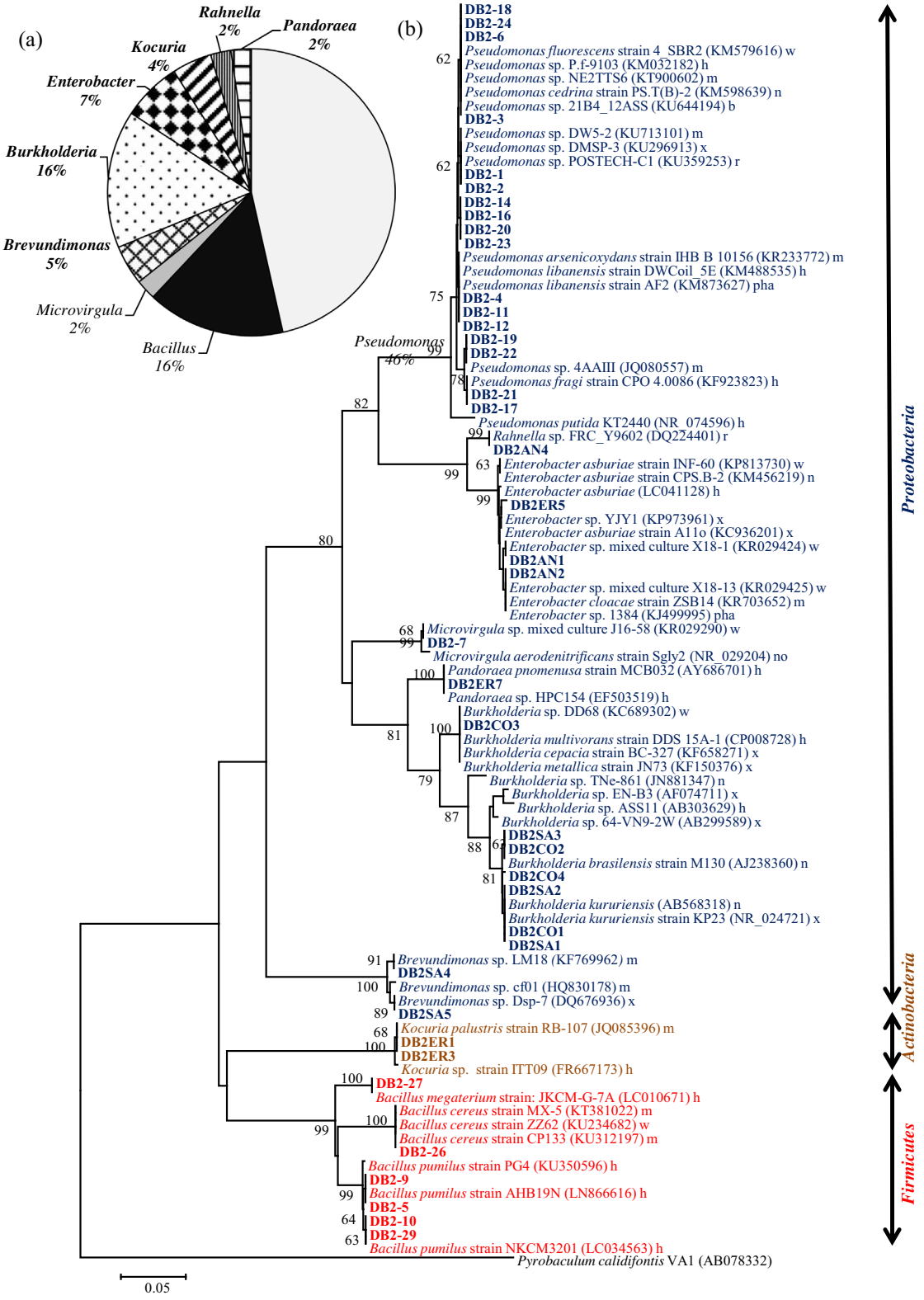


Fig. 1. 16S rRNA gene based molecular identification of isolated bacterial strains. Taxonomic affiliation to different genera (a); and their phylogenetic lineages to closest taxonomic relatives (Neighbour-joining tree based) (b). Sequences obtained in this study were marked in bold. Sequences of the test strains were compared with members of the same taxa obtained from diverse contaminated habitat or possessing specific catalytic activities. Sequences obtained for isolates from diverse contaminated habitats were prefixed with alphabetical notations [h-hydrocarbon contaminated, m-heavy metal contaminated, b-biogas plants, n-nitrate reducing, r-radioactive compound, x-xenobiotic compound contaminated niches and w-wastewater]. Sequences from bacteria with varied metabolic abilities were also indicated: no-nitric oxide respiring and pha-polyhydroxybutyrate (PHA) accumulating.

JKCM-G-7A), multimetal resistant and waste water inhabiting (*B. cereus* MX-5) and with aromatic hydrocarbon/polyester degrading *B. pumilus* spp. Two strains isolated from enrichment culture were identified as members of the genus *Kocuria* and showed close identity with chlorophenol degrading and Cu resistant *Kocuria* spp.

Members of the genera *Pseudomonas*, *Bacillus*, *Burkholderia*, *Enterobacter, Kocuria, Pandoraea* etc. isolated from this waste showed close lineage with previously reported HC degrading members of same genera and most of them have been reported earlier from many petroleum impacted habitats (Benedek et al., 2013; Bhattacharya et al., 2003; Brooijmans et al., 2009; Cheema et al., 2014; Mahjoubi et al., 2013; Uhlik et al., 2012). Our observation on isolation of organisms affiliated to entirely different taxonomic groups following enrichment clearly indicated the effect of incubation with HCs. Detection of *Pseudomonas* and *Bacillus* as sole representatives of populations obtained through direct isolation could be attributed to the much higher cultivability of these two genera in diverse physical settings (Jurelevicius et al., 2009). The present observation on the entirely different set of populations with abundance of *Burkholderiaceae* members and *Enterobacter* following enrichment is in good agreement with earlier reports indicating superior degradative abilities of these members (Bell et al., 2013; Zafra et al., 2014). In the present case, the community was inhabiting a heavily HC rich environment; and amended HC substrates appeared necessary for maintaining the metabolically active populations in enrichment culture (Païssé et al., 2010).

3.4. Metabolic characterization of bacterial strains

Bacterial strains were characterized by a set of general physiological characters as well as for traits relevant for their survival and activities in hydrocarbon enriched condition (Fig. 2). Tolerance to broad pH (3.0–9.0) and temperature (5–40 °C) ranges was observed for most of the strains. Thirty strains affiliated to genera *Bacillus, Pseudomonas* and *Microvirgula* showed their ability to grow even at pH 3.0. Strains of *Enterobacter* and *Kocuria* showed their ability to grow at pH 10.0, followed by a few *Bacillus* and many *Pseudomonas* strains which could grow at pH 9.0. Members of *Burkholdera* remained more sensitive to higher alkalinity and could not grow beyond pH 8.0. *Bacillus* strains showed the broadest (5–45 °C) temperature tolerance followed by *Pseudomonas* strains (5–40 °C). With respect to osmotic stress tolerance, all the 42 strains could withstand a concentration up to 2% NaCl, but few strains affiliated to genera *Burkholderia*, *Pandoraea* could grow up to 4% NaCl and two *Kocuria* strains up to 5% NaCl concentration. Catalase and oxidase activities, motility, production of -biosurfactant, -siderophore and ability to grow under anaerobic condition utilizing diverse alternate terminal electron acceptors (TEAs) were found to be omnipresent among the isolated strains and across diverse taxonomic groups. Among the TEAs tested preference towards nitrate over sulfate followed by iron observed and this could be due to thermodynamic favorability and high reduction potential of nitrate under anoxic conditions (Sarkar et al., 2016). Utilization of all the three TEAs as respiratory substrate was present in *Burkholderia, Kocuria, Enterobacter* and *Pandoraea* strains. The abundance of biosurfactant and siderophore production ability illuminates the necessity of such catabolic processes in solubilizing different HCs and/or accessing iron for cellular metabolism from the relatively not available states (Ahmed and Holmström, 2014; Antoniou et al., 2015).

Preliminary HC utilization data suggested the preference for BTEX by all pseudomonads, whereas members of *Burkholderia* showed an affinity towards hexadecane and naphthalene but not BTEX. BTEX being a volatile compound gets exhausted at early phases of contamination and preference to this compound by certain genera indicates that they may play preliminary role in the degradation of simple hydrocarbons. Aerobic or anaerobic degradation of BTEX by pseudomonads and some members of β-, δ-, or ε-*Proteobacterial* genera is a well illustrated fact (Weelink et al., 2010). Members of the genera *Burkholderia*, *Kocuria, Enterobacter*, *Pandoraea* along with few *Bacillus* and *Pseudomonas* could metabolize crude oil. Most of the strains showed their ability to withstand Hg^{2+} followed by Ni^{2+}, while resistance to Cd^{2+} was more pronounced among the *Bacillus* and *Pseudomonas* strains. Pb^{2+} or As^{3+} resistance remained confined to *Burkholderia*, *Enterobacter, Kocuria* and/or a few *Pseudomonas* strains (for Pb^{2+} only). Capacity to withstand multiple heavy metals simultaneously was found to be restricted only to the members of *Burkholderia, Enterobacter* and *Pandorea*. We have compared the metabolic properties of our strains with validly described members of the same taxa, except *Pseudomonas* strains rest all showed significant dissimilarity to their respective taxonomic neighbors (Supplementary Tables 2 and 3). This analysis indicated that the isolated strains though bear taxonomic relatedness with their neighbours; functionally they could be more different.

Presence of superior metabolic properties relevant to resistance to toxic heavy metals and utilization of wide array of petroleum hydrocarbons within all the strains isolated through enrichment possibly demonstrates induction of an enhanced genetic and enzymatic repository within them during the course of enrichment that perhaps acted as strong selection pressure (Kostka et al., 2011; Leahy and Colwell, 1990).

Propinquity among the bacterial strains concerning to the observed metabolic properties was analyzed through Jaccard similarity coefficient (Fig. 3). A heat map was first drawn to represent co-occurrence pattern of major metabolic properties evaluated (Fig. 3a). It could be easily observed that a number of properties of the isolates such as growth in broad ranges of pH, temperature, osmolarity (NaCl concentration), resistance to Cd^{2+}, Hg^{2+}, Ni^{2+} and Pb^{2+}, production of biosurfactant, siderophore, catalase-, oxidase-activities and nitrate-, sulphate- reduction remained more or less consistent among all the strains. Whereas growth at very high pH 10.0, temperature $\geq$45 °C; tolerance to >3% NaCl, resistance to As^{3+} and multiple heavy metals together, reduction of Fe^{3+} (as TEA) and utilization of naphthalene, remained constricted to fewer strains only. The hierarchical clustering method based on Jaccard algorithm verified the statistical interrelationship between the taxonomic affiliation and metabolic properties of the strains (Fig. 3b). The resemblance dendogram indicated that the strains can be broadly divided into distinct two clusters. Notably, cluster one consisted of strain affiliated to genera *Burkholderia*, *Brevundimonas*, *Enterobacter*, *Kocuria* and *Pandoraea*; all isolated through enrichment and the second cluster consisted of strains isolated through direct cultivation, *viz, Bacillus*, *Microvirgula* and *Pseudomonas*. The observed result clearly indicate a role of positive selection pressure during enrichment enabling the proliferation of not only taxonomically distinct and metabolically more superior populations but also diminishing the more popular cultivable and easy growing genera like *Pseudomonas* and *Bacillus*. Distribution of metabolic traits were also analyzed through two component PCA (Fig. 3c) It was observed that except a few traits related to growth/survival at relatively extreme conditions and utilization/resistance to few substrates responses of the strains to all other properties remained in good agreement.

3.5. Selection of representative strains for bioremediation study

Bacterial strains were screened for selecting the most efficient organisms for bioremediation study. The ability of the strains to metabolize and grow with different aliphatic and aromatic compounds was used as one of the selection criteria and the strains showing superior growth (CFU/ml) utilizing more diverse types

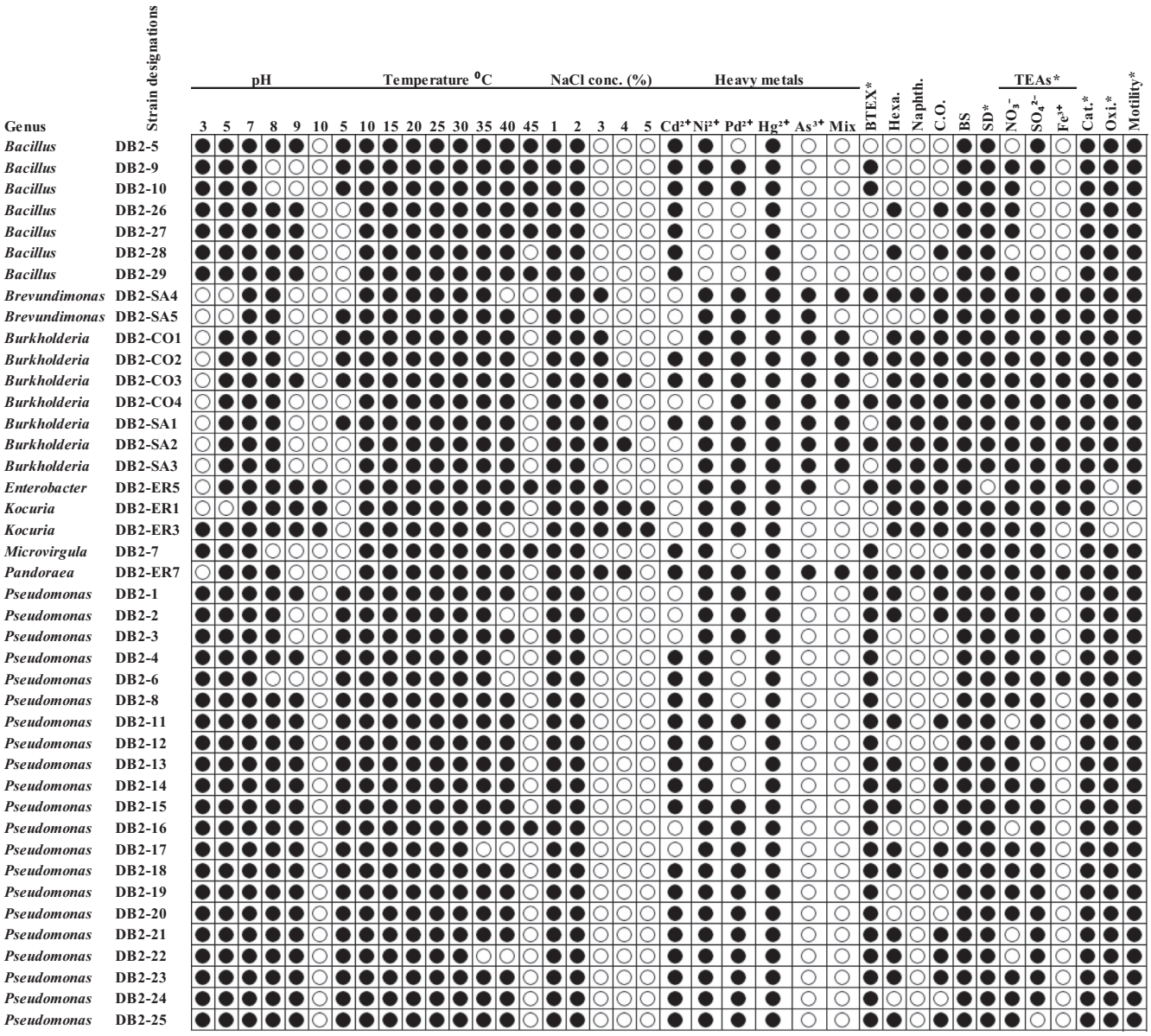


Fig. 2. Consolidated ecophysiological and metabolic properties of the strains. Ecophysiological and metabolic properties of the strains were presented with positive (black) and negative (white) responses for each of the characters tested (abbreviations: HMM-heavy metal mixture; BTEX-benzene, toluene, ethylbenzene, xylene; Hexa.-hexadecane; Naphth.-naphthalene; C.O.-crude oil; BS-biosurfactant; SD-siderophore; cat.-catalase, oxi.-oxidase). *Mark denotes the metabolic characters which were tested in solid agar plates. In case of the tests performed in liquid media, growth was considered positive with an OD_{600} of ≥0.3 following 24–72 h of incubation and for solid media positive growth comprised of visible bacterial colonies.

of substrates were chosen (Fig. 4). E24 index for biosurfactant production was used as the second criteria. HC utilization data showed that a number of strains affiliated to genera *Burkholderia*, *Enterobacter*, *Kocuria* and *Pandoraea* were able to grow very well metabolizing all the seven aliphatic compounds of diverse carbon chain length and polyaromatic compounds tested. Noticeably, most of the *Bacillus* strains showed a very poor response (able to grow weekly only with one or few compounds). Inability of these strains to grow with PAHs, or ability to grow with either aliphatic or aromatic compounds but not both was observed. Surprisingly, the *Pseudomonas* strains although showed weak growth in the presence of some of the compounds tested, they all were incompetent in utilizing PAHs. Few *Bacillus* strains failed to grow with any of the compounds tested. Biosurfactant production levels (E24 index) as monitored for all strains during their growth with alkanes and PAHs showed (a) significantly positive response only for isolates obtained through enrichment (b) during their growth with alkanes and (c) elevated values with increase in carbon chain length. *Burkholderia*, *Enterobacter*, *Kocuria* and *Pseudomonas* are well known for their ability to metabolize diverse aliphatic and aromatic HCs (Zafra et al., 2014) whereas *Pandoraea* sp. are more associated with aromatic hydrocarbons degradation (Colbert et al., 2013). Superior growth performance of *Burkholderia, Enterobacter* and *Pandoraea* members all isolated through enrichment could be attributed to their preferential selection during enrichment with wide range of aliphatic and aromatic compounds. However, considering the hydrocarbon enriched state of the original sample, it is surprising that *Pseudomonas* strains are unable to grow adequately. Members of the genus *Pseudomonas* are well-known biodegrader, capable of metabolizing a range of compounds; however, their abundance and activity towards HC mineralization in complex petroleum waste were not in favor. Recent studies with

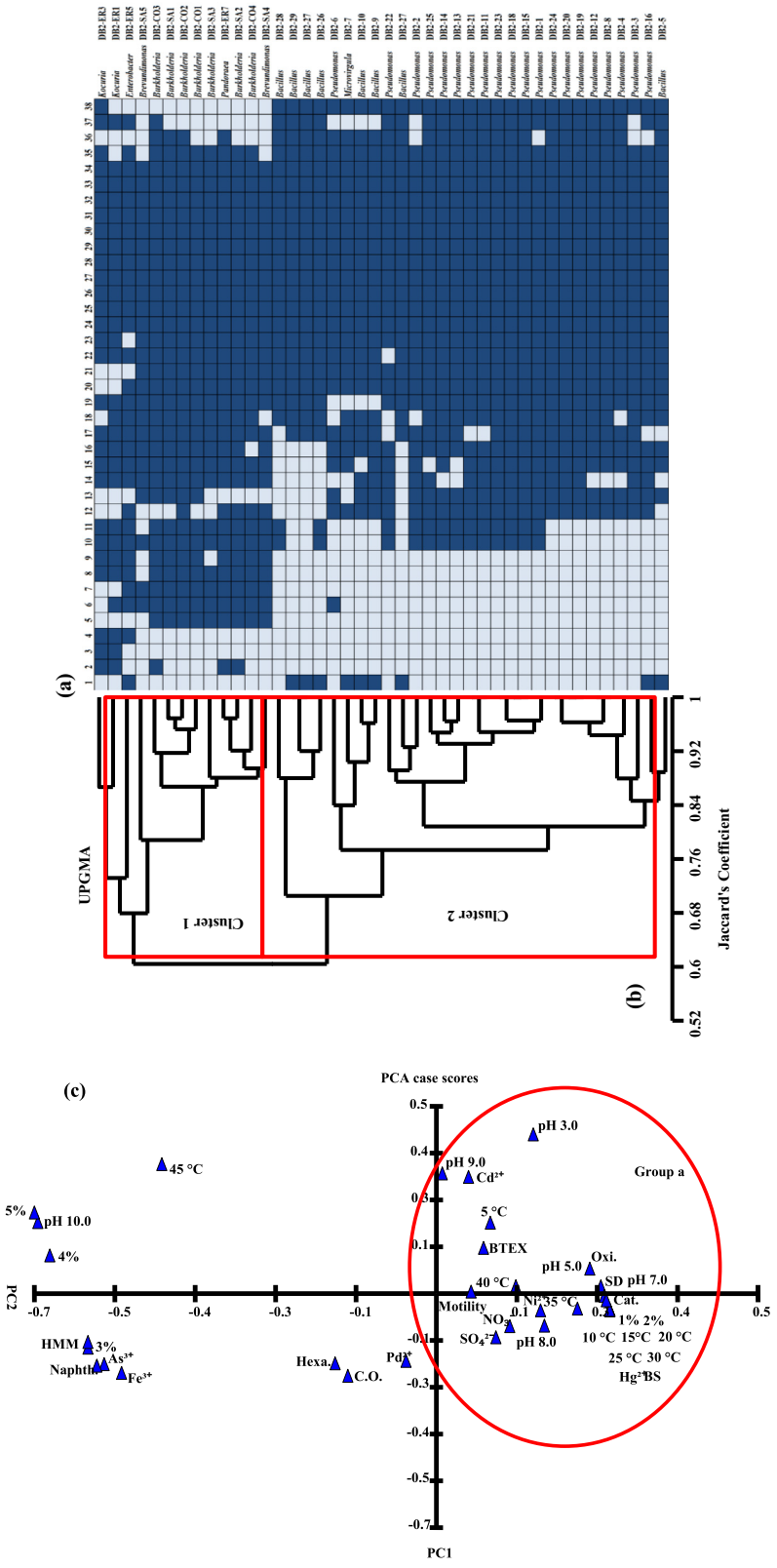


Fig. 3. Correlation analysis among the bacterial taxonomic groups and their metabolic traits observed. Responses of the organisms [positive (deep blue) and negative (light blue)] against 38 ecophysiological/metabolic properties were compiled (a). Unweighted pair group method with arithmetic mean (UPGMA) based hierarchical clustering of the strains performed (b), along with principal component analysis of metabolic traits (c). [Following metabolic tests were considered, presented in accordance to their appearance in (a): 1-growth at 45 °C, 2-growth at 4% NaCl, 3-growth at 5% NaCl, 4-growth at pH10.0, 5-growth with heavy metal mixture, 6-Fe^{3+} as terminal electron acceptor, 7-growth with As^{3+}, 8-naphthalene utilization, 9-growth with 3% NaCl, 10-crude oil utilization, 11-Hexadecane utilization, 12-BTEX utilization, 13-growth with 5 °C, 14-growth with Pb^{2+}, 15-SO_4^{2-} as terminal electron acceptor, 16-growth with Ni^{2+}, 17-NO_3^- as terminal electron acceptor, 18-growth with 40 °C, 19-growth at pH 8.0, 20-motility, 21-oxidase, 22–35 °C, 23-siderophore production, 24-bisurfactant production, 25-growth with Hg^{2+}, 26-catalase, 27-growth with 2% NaCl, 28-growth with 1% NaCl, 29-growth at 30 °C, 30-growth at 25 °C, 31-growth at 20 °C, 32-growth at 15 °C, 33-growth at 10 °C, 34-growth at pH 7.0, 35-growth at pH 5.0, 36-growth with Cd^{2+}, 37-growth at pH 9.0, 38-growth at pH 3.0]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

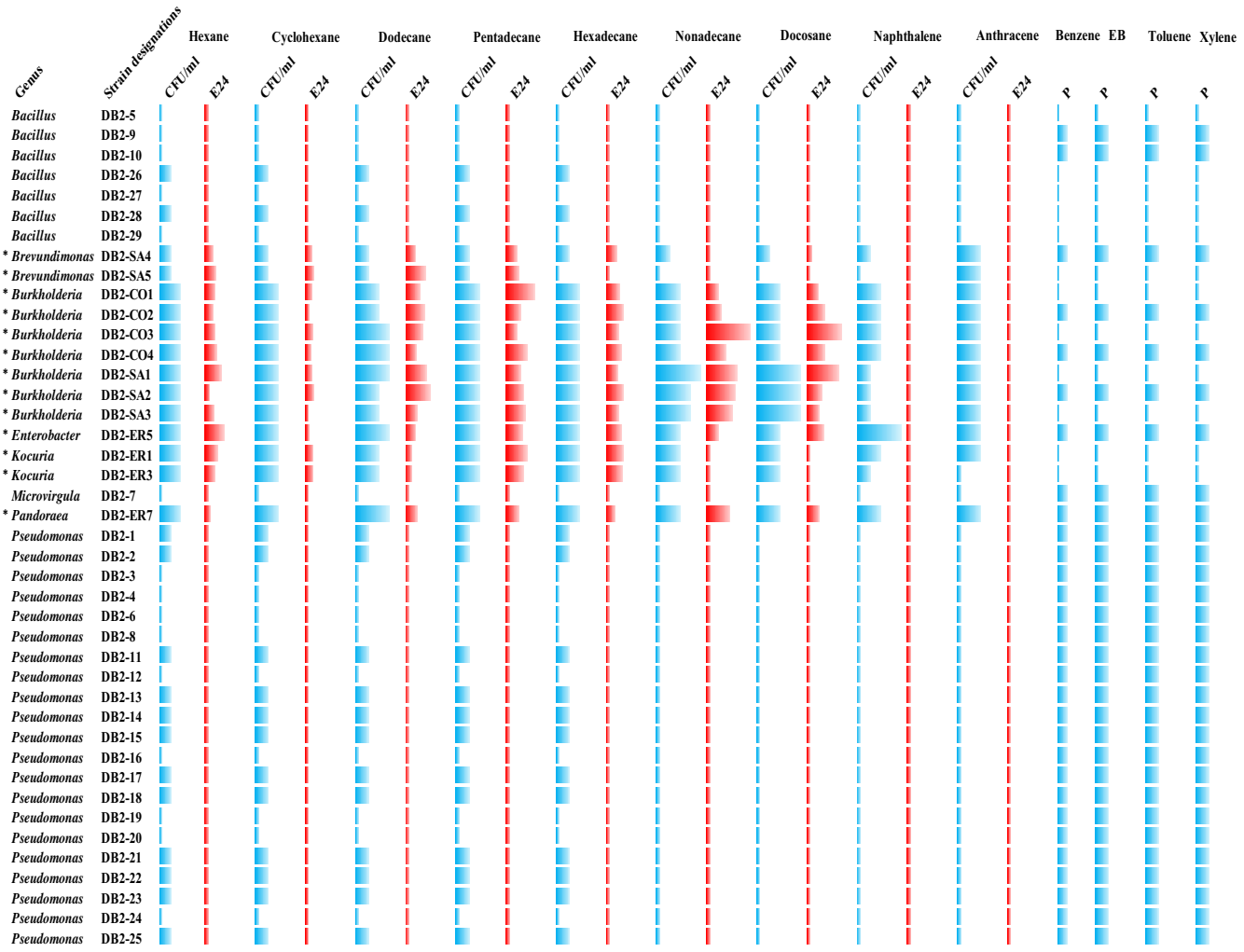


Fig. 4. Profiles of viable cell counts and biosurfactant production (E24 index) by the bacterial strains during growth on aliphatic and aromatic hydrocarbons. (Abbreviations: CFU/ml-Colony forming units/ml; EB-Ethyl benzene; P-visible growth on agar plates).

refinery wastes/oil spill indicated their low abundance and less proliferation during active bioremediation process (Bell et al., 2013; Sarkar et al., 2016). Furthermore, it has been reported that growth of *Pseudomonas* strains utilizing HCs required the presence of surfactants which they are incapable to synthesize and their isolation as single colony may not be possible all the time (Zhang et al., 2005). On the other hand, members of *Betaproteobactria* particularly *Burkholderiaceae* members have been reported to be genetically and physiologically more efficient and best predictors of degradation across contaminated niches, thus playing an active role in HC metabolism (Bell et al., 2013). Based on these results, 13 strains isolated through enrichment and showing excellent growth were considered for more critical evaluation wherein increase in CFU/ml was compared along with cell surface hydrophobicity index (MATH) assay. Highest cell proliferation was observed with dodecane as substrate by five strains belonging to genera *Burkholderia* (DB2-CO3, DB2-CO4, DB2-SA1), *Enterobacter* (DB2-ER5), and *Pandoraea* (DB2-ER7); followed by anthracene by *Burkholderia* strains (DB2-SA1, DB2-SA3 and DB2-SA4) and *Brevundimonas* (DB2-SA4 and DB2-SA5) (Supplementary Fig. 1). Preferential utilization of moderate chain aliphatics by bacterial populations in refinery waste has been reported recently and our observation on docosane utilization corroborates with that very well (Sarkar et al., 2016). Cell surface hydrophobicity index showed highest values (100%) for *Burkholderia* (DB2-SA1), *Brevundimonas* (DB2-SA4) and *Enterobacter* (DB2-ER5) followed by *Kocuria* (DB2-ER3) and *Pandoraea* (DB2-ER7). Hydrophobicity analysis depicted the involvement of bacterial cell membranes in uptake of hydrocarbon substrates for enhanced biodegradation. Based on the above results five strains *Burkholderia* (DB2-CO3, DB2-CO4, DB2-SA1), *Enterobacter* (DB2-ER5) and *Pandoraea* (DB2-ER7) were finally shortlisted for bioremediation study through microcosm.

3.6. Metabolism of alkane (dodecane) by bacterial strains

Among the various aliphatic and aromatic hydrocarbons tested highest cell proliferation was observed with dodecane as substrate by the five selected bacterial strains. Therefore dodecane metabolism was investigated. All the five strains were grown in medium containing dodecane and concentration of dodecane, cellular fatty acids and presence of *alk*B gene was assessed (Fig. 5). *Pandoraea* DB2-ER7 showed the highest degradation of 67% followed by *Burkholderia* DB2-CO4 and *Enterobacter* DB2-ER5 (65%), *Burkholderia* DB2-CO3 (56%) and *Burkholderia* DB2-SA1 (46%). Formation of intermediary metabolites of alkane metabolism was assessed. Total eighteen fatty acids (FAs) were detected in the strains and following growth with dodecane increase in abundance of some FAs or appearance of new ones were mostly noted (Fig. 5, Supplementary Fig. 2). Pentadecanoic, hexadecanoic, octadecanoic, nonadecanoic and octadecadienoic acids were ubiquitously produced

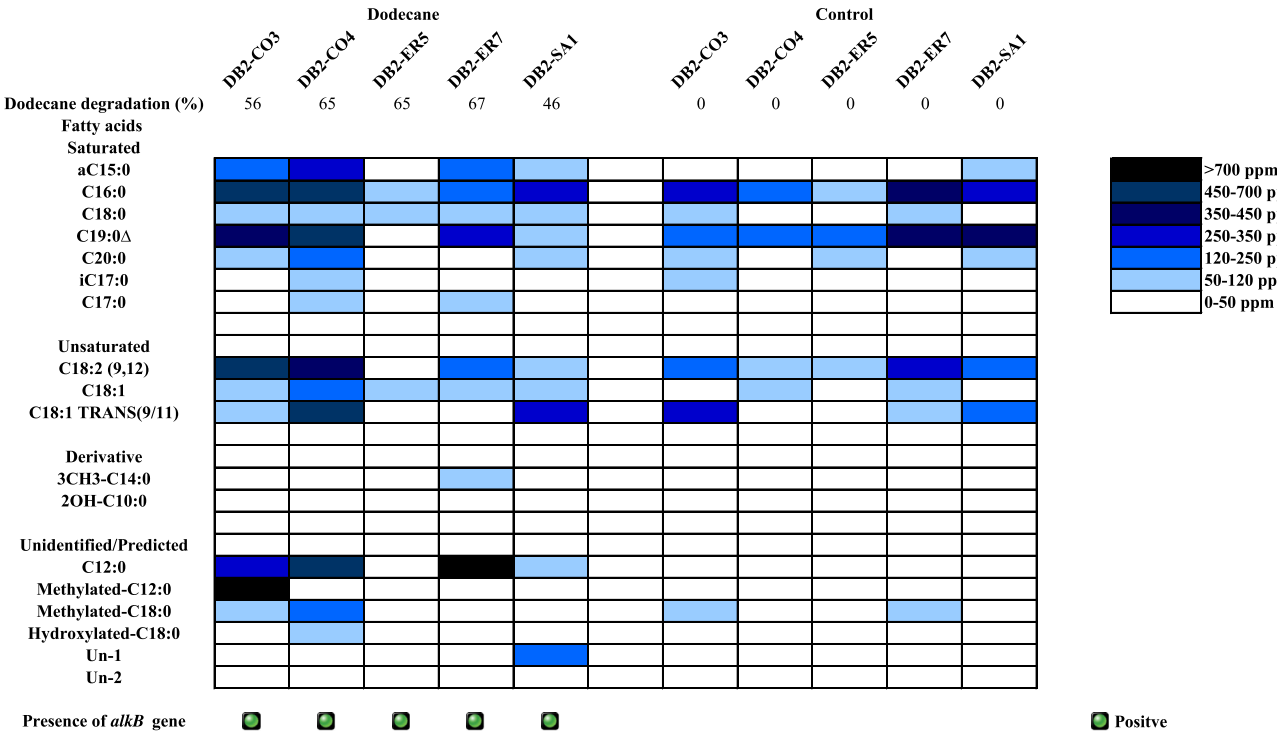


Fig. 5. Degradation of dodecane (a), fatty acid methyl esters (FAME) profiles (b) and presence of *alkB* gene (c) within selected bacterial strains.

suggesting them signature fatty acids within the cell membrane of these genera (Coenye et al., 2000). Eicosanoic acid remained restricted to *Burkholderia* (DB2-CO3, DB2-CO4 and DB2-SA1), *Enterobacter* (DB2-ER5) species only. The presence of heptadecanoic acid was observed in dodecane grown cells of two strains DB2-CO4 (*Burkholderia*) and DB2-ER7 (*Pandoraea*). Presence of essential biomarker membrane fatty acids was noted in case of each dodecane and yeast extract grown bacterial cells. These fatty acids help in maintaining basic cell membrane structure and fluidity of bacterial cells. Enhanced production of biomarker fatty acids *viz.* pentadecanoic, heptadecanoic acids, octadec-11-enoic acid following growth with dodecane as observed here is in line with previous reports on alkane metabolizing *Mycobacterium*, *Brevibacterium*, *Nocardia*, etc. (Aries et al., 2001). For DB2-ER7 methylated tetradecanoic acid was formed during growth on dodecane which could be corroborated to enhanced tolerance towards hostile growth conditions (alkane) by changing cell membrane fluidity. The presence of dodecanoic acid as observed in all the alkane grown cells (except DB2ER5) corroborates to its formation due to aerobic terminal or sub terminal activation during oxidation by alkane monoxygenases. The pathways lead to the formation of primary/secondary alcohols which eventually incorporates into the cell as corresponding FAs or fatty acid esters, respectively (Rojo, 2009). Our observations related to dodecane metabolism were further validated with the presence of 550 bp alkane metabolizing (*alkB)* gene in each of the strains.

3.7. Microcosm based bioremediation study

To assess the intrinsic bioremediation potentials of selected five bacterial strains as candidates for bioremediation agent, microcosm based approach was devised. The petroleum refinery waste was composed of multifarious hydrocarbon fractions as ascertained by GC–MS analysis. The waste sample was mostly comprised of mixed alkanes (eg. 2, 6, 10-trimethyldodecane, pentadecane, heneicosane, tricosane, etc.), 1, 5-diisopropyl-2, 3-dimethylcyclohexane and few naphthalene derivatives which rendered the sample hazardous in nature. Separate bioaugmentation trial microcosms for each of the five strains were set up with the waste sample. HC attenuation capacity by each strain was assessed by monitoring TPH level and biosurfactant production in each set at specific time intervals (Fig. 6a-b). At the end of the incubation change in COD content was determined to gain a comprehensive assessment of bioremediation performance. Remaining COD for DB2-ER5 amended microcosm was less (37 g/kg) followed by DB2-ER7 (96 g/kg), DB2-CO3 (114 g/kg), DB2-CO4 (114 g/kg), DB2-SA1 (204.6 g/kg), unamended control (210 g/kg) and killed control (246 g/kg). In case of the control set devoid of any bioaugmentation, significant ($p < 0.05$) attenuation of HC components was observed following specific bacterial amendments. The rate of HC degradation by all the five strain varied considerably for the entire period of incubation (with *Pandoraea* strain DB2-ER7 showing the most rapid biodegradation and *Burkholderia* strain DB2-SA1, the slowest) with an average of >90% TPH reduction being attained by 15 days. By this period highest amount (94%) of TPH reduction was achieved with *Pandoraea* strain DB2-ER7 and lowest (83%) with *Burkholderia* strain DB2-CO4. Subsequent incubation yielded more reduction that culminated to 98% TPH loss by *Enterobacter* strain DB2-ER5 followed by *Pandoraea* strain DB2-ER7. The data clearly revealed a strong positive effect of bioaugmentation with catabolically efficient bacteria in improving the overall bioremediation efficiency. Together with reduction of TPH, E24 emulsification index was determined for each set at each time points. Strains DB2-CO3, DB2-CO4, and DB2-SA1 showing slightly slower pace of biodegradation during the early phase of incubation (5 day) exhibited presence of elevated emulsifiers within the microcosms. While in strains DB2-CO3 and DB2-ER7 and DB2-SA1, E24 values declined over the time steadily; in other strains, relatively higher activities remained till end of incubation time. Levels of COD within each set was determined after 4 weeks and highest amount of carbon mineralization (low COD) was noted for the *Enterobacter* strain DB2-ER5, followed by *Pandoraea* strain

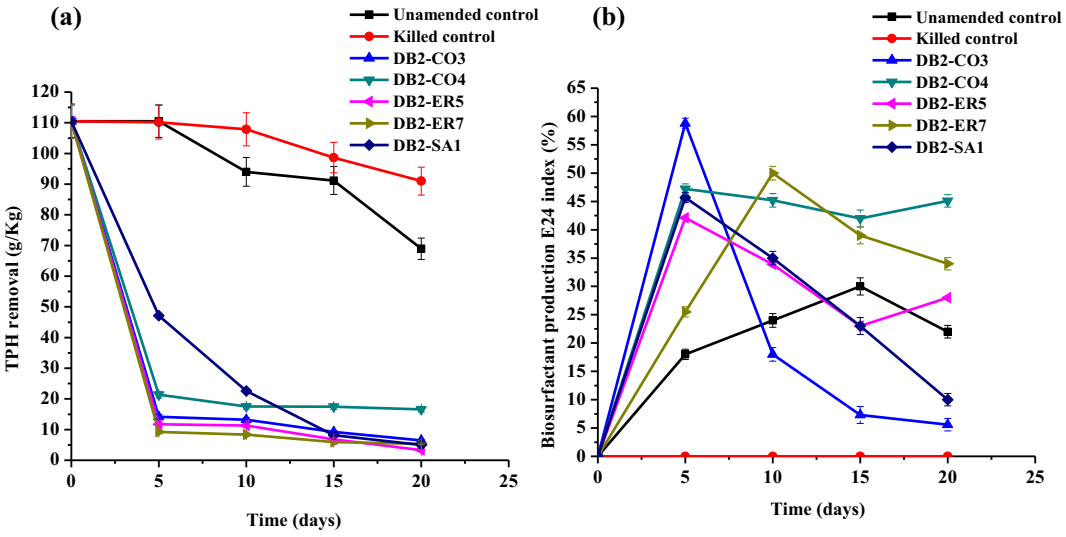


Fig. 6. Microcosm based biodegradation study on TPH removal following bioaugmentation with selected bacterial strains (a) and biosurfactant production (b).

DB2-ER7 and least in unamended control. Among the bioaugmentation sets, the one with *Burkholderia* strain DB2-SA1 showed relatively higher COD value.

The use of bioaugmentation as a pragmatic alternative for mitigation of petroleum wastes is consistent with other published reports where well proclaimed hydrocarbon degrading strains (such as *Pseudomonas*, *Bacillus*, *Rhodococcus,* etc.) proved to be useful in removal of total petroleum fractions within wastes (Agnello et al., 2016; Cerqueira et al., 2014; Varjani and Upasani, 2016). Efficacy of the members of *Beta-* and *Gamma- proteobacteria*, particularly *Burkholderiaceae* and *Enterobacteriaceae* in petroleum degradation (including PAHs and n-Alkanes ranging C_{12}-C_{26}) is well established (Bell et al., 2013; Sarkar et al., 2016); although, reports on bioremediation by *Pandoraea* and *Enterobacteria* was not so frequent. The observed bioremediation performance of the test strains was compared with published literature on different bioaugmentation approaches (Supplementary Table 4) highlighting considerable TPH depletion within 25–30 days by application of single bacterial strains (Abou-Shanab et al., 2016; Dellagnezze et al., 2016). The progression of biodegradation was associated with concomitant production and release of biosurfactant which indicated involvement of active biosurfactant production by the applied strain within the petroleum waste. HC degradation achieved with unamended control could be attributed to the catalytic activities of indigenous organisms of the waste which perhaps activated further due to presence of high moisture and medium components.

3.7.1. Kinetic modeling of the biodegradation process

Kinetic modeling performs a critical role in predicting the process of biodegradation. The bioremediation process was mathematically scrutinized utilizing first order kinetic model equation. Subsequently, biodegradation rate (k) and half life ($t_{1/2}$) of total petroleum hydrocarbons were hypothesized using Eqs. (1) and (2) respectively. The kinetic equations suggest that higher the biodegradation rate the faster will be the biodegradation and consequently the half life times will be reduced. It was observed from our experiment that compared to unamended control set (without any bioaugmentation), specific bioaugmentation allowed >4 fold increase in degradation rate (Table 1). According to the observed half lives the overall velocity of degradation process in microcosms was found to be in the order of following amendments: *Enterobacter* > *Burkholderia* > *Pandoraea*. Evaluation of kinetic parameters is necessary for understanding biodegradation processes in nature. Our investigation on kinetic analysis of the biodegradation process using single bacterial strain revealed a remarkable decrease in half-life of petroleum hydrocarbons which signified the role of the active hydrocarbon degrading strains having an interactive effect on existing microbial populations in mitigating petroleum refinery wastes.

Table 1
Details of kinetic parameters of the biodegradation process by the bacterial strains.

Treatments	Biodegradation rate (day^{-1})	Half life (days)
Unamended control	0.015	46.2
Killed control	0.005	138.6
DB2-CO3	0.046	15.06
DB2-CO4	0.045	15.4
DB2-ER5	0.066	10.5
DB2-ER7	0.049	14.1
DB2-SA1	0.067	10.3

4. Conclusion

Burkholderia, *Kocuria, Enterobacter* and *Pandoraea* strains with versatile metabolic abilities represent the major hydrocarbon-degrading cultivable populations of petroleum refinery waste. Their isolation only through enrichment indicates obligatory relationship of indigenous bacteria of refinery waste with hydrocarbons for cultivability. The study provides evidence on intrinsic ability of refinery waste bacteria in facilitating enhanced biodegradation which could be leveraged during bioremediation of other hydrocarbon rich wastes. Based on our study we recommend that refinery waste could be a good resource for recovering petroleum hydrocarbon bioremediation relevant bacteria and enrichment based isolation is a potent method to get those strains as pure culture.

Acknowledgement

The authors would like to acknowledge Department of Biotechnology, Government of India under NER Twinning project (BT/226/NE/TBP/2011) for financial aid. Generous help from Indian Oil Corporation Limited, Digboi refinery, Assam, India for providing the waste samples is thankfully acknowledged. Poulomi Sarkar and

Ajoy Roy acknowledge financial support (institute fellowship) received from Indian Institute of Technology, Kharagpur, India and National Institute of Technology, Durgapur, India respectively. Siddhartha Pal and Balaram Mohapatra are recipients of DBT project fellowship and DST Inspire doctoral fellowship, respectively.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2017.05.010.

References

Abed, R.M.M., Al-Sabahi, J., Al-Maqrashi, F., Al-Habsi, A., Al-Hinai, M., 2014. Characterization of hydrocarbon-degrading bacteria isolated from oil-contaminated sediments in the Sultanate of Oman and evaluation of bioaugmentation and biostimulation approaches in microcosm experiments. Int. Biodeterior. Biodegrad. 89, 58–66. http://dx.doi.org/10.1016/j.ibiod.2014.01.006.

Abou-Shanab, R.A.I., Eraky, M., Haddad, A.M., Abdel-Gaffar, A.R.B., Salem, A.M., 2016. Characterization of crude oil degrading bacteria isolated from contaminated soils surrounding gas stations. Bull. Environ. Contam. Toxicol. 97, 684–688. http://dx.doi.org/10.1007/s00128-016-1924-2.

Agnello, A.C., Bagard, M., van Hullebusch, E.D., Esposito, G., Huguenot, D., 2016. Comparative bioremediation of heavy metals and petroleum hydrocarbons co-contaminated soil by natural attenuation, phytoremediation, bioaugmentation and bioaugmentation-assisted phytoremediation. Sci. Total Environ. 563–564, 693–703. http://dx.doi.org/10.1016/j.scitotenv.2015.10.061.

Ahmed, E., Holmström, S.J.M., 2014. Siderophores in environmental research: Roles and applications. Microb. Biotechnol. 7, 196–208. http://dx.doi.org/10.1111/1751-7915.12117.

Antoniou, E., Fodelianakis, S., Korkakaki, E., Kalogerakis, N., 2015. Biosurfactant production from marine hydrocarbon-degrading consortia and pure bacterial strains using crude oil as carbon source. Front. Microbiol. 6, 1–14. http://dx.doi.org/10.3389/fmicb.2015.00274.

Aries, E., Doumenq, P., Artaud, J., Acquaviva, M., Bertrand, J.C., 2001. Effects of petroleum hydrocarbons on the phospholipid fatty acid composition of a consortium composed of marine hydrocarbon-degrading bacteria. Org. Geochem. 32, 891–903. http://dx.doi.org/10.1016/S0146-6380(01)00052-3.

Barker, D.J., Mannucchi, G.A., Salvi, S.M.L., Stuckey, D.C., 1999. Characterisation of soluble residual chemical oxygen demand (COD) in anaerobic wastewater treatment effluents. Water Res. 33, 2499–2510. http://dx.doi.org/10.1016/S0043-1354(98)00489-8.

Bell, T.H., Yergeau, E., Maynard, C., Juck, D., Whyte, L.G., Greer, C.W., 2013. Predictable bacterial composition and hydrocarbon degradation in Arctic soils following diesel and nutrient disturbance. ISME J. 7, 1200–1210. http://dx.doi.org/10.1038/ismej.2013.1.

Benedek, T., Vajna, B., Táncsics, A., Márialigeti, K., Lányi, S., Máthé, I., 2013. Remarkable impact of PAHs and TPHs on the richness and diversity of bacterial species in surface soils exposed to long-term hydrocarbon pollution. World J. Microbiol. Biotechnol. 29, 1989–2002. http://dx.doi.org/10.1007/s11274-013-1362-9.

Bhattacharya, D., Sarma, P.M., Krishnan, S., Mishra, S., Lal, B., 2003. Evaluation of Genetic Diversity among Pseudomonas citronellolis Strains Isolated from Oily Sludge-Contaminated Sites Evaluation of Genetic Diversity among Pseudomonas citronellolis Strains Isolated from Oily Sludge-Contaminated Sites. Appl. Environ. Microbiol. 69, 1435–1441. http://dx.doi.org/10.1128/AEM.69.3.1435.

Brima, A., Yao, J., Sandy, E.H., Zheng, S., Zaray, G., Koroma, B.M., 2010. Cell surface hydrophobicity (CSH) of Escherichia coli, Staphylococcus aureus and Aspergillus niger and the biodegradation of Diethyl Phthalate (DEP) via Microcalorimetry. 6, 78–88.

Brooijmans, R.J.W., Pastink, M.I., Siezen, R.J., 2009. Hydrocarbon-degrading bacteria: The oil-spill clean-up crew. Microb. Biotechnol. 2, 587–594. http://dx.doi.org/10.1111/j.1751-7915.2009.00151.x.

Cerqueira, V.S., Hollenbach, E.B., Maboni, F., Vainstein, M.H., Camargo, F.A.O., Peralba, M.do C.R., Bento, F.M., 2011. Biodegradation potential of oily sludge by pure and mixed bacterial cultures. Bioresour. Technol. 102, 11003–11010. http://dx.doi.org/10.1016/j.biortech.2011.09.074.

Cerqueira, V.S., Peralba, M.do C.R., Camargo, F.A.O., Bento, F.M., 2014. Comparison of bioremediation strategies for soil impacted with petrochemical oily sludge. Int. Biodeterior. Biodegrad. 95, 338–345. http://dx.doi.org/10.1016/j.ibiod.2014.08.015.

Cheema, S., Lavania, M., Lal, B., 2014. Impact of petroleum hydrocarbon contamination on the indigenous soil microbial community. Ann. Microbiol. 359–369. http://dx.doi.org/10.1007/s13213-014-0868-1.

Ciric, L., Philp, J.C., Whiteley, A.S., 2010. Hydrocarbon utilization within a diesel-degrading bacterial consortium. FEMS Microbiol. Lett. 303, 116–122. http://dx.doi.org/10.1111/j.1574-6968.2009.01871.x.

Coenye, T., Falsen, E., Hoste, B., Ohlén, M., Goris, J., Govan, J.R.W., Gillis, M., Vandamme, P., 2000. Description of Pandoraea gen. nov. with Pandoraea apista sp. nov., Pandoraea pulmonicola sp. nov., Pandoraea pnomenusa sp. nov., Pandoraea sputorum sp. nov. and Pandoraea norimbergensis comb. nov. Int. J. Syst. Evol. Microbiol. 50, 887–899. http://dx.doi.org/10.1099/00207713-50-2-887.

Colbert, C.L., Agar, N.Y.R., Kumar, P., Chakko, M.N., Sinha, S.C., Powlowski, J.B., Eltis, L.D., Bolin, J.T., 2013. Structural Characterization of Pandoraea pnomenusa B-356 Biphenyl Dioxygenase Reveals Features of Potent Polychlorinated Biphenyl-Degrading Enzymes. PLoS ONE 8. http://dx.doi.org/10.1371/journal.pone.0052550.

Das, R., Kazy, S.K., 2014. Microbial diversity, community composition and metabolic potential in hydrocarbon contaminated oily sludge: Prospects for in situ bioremediation. Environ. Sci. Pollut. Res. 21, 7369–7389. http://dx.doi.org/10.1007/s11356-014-2640-2.

Dellagnezze, B.M., Vasconcellos, S.P., Angelim, A.L., Melo, V.M.M., Santisi, S., Cappello, S., Oliveira, V.M., 2016. Bioaugmentation strategy employing a microbial consortium immobilized in chitosan beads for oil degradation in mesocosm scale. Mar. Pollut. Bull. 107, 107–117. http://dx.doi.org/10.1016/j.marpolbul.2016.04.011.

Dueholm, M.S., Marques, I.G., Karst, S.M., D'Imperio, S., Tale, V.P., Lewis, D., Nielsen, P.H., Nielsen, J.L., 2015. Survival and activity of individual bioaugmentation strains. Bioresour. Technol. 186, 192–199. http://dx.doi.org/10.1016/j.biortech.2015.02.111.

Fuentes, S., Méndez, V., Aguila, P., Seeger, M., 2014. Bioremediation of petroleum hydrocarbons: Catabolic genes, microbial communities, and applications. Appl. Microbiol. Biotechnol. 98, 4781–4794. http://dx.doi.org/10.1007/s00253-014-5684-9.

Hilyard, E.J., Jones-Meehan, J.M., Spargo, B.J., Hill, R.T., 2008. Enrichment, isolation, and phylogenetic identification of polycyclic aromatic hydrocarbon-degrading bacteria from Elizabeth River sediments. Appl. Environ. Microbiol. 74, 1176–1182. http://dx.doi.org/10.1128/AEM.01518-07.

Hu, G., Li, J., Zeng, G., 2013. Recent development in the treatment of oily sludge from petroleum industry–a review. J. Hazard. Mater. 261, 470–490. http://dx.doi.org/10.1016/j.jhazmat.2013.07.069.

Jurelevicius, D., Korenblum, E., Casella, R., Vital, R.L., Seldin, L., 2009. Polyphasic analysis of the bacterial community in the rhizosphere and root of Cyperus rotundus L. grown in a petroleum-contaminated soil de. J. J. Microbiol. Biotechnol. 48, 5643. http://dx.doi.org/10.4014/jmb.0910.10012.

Kloos, K., Munch, J.C., Schloter, M., 2006. A new method for the detection of alkane-monooxygenase homologous genes (alkB) in soils based on PCR-hybridization. J. Microbiol. Methods 66, 486–496. http://dx.doi.org/10.1016/j.mimet.2006.01.014.

Koichiro, T., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis. Mol. Biol. Evol. 28, 2731–2739. http://dx.doi.org/10.1111/j.1365-313X.2005.02611.x.

Kostka, J.E., Prakash, O., Overholt, W.A., Green, S.J., Freyer, G., Canion, A., Delgardio, J., Norton, N., Hazen, T.C., Huettel, M., 2011. Hydrocarbon-degrading bacteria and the bacterial community response in Gulf of Mexico beach sands impacted by the deepwater horizon oil spill. Appl. Environ. Microbiol. 77, 7962–7974. http://dx.doi.org/10.1128/AEM.05402-11.

Kuppusamy, S., Thavamani, P., Megharaj, M., Naidu, R., 2016. Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by novel bacterial consortia tolerant to diverse physical settings - Assessments in liquid- and slurry-phase systems. Int. Biodeterior. Biodegrad. 108, 149–157. http://dx.doi.org/10.1016/j.ibiod.2015.12.013.

Lamendella, R., Strutt, S., Borglin, S., Chakraborty, R., Tas, N., Mason, O.U., Hultman, J., Prestat, E., Hazen, T.C., Jansson, J.K., 2014. Assessment of the deepwater horizon oil spill impact on gulf coast microbial communities. Front. Microbiol. 5, 1–13. http://dx.doi.org/10.3389/fmicb.2014.00130.

Leahy, J.G., Colwell, R.R., 1990. Microbial degradation of hydrocarbons in the environment. Microbiol. Rev. 54, 305–315.

Mahjoubi, M., Jaouani, A., Guesmi, A., Ben Amor, S., Jouini, A., Cherif, H., Najjari, A., Boudabous, A., Koubaa, N., Cherif, A., 2013. Hydrocarbonoclastic bacteria isolated from petroleum contaminated sites In Tunisia: Isolation, identification and characterization of the biotechnological potential. N. Biotechnol. 30, 723–733. http://dx.doi.org/10.1016/j.nbt.2013.03.004.

Mnif, S., Chamkha, M., Labat, M., Sayadi, S., 2011. Simultaneous hydrocarbon biodegradation and biosurfactant production by oilfield-selected bacteria. J. Appl. Microbiol. 111, 525–536. http://dx.doi.org/10.1111/j.1365-2672.2011.05071.x.

Païssé, S., Goñi-Urriza, M., Coulon, F., Duran, R., 2010. How a bacterial community originating from a contaminated coastal sediment responds to an oil input. Microb. Ecol. 60, 394–405. http://dx.doi.org/10.1007/s00248-010-9721-7.

Paul, D., Kazy, S.K., Gupta, A.K., Pal, T., Sar, P., 2015. Diversity, metabolic properties and arsenic mobilization potential of indigenous bacteria in arsenic contaminated groundwater of West Bengal. India. PLoS One 10. http://dx.doi.org/10.1371/journal.pone.0118735.

Rodriguez-R, L.M., Overholt, W.A., Hagan, C., Huettel, M., Kostka, J.E., Konstantinidis, K.T., 2015. Microbial community successional patterns in beach sands impacted by the Deepwater Horizon oil spill. ISME J. 1–13. http://dx.doi.org/10.1038/ismej.2015.5.

Rojo, F., 2009. Degradation of alkanes by bacteria: Minireview. Environ. Microbiol. 11, 2477–2490. http://dx.doi.org/10.1111/j.1462-2920.2009.01948.x.

Sarkar, J., Kazy, S.K., Gupta, A., Dutta, A., Mohapatra, B., Roy, A., Bera, P., Mitra, A., Sar, P., 2016. Biostimulation of indigenous microbial community for bioremediation of petroleum refinery sludge. Front. Microbiol. 7, 1–20. http://dx.doi.org/10.3389/fmicb.2016.01407.

Solanki, P., Kumar, A., Laura, J.S., 2015. Original Research Article Isolation and assessment of biodegradation potential of fungal species isolated from sludge contaminated soil 3, 197–204.

Tao, K., Liu, X., Chen, X., Hu, X., Cao, L., Yuan, X., 2016. Biodegradation of crude oil by a defined co-culture of indigenous bacterial consortium and exogenous Bacillus subtilis. Bioresour. Technol. 327–332. http://dx.doi.org/10.1016/j.biortech.2016.10.073.

Uhlik, O., Wald, J., Strejcek, M., Musilova, L., Ridl, J., Hroudova, M., Vlcek, C., Cardenas, E., Mackova, M., Macek, T., 2012. Identification of bacteria utilizing biphenyl, benzoate, and naphthalene in long-term contaminated soil. PLoS ONE 7, 1–10. http://dx.doi.org/10.1371/journal.pone.0040653.

Varjani, S.J., Upasani, V.N., 2016. Biodegradation of petroleum hydrocarbons by oleophilic strain of Pseudomonas aeruginosa NCIM 5514. Bioresour. Technol. 222, 195–201. http://dx.doi.org/10.1016/j.biortech.2016.10.006.

Weelink, S.A.B., van Eekert, M.H.A., Stams, A.J.M., 2010. Degradation of BTEX by anaerobic bacteria: Physiology and application. Rev. Environ. Sci. Biotechnol. 9, 359–385. http://dx.doi.org/10.1007/s11157-010-9219-2.

Zafra, G., Absalón, Á.E., Cuevas, M.D.C., Cortés-Espinosa, D.V., 2014. Isolation and selection of a highly tolerant microbial consortium with potential for PAH biodegradation from heavy crude oil-contaminated soils. Water. Air. Soil Pollut. 225. http://dx.doi.org/10.1007/s11270-013-1826-4.

Ze, P., Ngassoum, M.B., Solange, C., Ekoka, J., 2016. Characterization of Oily Sludge from Cameroon Petroleum Refinery 4, 34–38.

Zhang, G., Wu, Y., Qian, X., Meng, Q., 2005. Biodegradation of crude oil by Pseudomonas aeruginosa in the presence of rhamnolipids. J. Zhejiang Univ. Sci. B 6, 725–730. http://dx.doi.org/10.1631/jzus.2005.B0725.