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RESEARCH PAPER

Biodegradation of Selected Polycyclic Aromatic Hydrocarbons by Axenic Bacterial Species Belonging to the Genera *Lysinibacillus* and *Paenibacillus*

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Abstract The quest for competent degraders of recalcitrant polynuclear aromatic hydrocarbons (PAHs) for use in sustainable bioremediation technology has justified the execution of this work. In this study, three bacterial strains (FB-1, FB-2 and FB-3) were isolated from a former industrial site in Bloomington, Indiana. The catabolic versatility of these obtained strains was evaluated on some selected PAH-naphthalene, anthracene, fluoranthene and pyrene. Using the 16S rRNA sequencing analyses, our strains belonged to the family Firmicutes whereby strain FB-1 was identified as *Lysinibacillus* sp. FB-1, strain FB-2 as Bacterium FB-2 and strain FB-3 as *Lysinibacillus fusiformis* FB-3. The biodegradation of the selected PAHs was determined using gas chromatography, and the calculated percentage utilization of the selected PAHs varied between 97 and 4%. We further determined the mean biodegradation rates for fluoranthene when supplemented with molasses. The mean biodegradation rates were between (mg L^{-1}) 0.214 ± 0.006 and 0.318 ± 0.002, while MS-fluoranthene only ranged from (mg L^{-1}) 0.210 ± 0.056 to 0.437 ± 0.176. However, with ANOVA at 5% ($P < 0.05$) there seemed to be no significant difference in the mean biodegradation rates between both media. These findings may have practical and ecological prospects in designing and improving bioremediation of polycyclic aromatic hydrocarbon contaminated sites.

Keywords Competent degraders · Bioremediation · Molasses · PAHs · 16S rRNA

1 Introduction

Polynuclear aromatic hydrocarbons or polycyclic aromatic hydrocarbons (PAHs) have been classified as priority environmental pollutants by the US Environmental Protection Agency (US EPA) and the European Community (Wattiau 2002). This is because of their cytotoxic, mutagenic, toxic and genotoxic risks to human health (Kanaly and Harayama 2000). PAHs are important components of coal tar, crude oil, asphalt and creosote. They are introduced into the environment through various routes such as from volcanoes, forest fires, residential wood burning and exhaust from automobiles and trucks. PAHs are ubiquitous in the environment and are found in high concentrations on many industrial sites particularly those associated with the petroleum and wood preserving industries. PAHs are known for their low aqueous solubility and high soil affinity. This property results in their slow intrinsic degradation and continuous accumulation in nature (Anyakora and Coker 2006). Reda (2009) reported that low molecular weight (LMW PAHs) are toxic, while high molecular weight (HMW PAHs) are genotoxic. Mishra et al. (2001) and Bach et al. (2005) confirmed this fact that PAHs are potent immunotoxicants.

In the environment, PAHs can leak into aquifers, which often lead to concentrations of dissolved hydrocarbons far

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in excess of regulatory limits. This could lead to the destruction of hydrophytes, microflora, fauna, and fishes and reduced outputs of wetlands. In addition, there could be huge demands on the inorganic nutrient sources and oxygen, leading to ecological imbalance.

In soils and sediments, PAHs bind with soil particles and organic particles thus becoming less bioavailable for biological uptake. PAHs usually decrease microbial diversity, with a consequent disruption of the ecosystem dynamics arising from the alteration of soil organic matter mineralization and nutrient mineralization ratio. PAHs are difficult to remove from contaminated soil by employing treatments that have been used successfully to clean soils contaminated with more degradable or volatile organic compounds.

Conventional remediation methods such as dredging and incineration are not cost-effective. In addition, these methods can impact negatively on the soil structure and texture (Hilyard et al. 2008). Currently, bioremediation strategies have become widely accepted as a method for treatment of contaminated soils. Its success relies in part on characterizing the natural diversity that can utilize PAH and developing methods of manipulating the diversity to improve their catabolic versatility (Mueller et al. 1997). PAH degradation is most prevalent under aerobic conditions but also occurs in anaerobic systems (Davidova et al. 2007). The metabolism of PAHs by pure cultures have provided information on the types and diversity of metabolic pathways. However, more knowledge is required for understanding the microbial processes in polluted soils. It has also been noted that bacterial communities in creosote-contaminated sites are correlated to the type of PAH present rather than with PAH concentration (Muckian et al. 2007). For example, community structure in the presence of naphthalene (2-ring) was quite distinct from those in soil containing multicomponent mixtures of PAHs 2, 3 and 4 rings. Although a number of bacterial groups appear to be associated with PAH contamination, their function in PAH metabolism in situ remains unclear. The paucity of sufficient information on PAH degradation principles has caused failures of bioaugmentation and bioremediation strategies (Johnsen et al. 2005). Thus for effective application of bioremediation strategies, culture-dependent microorganisms are interesting and imperative toward revealing the enzymes involved in PAH degradation. In addition, the metabolic and phylogenetic characterization of bacteria that grow on certain PAHs would be essential in establishing the categories of bacteria in environment that may be most effective in PAH degradation.

Bacterial strains indigenous to a PAH-contaminated site have been shown to outcompete other artificially introduced strains in bioremediation investigations of contaminated sites (Bogardt and Hemmingsen 1992; Iwabuchi et al. 1997). It is evident that detailed knowledge of the indigenous microbial ecology would be of immense importance in the design and implementation of sustainable remediation.

Hydrocarbons may not be the best source of nitrogen and phosphorus (Obayori et al. 2010). Nonetheless, these nutrients are usually limited in contaminated sites. Stimulation with inorganic nutrients could enhance the activities of these hydrocarbon degrading bacteria. However, even among the hydrocarbon degrading bacteria the choices of these nutrients are exclusive to the type of strain or species that is present in the polluted soil (Heitkamp et al. Heitkamp et al. 1988a, b; Jimenez and Bartha 1996; Kastner et al. 1994). Many workers have reported that even with supplementation of nutrients in hydrocarbon contaminated sites, there are failures or even death of the hydrocarbon degrading bacteria due to toxicity (Carmichael and Pfaender 1997; Seklemova et al. 2001; Chaineau et al. 2005). Alternately, the use of organic waste products such as molasses may circumvent these problems encountered when using inorganic nutrient sources.

Blackstrap molasses contain about 35–40 wt% sucrose, 15–20 wt% invert sugars such as glucose and fructose and 28–35 wt% of non-sugar solids. Molasses contain a significant amount of Vitamin B6, calcium, magnesium, iron and manganese. To the best of our knowledge, studies have not been carried out in assessing the improvement of PAH degradation using molasses.

In this study, we report the isolation and characterization of bacterial strains *Lysinibacillus* sp. FB1, Bacterium FB2 and L*ysinibacillus fusiformis* strain FB3 using fluoranthene sprayed on minimal salt media. The strains were, respectively, screened for their catabolic activity on naphthalene, anthracene, pyrene and fluoranthene in liquid cultures. We used molasses, a cheap available nutrient source to evaluate the potentials of our strain to utilize fluoranthene in liquid cultures.

2 Materials and methods

2.1 Bacterial strains and culture conditions

Lysinibacillus sp. FB1, Bacterium FB2 and L*ysinibacillus fusiformis* strain FB3 were isolated from Mc Doel switchyard contaminated soils in Bloomington, Indiana, after periodic enrichment. To prevent loss of catabolic plasmids from our strains, we made use of minimal salt (MS) agar medium supplemented with fluoranthene to maintain selective pressure. Unless otherwise stated, incubation was performed at (30 ± 2.0 °C). The identification of these strains was based on sequencing of the 16S rRNA gene as previously described by Nwinyi et al. (2013).

2.2 Chemicals

Analytical grades of naphthalene, fluoranthene, pyrene and anthracene were supplied from Sigma-Aldrich Corp. (St Louis, MO. USA). The PAH analytical standards were procured from Accustandard Inc (New Haven, CT 06513). Sodium benzoate (>99% purity), 2,2,4,4,6,8,8-heptamethylnonane (HMN) (>99% purity) and all other organic solvents were obtained from Fisher Scientific Co.(Springfield, NJ, USA). Hexane, a high purity solvent for GC-chromatograph was obtained from EMD Chemicals Inc., Merck.

2.3 Preparation of Inoculum

Bacterial strains were incubated overnight in minimal salts (MSM) medium supplemented with 2.5 mM sodium benzoate at (30 ± 2.0 °C). The minimal salts used were as described by Nwinyi et al. (2013). For this, the medium consisted of (g) 0. 5 $(NH_4)_2SO_4$, 0.1 $MgSO_4{\cdot}7H_2O$, 0.076 $Ca(NO_3)_2{\cdot}4H_2O$ and 1.0 mL each of trace metal and vitamin solutions per liter of 40 mM phosphate buffer (pH 7.25). Cells were harvested by centrifugation using Beckman GS-6 series centrifuge. The cells were washed twice in MS medium without benzoate and re-suspended in the same medium at an optical density of 0.6 at 600 nm. The inoculum was used for the growth and transformation of PAH experiments.

2.4 Stock solutions preparation

Naphthalene was prepared in 2,2,4,4,6,8,8-heptamethylnonane, a non-degradable carrier to provide an initial concentration of >116 ppm. The concentration represents the total mass in both the aqueous and HMN phases, divided by the aqueous volume. In tandem, anthracene, fluoranthene and pyrene stock solutions were prepared by dissolving the weighted test compounds in acetone, respectively. From the prepared stock solutions of fluoranthene, anthracene and pyrene; Hamilton gastight syringe (250-µL) was used to add the test compounds into different Balch tubes. This provided the following test compound concentrations ca. 103 ppm for fluoranthene, ca. 21 ppm for anthracene and pyrene ca. 103 ppm in the final medium. Solid MS medium was made by the addition of 1.8% Bacto-agar (Difco Laboratories, Detroit, MI, USA). From the naphthalene-HMN stock solution, naphthalene was also added into the Balch tubes using Hamilton gastight syringe (250 µL aliquots) to provide test compound concentration of ca. 117 ppm in the final medium. The MS medium was supplemented with the test compound to achieve an experiment dependent concentration. The cultures were incubated at ambient temperature on a shaker Table (100 rpm) to aid slow mass transfer of the naphthalene into the aqueous phase. An initial investigation, using HMN as the sole carbon and energy sources revealed that HMN did not serve as growth substrate.

2.5 Characterization using 16S rRNA sequencing

The genomic DNA was isolated from overnight cultures of isolates growing on 2.5 mM benzoate using an UltraClean Microbial DNA Isolation kit (Mo Bio Laboratories, Solana Beach CA, USA). For the amplification of 16S rRNA gene, three eubacterial PCR primers, forward primer 8 fm (AGAGTTTGATCMTGGTCAG) and reverse primers 926r (CCGTCAATTCCTTTRAGTTT) and 1387r (GGGCGGWGTGTACAAGGC), were used. The polymerase chain reaction (PCR) program cycle for the reaction mixtures was incubated at 95 °C for 2.5 min and then cycled 33 times through the following temperature profile: 95 °C for 30 s, 48 °C for 30 s and 72 °C for 1.5 min, followed by a single 10 min incubation at 72 °C. About 2 µl of each amplification mixture was analyzed by agarose gel electrophoresis 10.0 µg ml^{-1} (w/v) ethidium bromide to determine that amplicons were of the expected length. The PCR amplicons were subsequently cleaned using QIAquick Nucleotide Removal kit from Qiagen Inc. (Turnberry lane, CA 91355). The PCR products were sequenced following an ABI Big Dye Terminator Cycle Sequencing reaction using an Applied Biosystems 3730 automated sequencing system (Applied Biosystems, Inc., Foster City, CA, USA). The resultant sequences were edited and aligned using CodonCode Aligner v. 2.0.6 (CodonCode Corporation, Dedham, MA, USA). Sequences were subsequently compared with deposited sequences in GenBank database using the BLAST algorithm. The 16S rRNA gene sequences have been deposited with the following accession numbers under GenBank database JN624747, JN983822, JN624748 for strains FB-1, FB-2 and FB-3.

2.6 Physicochemical composition of blackstrap molasses

The composition of blackstrap molasses was carbohydrates 8.31 g, total sugar 5.85 g, water 3.92 g, ash 1.12 g, total mineral 506.07 mg and total weight 13.67 g.

2.7 Growth on different carbon and energy sources

Replicate Balch tubes (triplicate tubes) of 10 ml of MSM containing 103 ppm of pyrene were inoculated with strains of FB-1, FB-2 and FB-3 at cell concentrations of 10^7 cells/ml. The cells were washed in phosphate buffer (pH 7.25) having been pre-grown in 2.5 mM benzoate. In each Balch

tube, 15 ml air headspace was maintained for aerobic conditions. The same procedure and conditions were adopted for the different test compound. For the naphthalene experiment, the final concentration was ca. 123 ppm; anthracene ca. 23 ppm and fluoranthene ca. 106 ppm. All the Balch tubes were crimp-sealed with Teflon-coated, butyl rubber stoppers to prevent losses due to volatilization or sorption. The tubes were incubated horizontally on a shaker table at (120 rev/min) at ambient temperature. Growth was monitored by total viable count. The total viable count was carried out by counting the cells numbers using replicate tubes via epifluorescence microscopic examination. Visual examinations in concurrence with periodic GC analyses to measure the residual test compound were done. In this study, growth was positive when there is an increase in turbidity greater than the killed or abiotic controls. For minimization of error at each time point, the entire contents of the Balch tubes were sacrificed.

2.8 Biodegradation of PAH compounds-naphthalene, fluoranthene, pyrene and anthracene experiments

The degradation experiments of naphthalene, fluoranthene, pyrene and chrysene were likewise conducted in the Balch tubes. The procedures and conditions adopted for the growth evaluation were maintained during this study. The tubes were inoculated differently with strains FB-1, FB-2 and FB-3, crimp-sealed and incubated horizontally on the shaker table at ambient temperature. The degradation study was stopped by the addition of 5 ml of hexane, vortexing for 1–2 min and subsequently, mixed continuously on a tube rotator for 12 h. The residual test compounds were extracted by liquid–liquid extraction using hexane as solvent. The hexane extracts and aqueous phases were separated by centrifugation at 2190 rpm for 20 min using Beckman GS-6 series centrifuge. The hexane extracts were collected using vial target crimp-sealed with Teflon stoppers and preserved at 4 °C prior to further analysis.

2.9 Analytical methods

2.9.1 GC analysis

Hexane extracts (5 µL injection volume) were analyzed with HP 5890 Series II gas chromatography GC (Hewlett Packard Co., Palo Alto, CA, USA) fitted with an HP 3396 series II integrator and equipped with a flame ionization detector (FID) and 30 m HP-5 megabore fused-silica capillary column (J & W Scientific, Folsom, CA, USA; 0.32 mm id, 0.25 µm film thickness) as described by (Nwinyi et al. 2013).

2.10 Statistical analysis

Statistical tests were performed using the Prism 4.0 computer software program (Graph Pad Software, San Diego, CA, USA).

3 Results

3.1 Isolation of bacterial strains in the fluoranthene and their characterization

Fluoranthene was selected as model for tetracyclic aromatic hydrocarbon (PAH) and is among the US Environmental Protection Agency's sixteen (16) priority pollutant polycyclic aromatic hydrocarbons (PAHs). The sole carbon source screening approach led to the isolation of three bacterial strains able to grow on fluoranthene as sole carbon and energy source. The isolates include: *Lysinibacillus* sp. FB1, Bacterium FB2 and *Lysinibacillus fusiformis* strain FB3. We further screened the individual isolates for growth on MS salicylic acid and MS-benzoate. The colony morphology of these isolates observed under the fluorescent microscope showed non-spore-forming straight and slightly curved rods.

3.2 Degradation and growth profile on Naphthalene

We observed higher competence for naphthalene degradation for strain Bacterium FB2 compared to *Lysinibacillus* sp. FB1 and L*ysinibacillus fusiformis* strain FB3 (see Fig. 1a). In the growth study of Bacterium FB2, in the presence of naphthalene as sole carbon and energy source, the organism exhibited an increase in cell numbers from 5.0×10^7 to 12.0×10^7 after a 1 week incubation, with a resultant decrease in naphthalene concentration. After 14 days of incubation, strain FB-2 consumed about 97% of the naphthalene from the initial concentration of naphthalene of 117 ppm. The mean biodegradation rate of naphthalene by strain FB-2 was 8.127 ± 0.074 mg l^{-1} day^{-1}. The strains FB-1 and FB-3 degraded 6.18 and 11.36% from the initial concentration of naphthalene ca. 117 ppm. The mean biodegradation rates of strain FB-1 were 0.513 ± 0.061 mg l^{-1} day^{-1}, while strain FB-3 was 0.958 ± 0.388 mg l^{-1} day^{-1}. In the growth profile study (Fig. 1b), the strains FB-1, FB-2 and FB-3 showed no lag phase, but continued to increase in cell numbers until after the second week of incubation. The increase in cell numbers was evident by increase in turbidity of the cultures, especially strain FB-2. In the control cultures (abiotic and killed), there was no appreciable difference in the naphthalene concentration (data not shown). It was observed

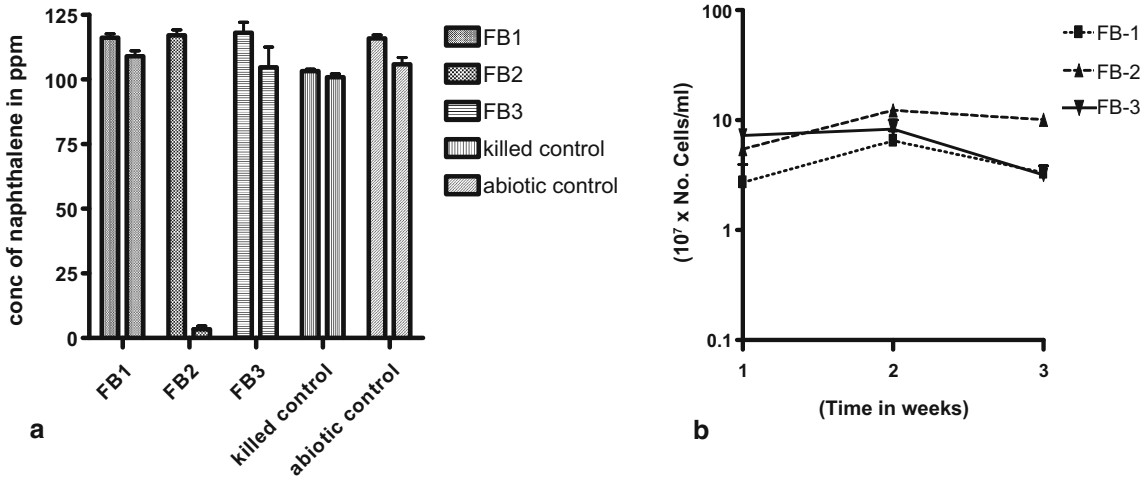

Fig. 1 **a** Degradation of naphthalene by MS-benzoate grown cells of FB-1, FB-2 and FB-3, incubated for 14 days. Data represent the mean and standard deviation of triplicate determination of initial and final concentration, respectively. The error bars (Stand. Dev.) were due to differential response of cells in triplicate tubes. **b** Naphthalene-dependent growth and cell numbers distribution of strains FB-1, FB-2 and FB-3 in naphthalene incubated for 14 days. Data represent the mean of replicates tubes for initial time (0) represented as (1), week one represent by (2), and the second week represented as (3), respectively. The x-axis value range was chosen as such to allow for even spread of the growth curve. The error bars (Stand. Dev.) were due to differential response of cells in triplicate tubes

that after some days, the culture medium developed slightly to a moderately turbid yellow color.

3.3 Degradation and growth profile on Anthracene

Higher growth rates were observed for strains FB-1, FB-2 and FB-3 on anthracene at low concentration 23 ppm. From the preliminary study in the liquid culture MS medium, we observed our strains inability to utilize 100 ppm when supplemented in the medium (data not shown). In all the strains, the highest population densities were attained on the first week of incubation, Fig. 2b showed that the population densities declined between the second and 4th week. The percentage net reduction in anthracene for strain FB-1 was 88.43% with the mean biodegradation rate of 0.933 ± 0.019 mg l^{-1} day^{-1}. Strain FB-2 had 87.34% net reduction in anthracene with the mean biodegradation rate of 0.873 ± 0.046 mg l^{-1} day^{-1}. Strain FB-3 utilized 58.18% of anthracene at the mean biodegradation rate of 0.581 ± 0.254 mg l^{-1} day^{-1}. The differences in our strains degradative behavior are as shown by the growth curve in Fig. 2b. Strain FB-1 exhibited a pseudo-stationary phase until the end of the experiment (Fig. 2b).

3.4 Degradation and growth profile on Pyrene

The growth curve of the strains FB-1, FB-2 and FB-3 in the course of pyrene degradation is as shown in Fig. 3b. The strains demonstrated very slow growth with concomitant recovery of high concentrations of pyrene at the end of incubation (Fig. 3a). The initial concentration of the pyrene used was ca. 103 ppm. After 21 days incubation, the percentage net reduction in pyrene by strains FB-1 was 6.39% with the mean biodegradation rate of 0.330 ± 0.085 mg l^{-1} day^{-1}. The strain FB-2 consumed 8.72% of pyrene at the mean biodegradation rate 0.434 ± 0.046 mg l^{-1} day^{-1}, while FB-3 utilized 0.530% at the mean biodegradation rate of 0.024 ± 0.069 mg l^{-1} day^{-1}. The growth curve fluxes showed the same pattern of decline in the cell numbers over the course of the study. It is apparent that our strains were not able to degrade pyrene in high concentrations.

3.5 Degradation and growth profile of Fluoranthene

Strains FB-1, FB-2 and FB-3 growth and degradation of fluoranthene are as shown in Fig. 4a, b. An initial fluoranthene concentration of ca. 103 ppm was used in this study. As shown in Fig. 4a, the net percentage reduction in the total fluoranthene content in (%) was 8.42, 4.24 and 4.77 for strain FB-1, FB-2 and FB-3, respectively. The mean biodegradation rate of strain FB-1 was 0.437 ± 0.176 mg l^{-1} day^{-1}. Strain FB-2 consumed fluoranthene at the rate of 0.210 ± 0.056 mg l^{-1} day^{-1}, while strain FB-3 utilized fluoranthene at the rate of 0.218 ± 0.172 mg l^{-1} day^{-1}. There was no rapid increase in cell numbers as anticipated, having been pre-grown on the MS-agar amended with sprayed fluoranthene. In Fig. 4b, the graph illustrates that strains FB-1 and FB-3 had similar growth pattern with cell numbers declining from $6.8–1.2 \times 10^7$ for FB-1, while $7.1–1.8 \times 10^7$ after the 1st

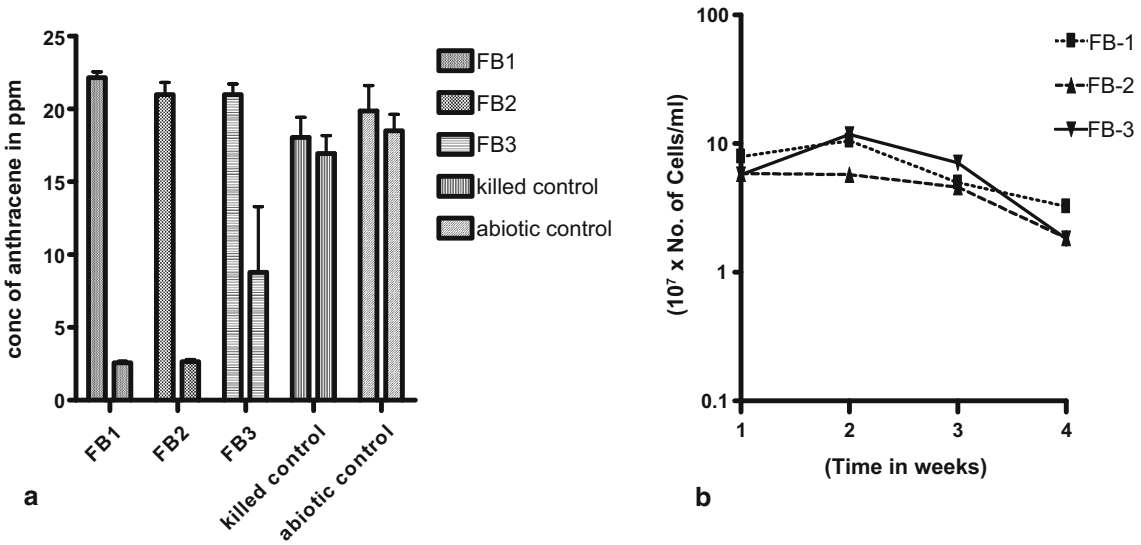

Fig. 2 **a** Degradation of anthracene by MS-benzoate grown cells of FB-1, FB-2 and FB-3 incubated for 21 days. Data represent the mean and standard deviation of triplicate determination of initial and final concentration, respectively. The error bars (Stand. Dev.) were due to differential response of cells in triplicate tubes. **b** Anthracene-dependent growth and cell numbers distribution of strains FB-1, FB-2 and FB-3 in anthracene incubated for 21 days. Data represent the mean of replicates tubes for initial time (0) represented as (1), week one represented (2), week two represent (3) and the 3rd week (21 days) represented as (4), respectively. The x-axis value range was chosen as such to allow for even spread of the growth curve. The error bars (Stand. Dev.) were due to differential response of cells in triplicate tubes

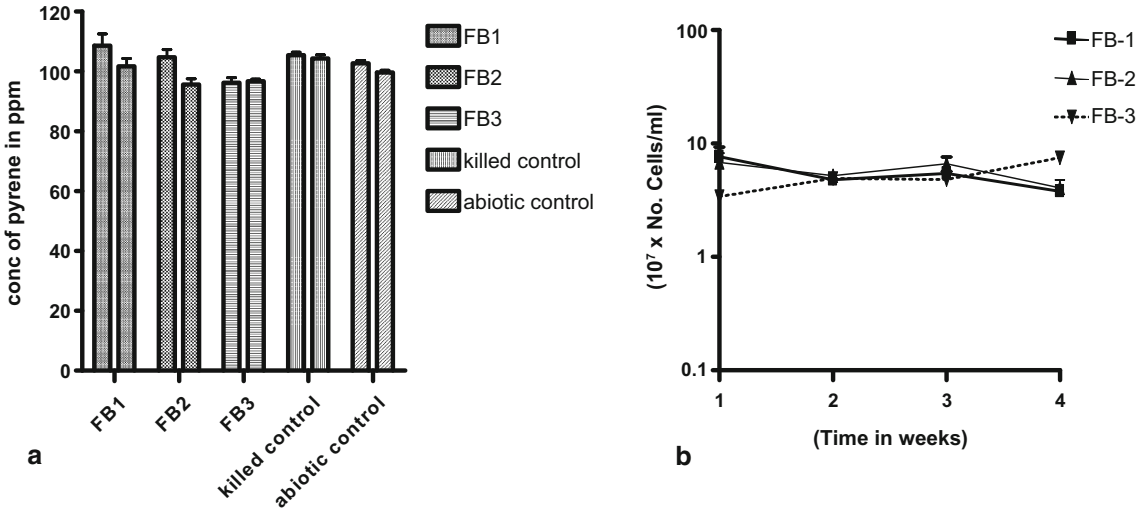

Fig. 3 **a** Degradation of pyrene by MS-benzoate grown cells of FB-1, FB-2 and FB-3 incubated for 21 days. Data represent the mean and standard deviation of triplicate determination of initial and final concentration, respectively. The error bars (Stand. Dev.) were due to differential response of cells in triplicate tubes. **b** Pyrene-dependent growth and cell numbers distribution of strains FB-1, FB-2 and FB-3 in pyrene incubated for 21 days. Data represent the mean of replicates tubes for initial time (0) represented as (1), week one represented (2), week two represent (3) and the third week (21 days) represented as (4), respectively. The x-axis value range was chosen as such to allow for even spread of the growth curve. The error bars (Stand. Dev.) were due to differential response of cells in triplicate tubes

week of incubation; thereafter, the cell numbers increased. This change in the growth dynamics of strains FB-1 and FB-3 in the liquid medium presents an interesting phenomenon and may be due to evolution of different degradation mechanisms in terms of site of attack and bioavailability of fluoranthene in liquid medium. Strain FB-2 showed different growth pattern which was in consonance with the continuous decline in the cell numbers when assessed during the total viable count. This decline continued until the end of experiment.

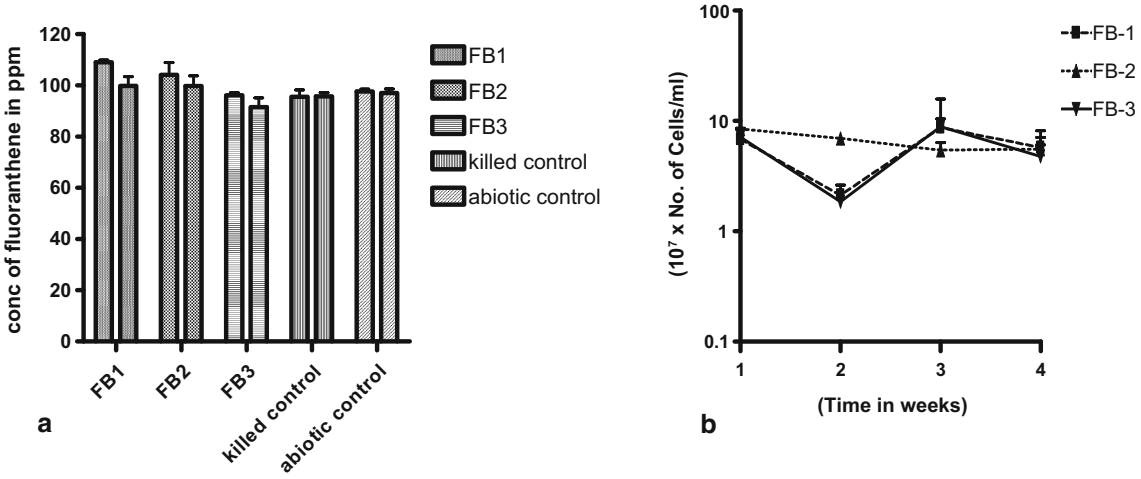

Fig. 4 **a** Degradation of fluoranthene by MS-benzoate grown cells of FB-1, FB-2 and FB-3 incubated for 21 days. Data represent the mean and standard deviation of triplicate determination of initial and final concentration, respectively. The error bars (Stand. Dev.) were due to differential response of cells in triplicate tubes. **b** Fluoranthene-dependent growth and cell numbers distribution of strains FB-1,FB-2 and FB-3 in fluoranthene incubated for 21 days. Data represent the mean of replicates tubes for initial time (0) represented as (1), week one represented (2), week two represent (3) and the third week (21 days) represented as (4), respectively. The x-axis value range was chosen as such to allow for even spread of the growth curve. The error bars (Stand. Dev.) were due to differential response of cells in triplicate tubes

3.6 Degradation of Fluoranthene Supplemented with BlackStrap Molasses

Replicates Balch tubes of 10 ml MSM containing ca. 103 ppm of fluoranthene and 1.4% of molasses were inoculated with strains FB-1, FB-2 and FB-3. The cell numbers were determined at 7 day interval for 21 days, while the residual fluoranthene was determined at the initial day and the final day of the incubation period. Our purpose was to determine whether the rate of fluoranthene utilization can be hastened when molasses is supplemented with fluoranthene. From this investigation, strain FB-1 could utilize 4.68% of the fluoranthene at the mean biodegradation rate 0.214 ± 0.006 mg L^{-1} day^{-1}. Strains FB-2 utilized 5.98% at the mean biodegradation rate of 0.296 ± 0.224 mg L^{-1} day^{-1}, while FB-3 consumed 6.36% from the initial concentration of fluoranthene at the mean biodegradation rate of 0.318 ± 0.002 mg L^{-1} day^{-1}. In Fig. 5b, strain FB1-3 showed an increase in their cell numbers following addition of molasses but without significant utilization of fluoranthene. The highest growth rate was recorded in the penultimate week before the termination of the study. The strain FB-2 showed the highest growth with cell numbers increasing from 7 to 13 (10^7 cells/ml). This suggested that in the liquid culture (MS) media, in the presence of another carbon source, our strains preferred the alternate carbon source (molasses) rather than the test compound (fluoranthene) served as sole carbon source. In addition, the increased cell numbers did not influence the utilization of fluoranthene as expected. There was only a slight increase in the percentage of fluoranthene utilized by strain FB-2 and FB-3, when compared with MS medium without molasses. Statistical evaluation using ANOVA at 5% showed no significant difference in the amount of fluoranthene used.

4 Discussion

Biological degradation is the major dissipation route for most organic pollutants in the soil environment. Nonetheless, the activity of the degrading microorganism is reliant upon contaminant uptake and bioavailability, concentration, toxicity, access to relevant nutrient and activated enzymes (Cerniglia 1992). Venkata Mohan et al. (2006) reported that microbial ecology, system microbiology, process operation conditions (microenvironment and configuration) and the presence/absence of indigenous activity will in turn govern the overall bioremediation efficiency. In this study, we report successful isolation of indigenous bacterial strains FB-1, FB-2 and FB-3 that were able to grow and utilize naphthalene, anthracene and partially pyrene and fluoranthene in liquid culture medium. Interestingly, these strains grew exceedingly when grown on solid MS medium sprayed with fluoranthene. PAH-contaminated soils provide a broad range of substrates and metabolites that create an environment that develops quite a complex microbial community. Most scavenging bacterial communities in contaminated soils tend to be dominated by the strains that can survive toxicity and thus are able to utilize the contaminant itself for growth (Zucchi et al. 2003).

During growth on naphthalene, strains FB-1 and FB-3 showed similar growth dynamics and possible degradation

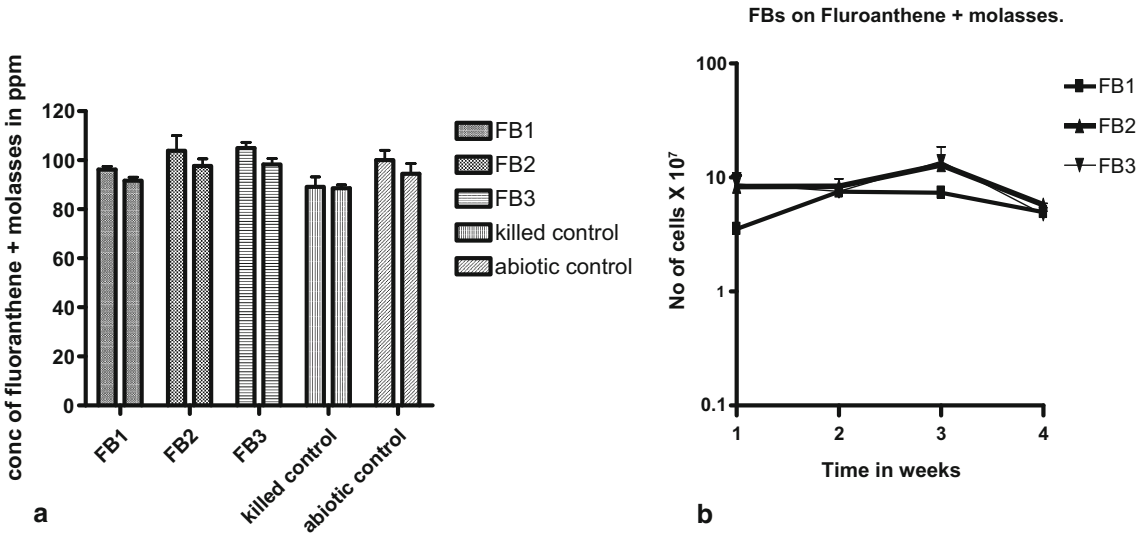

Fig. 5 **a** Degradation of fluoranthene supplemented with blackstrap molasses by MS-benzoate grown cells of strains FB-1 FB-2 and FB-3. Data represent the mean and standard deviation of triplicates determination of initial and final concentrations. The large error bars (Stand. dev) were due to differential response of cells in triplicate tubes. **b** Fluoranthene and blackstrap molasses-dependent growth and cell numbers of strains FB-1, FB-2 and FB-3 after 21-day incubation. Data represent the mean of replicates tubes for initial time (0) represented as (1), week one represented (2), week two represent (3) and the third week (21 days) represented as (4), respectively. The x-axis value range was chosen as such to allow for even spread of the growth curve. The error bars (Stand. dev) were due to differential response of cells in triplicate tubes

pattern. Strain FB-2 utilized significant proportion of the naphthalene present. It may therefore be that strain FB-2 might be entirely equipped with different catabolic pathway from that of FB-1 and FB-3. However, further work needs to be done to determine if our strains utilized the dioxygenase or monooxygenase enzymes to attack the aromatic ring of the naphthalene. Usually, the addition of oxygen by these enzymes is the rate limiting step in the degradation of PAHs (Kelley et al. 1990; Heitkamp et al. 1988a). The increase experienced in cell numbers of strain FB-2 correlates well with previous works on naphthalene degradation. Comparing the degradation performance of strains FB1 and FB-3, our strains showed slow utilization of naphthalene. Perhaps the low utilization of naphthalene may be due to toxicity of the test compound. It has been reported that the generation of toxic metabolites may be severe on the organisms, particularly when the test compound serves as the sole carbon and energy source (Adebusoye et al. 2008). Ramos et al. and Sikkema et al. demonstrated that bacteria associated with PAH metabolism face the task of acquiring carbon and energy sources from toxic compounds (Ramos et al. 2002; Sikkema et al. 1995). Thus, the inability of the potential biodegrading populations to tolerate the aromatic hydrocarbon toxicity contributes to the persistence of such pollutants in the environment. Previous workers (Park et al. 2004; Sikkema et al. 1995; Heipieper et al. 1994) believed that toxicity disrupts the biological membranes and in some cases causes the production of toxic metabolites. In addition, the lipophilic nature of aromatic hydrocarbons can alter membrane fluidity, permeabilize the membrane and cause swelling of the lipid bilayer. This alteration of membrane structure can disrupt energy transduction and possibly the activity of membrane-associated proteins that aid degradation of aromatic compounds (Heipieper et al. 1991; Sikkema et al. 1995).

Anthracene is a tricyclic PAH that is resistant to nucleophilic attack (Johnsen et al. 2005). Strains FB-1, FB-2 and FB-3 degradative efficiency were evaluated on anthracene (23 ppm) as sole carbon and energy sources. Strain FB-1 consumed 88.43%, FB-2 87.34% and FB-3 58.18%. Zhang et al. isolated a *Pseudomonas* Wphe1 that completely degraded phenanthrene and naphthalene but not anthracene. Only a *Paraccocus* sp. strain Ophe1 was capable of degrading anthracene (Zhang et al. 2004). Jacques et al. reported *Pseudomonas* 222A consumed about 71% of anthracene in 48 days (Jacques et al. 2005). In this study, our strains FB-1, FB-2 and FB-3 were able to utilize the anthracene but not as significantly as *Pseudomonas* reported by Jacques and co-workers who used 250 ppm of anthracene. Since all our strains were at exponential growth phase under the same condition, the initiation of pseudo-stationary growth phase by strain FB-2 in anthracene may not be related to inorganic nutrient limitation or accumulation of toxic substances. We observed rather that strain FB-2 exponential growth ceased. This may be due to the physical limitation to the maximum dissolution rate of anthracene within the cells.

The drop in total viable count of our strain over the incubation period on pyrene indicated their inability to utilize pyrene effectively in the MS liquid culture. We observed that the strain FB-1 utilized 6.39% of the pyrene, strain FB-2 utilized 8.72%, and FB-3 utilized 0.53%. It was apparent therefore that our axenic cultures did not degrade pyrene considerably. The data obtained were in agreement with Wang et al. (2008) who reported about their pure culture P1's inability to degrade pyrene as good as their consortium. Even when their strain P1 was screened on fluoranthene, it could not degrade the test compound as expected in a liquid medium. It was obvious that when our strains were incubated over MS-agar sprayed with fluoranthene, they thrived well. Following this interesting phenomenon, we investigated the abilities of our strain FB-1, FB-2 and FB-3 to degrade fluoranthene in the liquid culture medium. We recorded very minimal utilization of the fluoranthene where strain FB-1 utilized 8.24%, FB-2 4.24% and FB-3 4.77%. We further investigated supplementing the MS-fluoranthene with another carbon source molasses, having had a previous report of Obayori et al. (2010) that used corn steep liquor to improve the growth and degradation rate of pyrene by their organisms *Pseudomonas* sp. LP1 and *Pseudomonas aeruginosa* LP5. From the results obtained, it was obvious that the alternative carbon source did not increase the rate of fluoranthene degradation. The strains FB-1, FB-2 and FB-3 increased only in cell numbers without a decrease in fluoranthene concentration. Strain FB-1 utilized 4.68%, strain FB-2 utilized 5.98%, and strain FB-3 utilized 4.29%. By comparison, the unsupplemented fluoranthene (MS) medium had more degradation as fluoranthene served as the sole carbon and energy source. In this study, the degree of fluoranthene degradation in the two scenarios revealed that the organisms did not thrive well in the liquid culture medium. Possibly, their low utilization could be due to toxicity of fluoranthene. Our findings corroborate the report of Miller et al. (2004) who discovered that their organism readily degraded fluoranthene on plate, while it was difficult to be utilized in liquid culture. Also, Bastiaens et al. (2000) isolated an anthracene using *Mycobacterium* sp. from a PAH-sorbing membrane, but none of the anthracene utilizing strains were able to grow and degrade anthracene in a liquid enrichment medium. Muckian et al. (2009) noticed slow onset (15–20 days) in the degradation of fluoranthene. In this study, the recalcitrance of fluoranthene in liquid medium to microbial degradation could be due to fluoranthene hydrophobicity, high molecular weight and suitable site for microbial enzymes attack (Mueller et al. 1989; Hansen et al. 2004; Guerin and Boyd 1992). As a result of these aforementioned factors, it is possible that in our study site, fluoranthene may have been bound tightly to the soil thus reducing its mobility in the soil environment (Cerniglia 1993). We may suggest that our strain FB1-3 may have strongly adapted or attached to solid matrix, which they find difficult to grow in and utilize the high molecular weight PAHs (pyrene, fluoranthene) in the liquid cultures. Also, it is possible that due to sorption of fluoranthene and pyrene in the study site, our strains showed longer acclimation period with consequent low utilization of the substrates as observed.

From the microscopic examination using fluorescent microscope, the smear samples of our strains exhibited swarming form of movement at the edges. It is possible that our strains could thrive well under community structure rather than as individual cells. In line with this argument of possible community existence, our strains FB1, FB2 and FB3 could be growing as consortium in the form of biofilm communities, bioaggregates, microecosystems and other networks of interacting microorganisms. When strains FB1, FB2 and FB3 grow as a community consortium, they could exhibit exchange of primary and secondary metabolites and behavioral adaptations. The advantage of growing as consortium in solid matrix makes them more resilient to environmental stress, favorable collective growth constants (maximum specific growth rate, half saturation constant or cell yield) and broader habitat range. Hence, when our strains are in the liquid culture medium, the community structure–species composition network and architecture may be threatened.

In conclusion, three new strains of bacteria belonging to the genera *Lysinibacillus* species and *Paenibacillus* species which are capable of degrading PAHs were isolated and identified. Overall, the results obtained demonstrate that our strains *Lysinibacillus* sp. FB1, Bacterium FB2 and L*ysinibacillus fusiformis* strain FB3 may be excellent candidates with strong potential for future application in the bioremediation PAH-contaminated sites, particularly on solid soil matrices.

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Compliance with ethical standards

Conflict of interest Authors have declared that no competing interests exist.

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