Online ISSN: 1920-3853

Vol. 7, No. 2, June 2013

Print ISSN : 1715-9997

Canadian Journal of pure & applied sciences

an International Journal

SENRA
Academic Publishers
British Columbia

The Canadian Journal of Pure and Applied Sciences (CJPAS-ISSN 1715-9997) is a peer reviewed multi-disciplinary specialist journal aimed at promoting research worldwide in Agricultural Sciences, Biological Sciences, Chemical Sciences, Computer and Mathematical Sciences, Engineering, Environmental Sciences, Medicine and Physics (all subjects).

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Global Impact Factor for 2012 = 2.657
Index Copernicus Journals Evaluation for 2011 = 4.63

Frequency:
3 times a year (Feb, June and Oct.)

Editorial Office
E-mail: editor@cjpas.ca
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British Columbia V3X 0C5 Canada
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Print ISSN 1715-9997
Online ISSN 1920-3853

Volume 7, Number 2
June. 2013

CANADIAN JOURNAL OF PURE AND APPLIED SCIENCES

Board of Editorial Advisors

Member
CANADIAN ASSOCIATION OF LEARNED JOURNALS

SENRA Academic Publishers, British Columbia
Vol. 7, No. 2, pp. 2303-2314, June 2013
Online ISSN: 1920-3853; Print ISSN: 1715-9997

Canadian Journal of pure&applied sciences

AEROBIC DEGRADATION OF NAPHTHALENE, FLUORANTHENE, PYRENE AND CHRYSENE USING INDIGENOUS STRAINS OF BACTERIA ISOLATED FROM A FORMER INDUSTRIAL SITE

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ABSTRACT

Four bacterial strains were isolated from a former industrial site contaminated with organic and inorganic pollutants for decades. The isolation was done using naphthalene as sole source of carbon and energy during the enrichment. 16S rRNA gene sequence analyses of the four isolates (OC1, OC2, OC3, and OC4) assigned the strains to the genus, Enterobacter (OC1) and Pseudomonas (OC2, OC3, and OC4). The degradation and growth behavior of the four isolates was investigated on naphthalene, fluoranthene, pyrene and chrysene. All the strains utilized naphthalene, fluoranthene, chrysene but pyrene partially, as sole sources of carbon and energy. The time course studies using relative concentration > 100ppm, >115ppm, > 89ppm and > 12 ppm for naphthalene, fluoranthene, pyrene and chrysene respectively, resulted in rapid exponential increases in cell numbers and concomitant disappearance of the test substrates. Naphthalene was degraded between the range of 25 % and 99%, while chrysene degradation ranged between of 35 and 69%, pyrene 4 - 21% and fluoranthene 7 -19 %. Our results suggest that contaminated, former industrial sites contain a capable microbial community that may be used for bioremediation of the site.

Keywords: 16S rRNA, contaminated sites, bioremediation, napthalene, fluoranthene, pyrene, chrysene

INTRODUCTION

Soil contaminated with polycyclic aromatic hydrocarbons presents a considerable public health hazard (Cerniglia and Heitkamp, 1989; Samanta *et al*., 2002), particularly where other pollutants are also present. The problems are compounded when heavy metals are present in significant concentration (Springael *et al*., 1993; Sipila *et al*., 2010). Furthermore, the presence of pollutants could lead to temporal and spatial negative changes in the distribution of autochthonous microorganisms at the site of pollution. In this scenario, natural remediation of such sites by the native isolates could be hampered. Alternately, pollutants able to be used as microbial substrates might enrich populations that enhance degradation. From previous studies, PAHs contamination can significantly alter a region's ecology and present the greatest ecological challenge when streams, rivers and groundwater are at risk of contamination. Several studies have emphasized that the physico-chemical properties of PAHs and sorption to soil components over time reduce contaminant availability and degradability (Semple *et al*., 2007). Microbial degradation is one of the principal means of PAH removal from soils (Macleod and Semple, 2002; Doick *et al*., 2005; Macleod and Semple, 2006; Li *et al*., 2008) and is affected primarily by contaminant bioavailability and catabolic ability of indigenous microbial populations. Adaptation processes, that occur as a result of an increase in the hydrocarbon-oxidising potential of the microbial community (Macleod and Semple, 2006), encourage the development of microbial populations with the ability to degrade PAHs.

Naphthalene, is one of the 16 PAHs classified as priority pollutants by US Environmental Protection Agency (USEPA, 1994, 2004). This bicyclic aromatic hydrocarbon and its methylated derivatives are considered as some of the more problematic water-soluble fraction of petroleum (Heitkamp *et al*., 1987). With increasing aging times in contaminated soils, naphthalene shows a reduction in extractability (Ncibi *et al*., 2007), due hydrophobicity and resultant high solid-liquid distribution ratio, characteristics which also limit bioavailability and biodegradation. Chrysene, a high molecular weight PAH, is also of environmental concern due to its toxicity, carcinogenic and mutagenic nature (Nwanna *et al*., 2006). It is highly recalcitrant in soils under normal conditions due, at least in part, to limited bioavailability. Although aerobic PAH biodegradation has been studied for several decades, isolation and characterization of novel organisms

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remain of value because of the potential for discovery of microorganisms with unique and diverse substrate ranges and study of novel catabolic pathways. Successful enrichment and isolation of PAH-degrading isolates offer more intrinsic value than using molecular techniques alone (Hedlund and Staley, 2006). Isolation of new strains allows more detailed studies of biodegradation kinetics, enzymology, and genes encoding biodegradation enzymes. In addition, comparison of homologies between catabolic genes of microbes isolated from the same environment can allow assessment of horizontal gene transfer. Thus, from such knowledge, a deeper understanding of microbial-mediated mechanisms of catalysis of PAHs will provide new strategies for development of effective bioremediation of PAH-contaminated sites.

In the present study, several bacteria belonging to the γ-proteobacteria were isolated and screened for naphthalene, chrysene, fluoranthene and pyrene degradation. For the isolation, contaminated soils were collected and enriched in a PAH supplemented medium. Because preliminary investigation of these organisms revealed interesting substrate ranges and characteristics, we characterized them phylogenetically on the basis of 16S rRNA gene analysis. Although organisms belonging to other genera were also isolated, the scope of this work is limited to understanding the dynamics of degradation of naphthalene, chrysene, fluoranthene and pyrene by γ-proteobacteria enriched from naphthalene-fed enrich-ments under aerobic conditions.

MATERIALS AND METHODS

Chemicals

Analytical grades of high purity (>99%) naphthalene fluoranthene, pyrene and chrysene were procured from Sigma Aldrich Corp. (St. Louis, MO, USA). Sodium benzoate (99+ % purity), 2,2,4,4,6,8,8-heptamethylnonane (HMN), and organic solvents were obtained from Fisher Scientific Co. (Springfield, NJ, USA). Hexane, was purchased from EMD Chemicals Inc (Gibbstown, NJ 08027). PAH analytical standards used were purchased from Accustandard Inc (New Haven, CT 06513).

Stock solutions and media

All the enrichment and degradation experiments were performed using minimal salts (MS) medium as described by (Kim and Picardal, 2000; Nwinyi *et al*., 2008; Nwinyi, 2010, 2011) . The medium consisted of 0. 5g $(NH_4)_2SO_4$, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.076 g $Ca(NO_3)_2.4H_2O$ and 1.0mL each of vitamin and trace mineral solutions per liter of 40mM phosphate buffer (pH 7.25). The naphthalene stock solution was prepared in HMN, a non-degradable carrier to provide an initial concentration of ca. 100ppm. This concentration represents the total mass in both the aqueous and HMN phases, divided by the aqueous volume. The appropriate stock solution was added using a gas-tight syringe in 250-µL aliquots to provide test compound concentration of ca.100ppm in the final medium. Concurrently, fluoranthene, chrysene and pyrene stock solution were prepared differently by dissolving the weighted test compounds in acetone respectively. Fluoranthene, chrysene and pyrene were added from the different stock solution of the test compound into the balch tubes using a gas-tight syringe in 250-µL aliquots, to provide test compound concentration of ca.116 ppm for fluoranthene, ca. 12ppm for chrysene, and pyrene ca 94ppm in the final medium. Solid MS medium was made by the addition of 1.8% Bacto-agar (Difco Laboratories, Detroit, MI, USA). MS medium was supplemented with the test compound, achieving an experiment dependent concentration of about 100ppm. The cultures were incubated at ambient temperature on a shaker table to aid mass transfer of the naphthalene into the aqueous phase. Preliminary investigations were carried out using MS medium supplemented with HMN as the sole carbon and energy sources to determine that HMN did not serve as growth substrate.

Enrichment of bacterial species

Enrichment of bacterial isolates were performed using soil samples collected from PAH- contaminated sites at the former McDoel Switchyard in Bloomington, Indiana, USA. The site had been previously contaminated with PAHs and other organic and inorganic pollutants. The soil samples were collected at three locations with indications of low to high level PAH-contamination based on a preliminary environmental audit. The first sample was collected from a soil thought to have moderate levels of PAH contamination. This sample and subsequent samples were placed in separate sterile jars and transported back to the lab at ambient temperatures. The second sample was collected underneath a creosote-coated log. The third sample was collected at an area of reported high PAH contamination from soils previously overlaid by a railroad ties. The soil was less sandy and darker in color than the other soils. PAH- degrading bacteria indigenous to the soils were enriched and isolated as follows: Five g of the different soil samples were weighed into 160-mL serum bottles containing 30mL of sterile MS medium. Due to the fact that existing PAHs in the contaminated soils may have limited bioavailability due to sorption and 'weathering', naphthalene (4mg) ca.133ppm was added to the enrichment bottles containing the MS medium as a supplemental carbon and energy source. All the 160-mL serum bottle bioreactors were set up in triplicates. The serum bottles were crimp-sealed with teflon-coated, butyl rubber stoppers to prevent losses due to volatilization and/or sorption. These were incubated horizontally on an orbital shaker table (Labline Instruments, Inc. Melrore Park, IL, USA) at ambient temperature. Air sparging was done weekly to re-aerate the headspace and biweekly

transfers were made using about 15% inoculum into new MS medium supplemented with the test PAHs. The procedure was repeated for seven successive times.

Isolation, purification and characterization of pure culture using 16S rDNA

Pure cultures from naphthalene-enriched media were isolated by directly plating aliquots (0.2mL) of highly-enriched cultures onto MS agar. Because we wished to prevent loss of catabolic plasmids from capable isolates, we used MS agar medium supplemented with naphthalene rather than nutrient agar to maintain selective pressure. The naphthalene was added to the medium using the spray plate technique as described by (Kiyohara *et al*., 1982). Immediately after spread-plating the 0.2mL aliquot of enrichment culture, an ethereal solution of naphthalene was uniformly sprayed onto the surface of the agar. The plates were sealed with parafilm film and incubated for 1 week at 30^{o}C. Naphthalene-degrading microorganisms were identified by cleared zones around an individual colony. The colonies were purified on MS agar sprayed with naphthalene and sustained on solid MS plates containing 2.5mM benzoate or 100ppm salicylic acid. For 16S rRNA gene analysis, genomic DNA was isolated from overnight cultures of isolates growing on 2.5mM benzoate using an UltraClean Microbial DNA Isolation kit (Mo Bio. Laboratories,Solana Beach CA,USA). Three eubacterial PCR primers; forward primer 8fm (AGAGTTTGATCMTGGTCAG) and reverse primers 926r (CCGTCAATTCCTTTRAGTTT) and 1387r (GGGCGGWGTGTACAAGGC) were used to amplify the 16S rRNA gene. The reaction mixtures were incubated at 95^{o}C for 2.5min and then cycled 33 times through the following temperature profile: 95^{o}C for 30s, 48^{o}C for 30s, and 72^{o}C for 1.5min, followed by a single 10min incubation at 72^{o}C. About 2µl of each amplification mixture was analyzed by agarose gel electrophoresis 10.0µg ml^{-1} (w/v) ethidium bromide to ascertain 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). For the 16S rRNA sequencing, 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 available at URL http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al*., 1990).

Growth on different carbon and energy sources

Pure cultures were assessed for their potential to grow on naphthalene, fluoranthene, pyrene and chrysene. The tests were carried out in MS medium supplemented with each PAH test compound as sole carbon source. Although we examined aerobic PAH degradation, we conducted experiments in crimp-sealed tubes (Balch tubes) usually utilized for anaerobic studies. Prior to use in each experiment, tubes were baked in muffle furnace at 500^{o}C to remove organic contaminants. Growth and degradation studies were performed in Balch tubes containing 10ml of MS medium, the tested PAH, inoculum, and approximately 15mL air headspace to maintain aerobic conditions. . Different tubes were supplemented with different PAHs respectively. Naphthalene was added from an HMN stock solution at a concentration of ca.100ppm as described above and inoculated with 10^5 cells/ml of phosphate buffer (pH 7.25) washed cells pre-grown in 2.5mM benzoate. Fluoranthene, chrysene and pyrene were added from the stock solution into the balch tubes using a gas-tight syringe in 250-µL aliquots, to provide test compound concentration ca.116 ppm for fluoranthene, ca. 12 ppm for chrysene, and pyrene ca. 94ppm in the final medium. 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. All the stock solutions were aseptically prepared before use. Growth was monitored by counting the cells numbers using replicate tubes via epifluorescence microscopic examination. The cells were stained with acridine orange stain after fixation with 5µL of glutraldehyde. Visual examinations in concurrence with periodic GC analyses to measure the test compound disappearance was also done. In this study growth was positive when there is an increase in turbidity greater than the killed or abiotic control that was used. For statistical evaluation, at least 10 microscopic fields were randomly chosen and a minimum of 1000 cells were counted. Data are presented as the mean cell numbers ± the SEM.

Transformation of PAH compounds- naphthalene, fluoranthene, pyrene and chrysene experiments

Degradation study of naphthalene, fluoranthene, pyrene and chrysene were correspondingly conducted in the Balch tubes. The tubes were inoculated with respective bacterial cultures, crimp sealed and incubated horizontally on the shaker table at ambient temperature. The degradation reactions were stopped after 14days for naphthalene while experiments with fluoranthene, pyrene and chrysene were stopped after 21days. The degradation study was stopped by the addition of 5mL of hexane, vortexing for 1-2min and subsequently, mixed continuously on a tube rotator for 12hrs. The hexane extracts and aqueous phases were separated by centrifugation at 2190rpm for 20minutes using a Beckman GS-6 series centrifuge. The hexane and aqueous extracts were separated and the hexane extract collected for further analysis. Extracts were stored in target vials with a headspace of 1mL and crimp sealed using an

11mm Teflon rubber stopper from National scientific and stored at 4°C prior to analysis.

Analytical methods

GC analysis

Hexane extracts were analyzed on an 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). Hexane extracts (5µL injection volume) were injected using a 10-µl Hamilton gas-tight syringe through a 30m HP-5 megabore fused-silica capillary column (J & W Scientific, Folsom, CA, USA; 0.32mm id, 0.25µm film thickness). The GC utilized Helium (He) as the carrier gas and was programmed for naphthalene at an initial temperature of 50°C; this was held for 5min then ramped at 30°C/min to 180°C for 2min, then ramped to 300°C at 40°C/min for 4min. Analytical standards of PAHs were prepared in hexane. Typical coefficients of correlation for standard curves were 0.98-0.99.

STATISTICAL ANALYSIS

Statistical tests was performed using the Prism 4.0 computer software programme (Graph Pad Software, San Diego, CA, USA) and Statistical Package for Social Scientists (SPSS) 15.0.

RESULTS

Isolation and phylogenetic characterization of the PAH degrading strains

Eleven different microbial colonies were selected from the MS agar plates following initial enrichment on naphthalene. Upon screening individual isolates for growth on MS salicyclic acid and MS benzoate, we selected four isolates for further study. The colony morphology of some isolates observed under the fluorescent microscope showed non-spore-forming straight and slightly curved rods about 0.5-0.7µm x 1-2.5µm. Based on partial 16S rRNA sequencing (approximately 1300 bp), phylogenetic analysis placed our strains OC-2, OC-3 and OC-4 within the genus *Pseudomonas* (Table 1). The closest relative of strain OC-1 was an *Enterobacter* species with 99% similarity. Strain OC-2 had 99% homology as *Pseudomonas putida* (AB513735). Strain OC-3 had 99% identity as *Pseudomonas putida* (AB513735) and Strain OC-4 with 100% homology as *Pseudomonas putida* strain SP2 (GQ 200822). We have classified our isolates as Bacterium OC-1, Bacterium OC-2 , *Pseudomonas* sp.strain OC3 and *Pseudomonas* sp.strain OC4 (GenBank database accession numbers JN624749 through JN 624751 and JN983823).

Degradation of naphthalene by the bacterial species

Strains OC-1 OC-2, OC-3 and OC-4 ability to degrade naphthalene were examined using washed, benzoate grown cells. No carbon source other than naphthalene was provided. After 14days incubation, the different isolates ability to degrade the naphthalene was assessed by comparing the GC peak areas of the initial day time (0) and the final time (t). Growth on naphthalene was evidenced by intense turbidity of the culture media and significant reduction in the concentration of naphthalene. Strains OC-1, OC-3 and OC-4 were able to completely degrade almost all added naphthalene. (Figure 1a) shows the values of the net reduction (percent reduction in total naphthalene content) in naphthalene concentration. These were 99, 25, 99 and 99% respectively for strains OC-1, OC-2, OC-3 and OC-4. The initial concentration of

Table 1. Cloned fragments of 16S r RNA genomic DNA of PAH degrading bacterial species.

Bacterial strain	Tentative identity	Confirmed identity	Closest relative	Bacterial subdivision	% ID[a] with closest relative	Genbank accession no.	Length (nt)[1]
OC-1	*Enterobacter* species	Bacterium OC-1	*Enterobacter* species	γ-proteobacteria	99	JN624749	1309
OC-2	*Pseudomonas putida*	Bacterium OC-2	*Pseudomonas putida* (AB513735)	γ-proteobacteria	99	JN983823	1228
OC-3	*Pseudomonas putida*	*Pseudomonas* sp.strain OC3	*Pseudomonas putida* (AB513735)	γ-proteobacteria	99	JN624750	846
OC-4	*Pseudomonas putida*	*Pseudomonas putida* strain OC4	*Pseudomonas putida* strain SP2 (GQ 200822)	γ-proteobacteria	100	JN624751	1298

[a] *ID* identity, [1] *nt* nucleotides

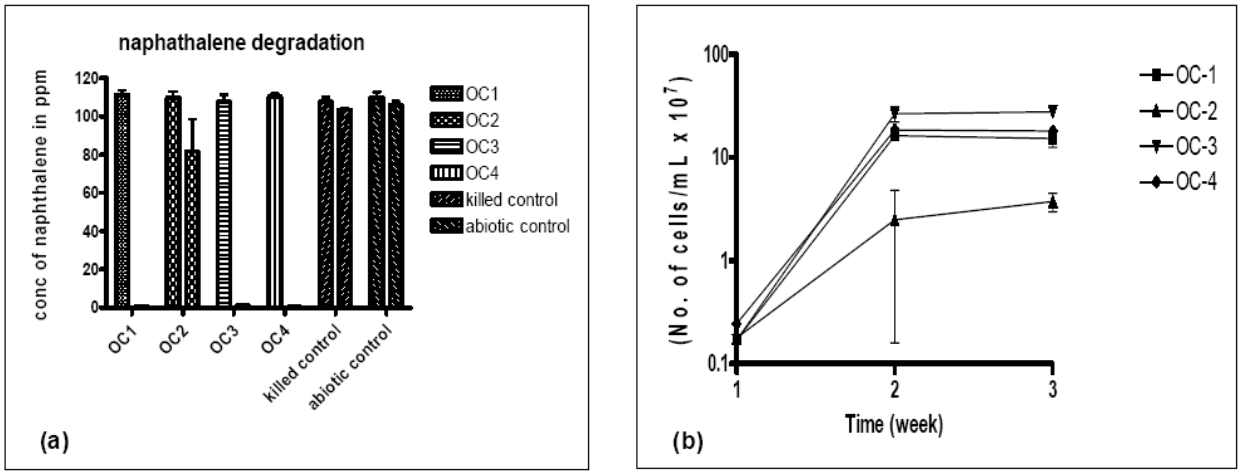

Fig. 1(a). Degradation of naphthalene by MS-benzoate grown cells of OC-1, OC-2, OC-3 and OC-4 incubated for 14 days. Data represent the mean and standard deviation of triplicate determination of initial and final concentration respectively. The large error bars (Stand. Dev.) were due to differential response of cells in triplicate tubes. (b) Naphthlane-dependent growth and cell numbers distribution of Strains OC-1, OC-2, OC-3 and OC-4 in naphthalene incubated for 14 days. Data represent the mean of replicates tubes for initial time (0) cell density represented as (1) and final time (14 days) represented as (3) respectively. The x-axis value range was chosen as such to allow for even spread of the growth curve. The large error bars (Stand. Dev.) were due to differential response of cells in triplicate tubes.

naphthalene at time zero was ca. 110 ppm while the final concentration ranged between 0.43–82 ppm. The mean biodegradation rate of naphthalene by strain OC-1 was 0.33 ± 0.01 mg L^{-1} h^{-1}. Strain OC-2 performance was less than that of strains OC-1, OC-3 and OC-4. The mean OC-2 biodegradation rate for naphthalene was 0.08 ± 0.06 mg L^{-1} h^{-1}. Strain OC-3 exhibited a biodegradation rate for naphthalene was 0.32 ± 0.01 mg L^{-1} h^{-1}. Strain OC-4 consumed naphthalene at the rate of 0.327 ± 0.01 mg L^{-1} h^{-1}. Following the end of the incubation period, a yellow colour was observed in some Balch tubes, suggesting that the strains may have incompletely degraded the naphthalene through a meta-cleavage pathway. Analyses were carried out by calibrating the HPLC with standards of intermediate products of naphthalene (salicylate, catechol and acetate) using an external standard method. When the aqueous extracts were analyzed for intermediate products, no product was detected, although the detection limit of the HPLC may have not allowed measurement of these limited intermediates. More likely, other intermediates may have been produced that were not detected with our HPLC method.

Growth of bacterial strains on naphthalene

We defined growth as an increase in cell numbers of at least one-order-of-magnitude and concomitant disappearance of the parent compound when compared to the abiotic and biotic control. Since naphthalene was dissolved in HMN, there was the need to determine that the observed growth was due to the presence of the test substrate naphthalene rather than the HMN. When HMN alone was added as the only carbon source in preliminary experiments, there was no appreciable growth observed for strains OC-1 OC-2, OC-3 and OC-4 over the time period of our experiments. A slight initial increase in cell numbers observed for some isolates fed HMN alone may possibly be due to continued cell division by the inocula or continued utilization of endogenous substrates. In all cases where growth occurred on the test substrates, cell numbers increased by a significant orders-of-magnitude more than tubes of the control (abiotic and biotic) HMN carrier. This clearly demonstrated growth on naphthalene. (Fig. 1b) shows the results of growth profiles of strains OC- 1-4. It shows a 1 to 2 orders-of-magnitude increase in cell numbers for all strains. Since cell numbers were counted after 7 days, it is not possible to ascertain if the benzoate-grown inoculum exhibited a lag period when presented with naphthalene as a substrate. Over the course of the experiments, strain OC-2 exhibited a smaller increase in cell numbers than did the other isolates. This is consistent with the lower naphthalene degradation rate of OC-2. Since naphthalene measurements were done at the end of the incubation, it is not clear if the cessation of growth after one week by OC-1, OC-2, and OC-4 was due to naphthalene depletion after 1 week.

Degradation of chrysene

All the strains OC-1-4, utilized chrysene as carbon and energy sources. However strain OC-4 was able to degrade more of the chrysene than other bacterial strains (Fig. 2a). The (mean and standard deviation values) of chrysene concentration used in this study was ca.12ppm. At the end

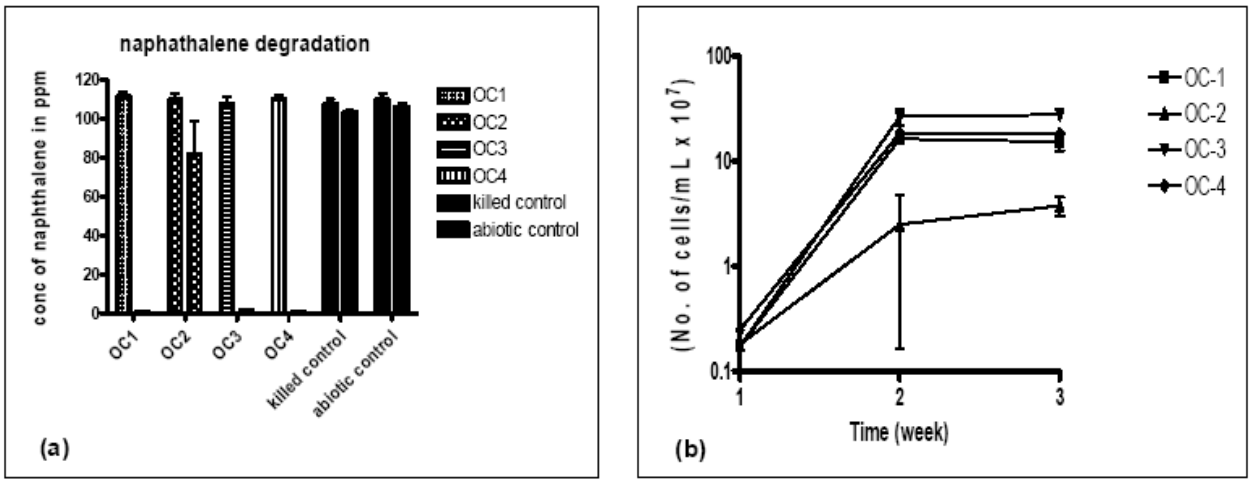

Fig. 2(a). Degradation of chrysene by MS-benzoate grown cells of OC-1, OC-2, OC-3 and OC-4 incubated for 21 days. Data represent the mean and standard deviation of triplicate determination of initial and final concentration respectively. The error bars (Stand. Dev.) were due to differential response of cells in triplicate tubes. (b) Chrysene-dependent growth and cell numbers distribution of Strains OC-1, OC-2, OC-3 and OC-4 in chrysene incubated for 21 days. Data represent the mean of replicates tubes for initial time (0) cell density represented as (1) and final time (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.

of the incubation period, the strains degraded about 36 - 69% of the chrysene. The final concentration (mean and standard deviation) assayed was between 3-7 ppm. Strain OC-1 utilized the chrysene at 36% at volume biodegradation rate of 0.01 ± 0.0002 mg L^{-1} h^{-1}. Strain OC-2 used 52% of its chrysene as carbon and energy source at volume biodegradation rate of 0.014 ± 0.01 mg L^{-1} h^{-1}. Strain OC-3 utilized 68 % of chrysene at the rate of 0.02 ± 0.02 mg L^{-1} h^{-1}. Strain OC-4 consumed 69 % chrysene at the rate of 0.02 ± 0.001 mg L^{-1} h^{-1}. From growth profile study (Fig. 2b) the strains entered into log phase. Among the organisms evaluated strain OC-4 degraded about 69% of the chrysene.

Degradation of Fluoranthene

The degradation of fluoranthene was evaluated using strains OC-1,OC-2 ,OC-3 and OC-4. It appeared that there was a general lag period in the growth profile of the organisms (Fig. 3b). This may be because the organisms were pre-grown with benzoate as a substrate which does not apparently induce the requisite enzymes to degrade the fluoranthene. The percentage net reductions for fluoranthene are 13, 13, 19 and 7% for strains OC-1, OC-2,OC-3 and OC-4 respectively. Representing the concentration in ppm the intial concentration ca. 94ppm and the final concentration ca. 81ppm thus this represents, at best, a minor utilization of the fluoranthene (Fig. 3a). The mean biodegradation rate of fluoranthene utilized by strain OC-1 was 0.03 ± 0.01mg L^{-1} h^{-1}. Strain OC-2 utilized fluoranthene at rate of 0.03 ± 0.01mg L^{-1} h^{-1}. Strain OC-3 volume biodegradation rate utilized per hour was 0.05 ± 0.001mg L^{-1} h^{-1}. Strain OC-4 utilized fluoranthene at the rate of 0.02 ± 0.001mg L^{-1} h^{-1}. In the killed and abiotic controls there were minimal losses. On comparison between the different strains, OC-3 utilized the fluoranthene more than strains OC-1, OC-2 and OC-4. Statistical analysis performed at P- value (0.05) with SPSS 15.0 (Fig. 3c) showed that there was no significant difference ($P < 0.05$) in the data obtained for our initial and final readings among our strains and the controls. This further validates the consistency in our experimental setup and goes to show that our organisms may have similar behaviour in degrading fluoranthene. Nonetheless, it doesn't mean that the strains didn't degrade fluoranthene when there is no difference between cultures and controls. In (Fig. 3c), variance analysis showed that results obtained for each strain in the degradation study tubes analysed showed a significant difference ($P>0.05$) from those obtained for killed and abiotic control tubes. In comparison, fluoranthene analysis of tubes inoculated with strain OC-3 showed an enhanced significance over other strains and the controls. There was no apparent difference in degradation in cultures of OC-4 and the killed control. The variance analysis showed that the data obtained for OC-4 did not show significant difference ($P>0.05$) from the killed control.

The growth profile exhibited by strain OC-2 and OC-3 were similar (Fig. 3b). There are expectations that the organisms may have similar growth patterns in fluoranthene unlike the patterns exhibited by isolates OC-1 and OC-4 . With the exception of strain OC-4, the organisms, however, were marginally able to degrade fluoranthene, a tetracyclic aromatic hydrocarbon. All the

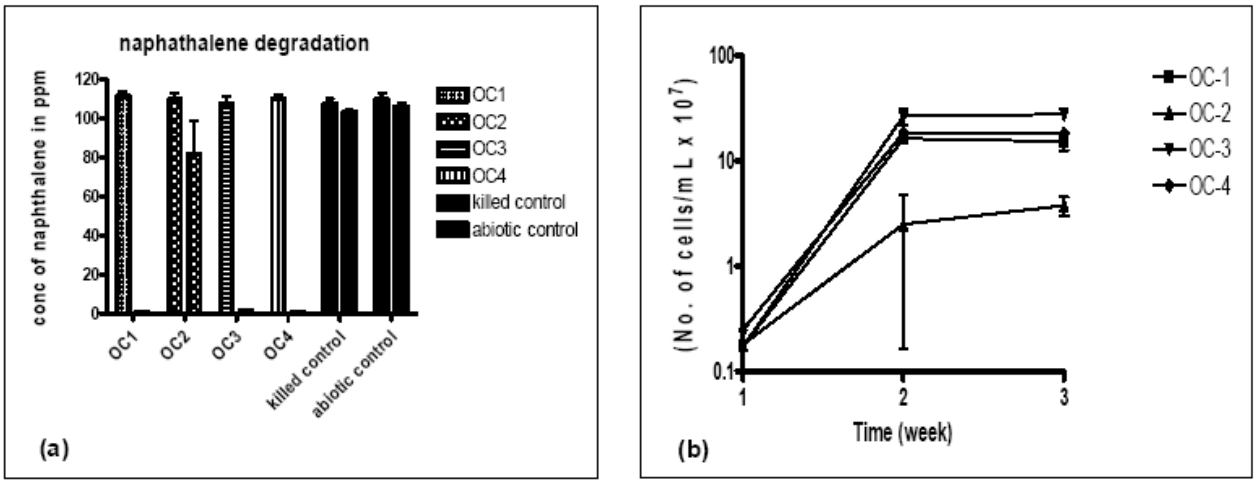

Fig. 3(a). Degradation of fluoranthene by MS-benzoate grown cells of strains OC-1, OC-2, OC-3 and OC-4 incubated for 21 days. Data represent the mean and standard deviation of triplicate determination of initial and final concentrations. The error bars (Stand. Dev.) were due to differential response of cells in triplicate tubes. (b) Fluoranthene-dependent growth and cell numbers distribution of Strins OC-1, OC-2, OC-3 and OC-4 incubated for 21 days. Data represent the mean of triplicate tubes for initial time (0) cell density represented as (1) and final time (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.

organisms lag phase followed the same dynamics but however with different time periods for entry into the log phase. After the third week of observed increases, there was a decline in cell numbers. This may suggest accumulation of a toxic metabolite, although this hypothesis needs further verification. However, the rate of cell decline for strains OC-2 and OC-3 were different from OC-1 and OC-4.

Figure 3c. Statistical analyses of Strains OC-1, OC-2, OC-3, and OC-4 cultures incubated with fluoranthene for 21days ,compared with that of the controls (abiotic and killed) at p-value 0.05 Values presented in y-axis represent the difference in mean of obtained data from the initial and final concentrations, compared with that of the abiotic and killed controls. Values presented are from triplicate samples.

Degradation of Pyrene

The abilities of strains OC-1, OC-2, OC-3 and OC-4 were evaluated for the extent of pyrene degradation in (Fig. 4a) and their growth patterns (Fig. 4b). It was evident, that the strains exhibited similar trend of growth profile in pyrene (Fig. 4b). There was no observed lag phase until after about a week of incubation. Following that, there was general decline and a lag phase period. It suggests that the organisms may have been utilizing endogenous substrates due to previous enrichment in MS-benzoate. Of course, the decline in cell number suggests that there was period of adaptation and synthesis of the enzymes for the utilization of pyrene. Conversely none of the isolates exhibited even 1-order-of-magnitude increase in cell numbers after 4 weeks. This shows that no clear growth occurred. The initial concentration of pyrene was ca. 94ppm and after the incubation period of 21 days, the final concentration ca. 81ppm. Strains of OC-1 were able to consume about 21% of pyrene at the biodegradation rate of 0.04 ± 0.003 mg L^{-1} h^{-1} , while OC-2 used , 11 %, at the rate of 0.02 ± 0.001 mg L^{-1} h^{-1}; strain OC-3, consumed 4.71% at the rate of 0.009 ± 0.002 mg L^{-1} h^{-1} and Strain OC-4, consumed 17 % of the pyrene added as sole source of carbon and energy. The rate of the biodegradation 0.032 ± 0.0001 mg L^{-1} h^{-1} . This suggests, that these organisms can slowly utilize pyrene but however, over a long adaptation period. The long period of adaptation observed may be due to the concentration of the pyrene which was ca. 94ppm. Among these strains OC-1 exhibited more degradative ability. Variance analysis using SPSS 15.0 in (Fig. 4c) showed that results obtained for each strain in the degradation study tubes analysed showed a significant difference ($P>0.05$) from those obtained for killed and abiotic control tubes.

DISCUSSION

Enrichment using the target substrates has always proved to achieve novel metabolic capabilities from indigenous microbial strains that can be explored in dealing with xenobiotics in the environment. In this contribution, we report for the first time the isolation and characterization bacterial strains from the McDoel Switchyard site. The soils collected during our studies were contaminated with

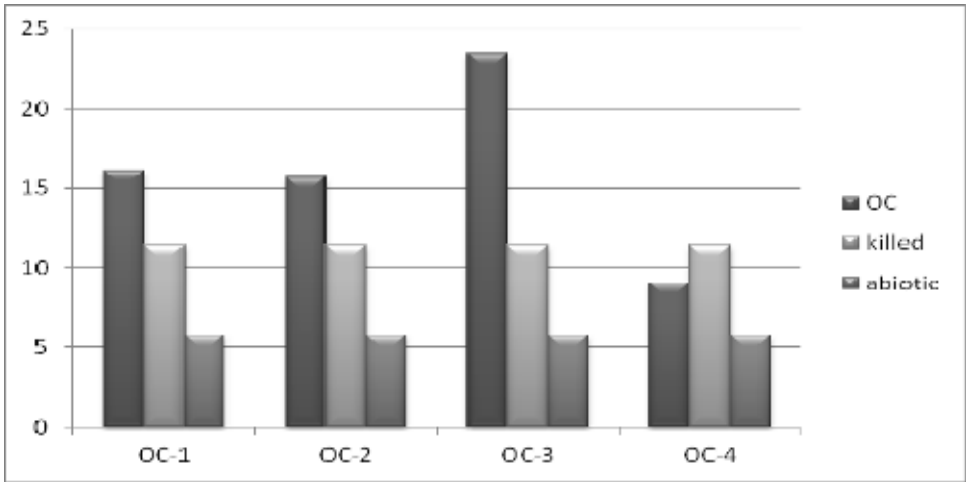

Fig. 3c. Statistical analyses of Strains OC-1, OC-2, OC-3, and OC-4 cultures incubated with fluoranthene for 21days, compared with that of the controls (abiotic and killed) at p-value 0.05. Values presented in y-axis represent the difference in mean of obtained data from the initial and final concentrations, compared with that of the abiotic and killed controls. Values presented are from triplicate samples.

inorganic and organic pollutants. The four bacterial strains that were isolated had the ability to degrade low molecular and high molecular weight PAHs – naphthalene chrysene, fluoranthene and pyrene partially. The identification of our strains to belong to the genus *Enterobacter* and *Pseudomonas* has continued to re-enforce the role the strain play in sustainable environmental management particularly in elucidation of naphthalene degradation pathways (Peng *et al*., 2008). The strains grew on MS salicylate and MS benzoate which are common intermediate products of naphthalene degradation.

According to reports of Ramos *et al*. (2002), most indigenous bacteria capable of utilizing aromatic hydrocarbons are challenged with securing carbon and energy sources from these compounds due to their potential toxicity and chemical stability. Thus this leads to persistence of such pollutants at sites of contamination. From previous studies, it is believed that toxicity of pollutants disrupts the cell membranes of most soil bacteria, influence production of toxic metabolites and can alter membrane fluidity, permeabilize the membrane and swelling of lipid bilayer (Sikkema *et al*., 1995; Park *et al*., 2004; Pepi *et al*., 2009). Furthermore, Heipieper *et al*., 1994; Sikkema *et al*., 1995 reported that with alteration of membrane structure, there could be a disruption of energy for transduction and the activity of membrane coupled proteins.

In this study, it was observed that naphthalene was volatile when dissolved in organic solvents such as dichloromethane and acetone. This however leads to inconsistency in the stock solution concentrations. This was investigated by measuring the stock solution in the GC FID chromatograph at different time intervals (data not shown). Thus with this anomaly, most workers using the aforementioned organic solvents may inadvertently be generating false positive results. Based on this we used 2,2,4,4,6,8,8 heptamethylnonane (HMN) to dissolve naphthalene and it yielded consistent values of the same concentration. HMN used as a carrier did not support significant or sustained increases in cell numbers in the abiotic and biotic controls. The changes in naphthalene concentrations without bacterial inoculation were very insignificant. It's believed that the HMN a highly branched alkane did not induce aromatic dioxygenase, thus functioned as intended i.e reduction in volatility and facilitated mass transfer of naphthalene into the medium. It may however, not be possible to discount that HMN may have influenced the result in an unknown manner.

Several workers have isolated bacterial species that can utilize naphthalene as a sole source of carbon and energy, most belong to the genera *Alcaligenes*, *Burkholderia*, *Mycobacterium*, *Polaromonas*, *Pseudomonas*, *Ralstonia*, *Rhodococcus*, *Sphingomonas*, and *Streptomyces* (Cerniglia, 1992; Auger *et al*., 1995; Story *et al*., 2001; Zhou *et al*., 2002; Kim *et al*., 2003; Pumphrey and Madsen, 2007). In the works of Pellizari *et al*. (1996), they reported of bacterial species isolated via naphthalene enrichment with ability to metabolize other organic pollutants. Thus the presence of naphthalene may be marginally effective in stimulating the cometabolism of other organic pollutants. This is due to the possibility of our strains to possess naphthalene dioxygenase that is known to be a versatile enzyme, able to catalyze a wide variety of other reactions. Our strains exhibited an ability to utilize a high molecular weight compound (chrysene) as carbon source. Chrysene, is a four condensed benzene

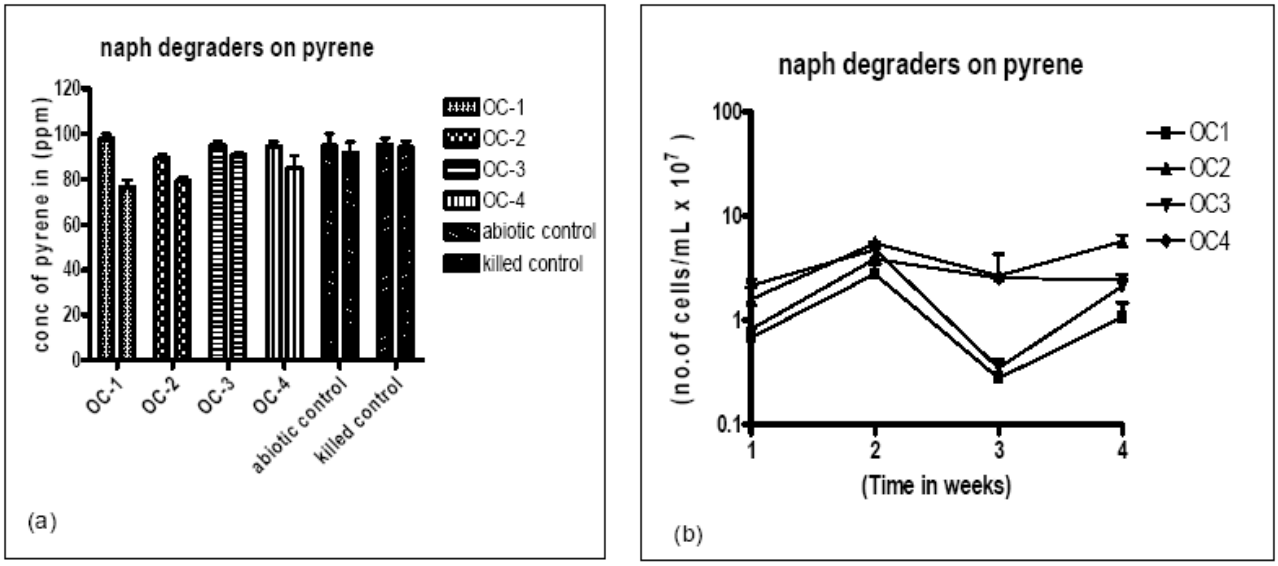

Fig. 4(a): Degradation of pyrene by MS-benzoate grown cells of strains OC-1, OC-2, OC-3 and OC-4 incubated for 21 days. Data represent the mean and standard deviation of triplicate determination of initial and final concentrations. The error bars (Stand. Dev.) were due to differential response of cells in triplicate tubes. (b) Pyrene-dependent growth and cell numbers of Strins OC-1, OC-2, OC-3 and OC-4 incubated for 21 days. Data represent the mean of triplicate tubes for initial time (0) cell density represented as (1) and final time (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 large error bars (Stand. Dev.) were due to differential response of cells in triplicate tubes.

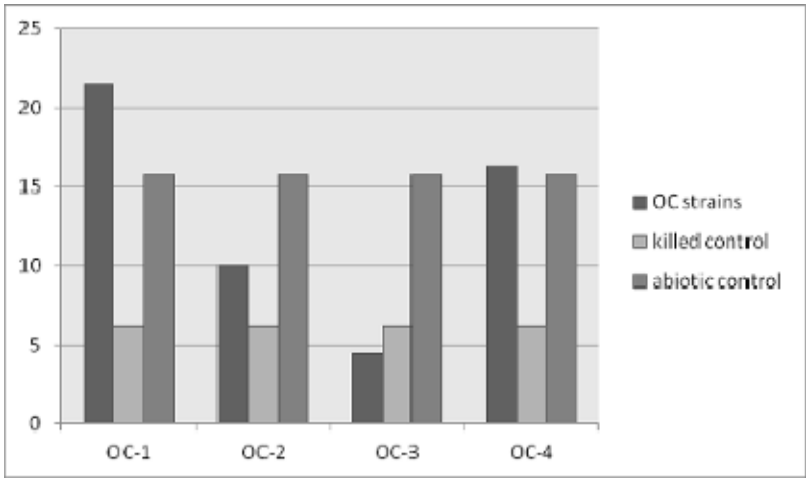

Fig. 4c. Statistical analyses of Strains OC-1, OC-2, OC-3, and OC-4 cultures incubated with pyrene for 21 days, compared with that of the controls (abiotic and killed) at p-value 0.05. Values presented in y-axis represent the difference in mean of obtained data from the initial and final concentrations, compared with that of the abiotic and killed controls. Values presented are from triplicate samples.

rings, produced via incomplete combustion of organic materials such as fossil fuels, other industrial processes, it's believed to be toxic, mutagenic and carcinogenic to humans. Microbial degradation is believed to be one of the major ways to clean up chrysene-contaminated environments. In the reports of (Tam *et al*., 2002; Hadibarata *et al*., 2009), microbial communities could have considerable potential to remedy aromatic hydrocarbon-contaminated sediment and remove chrysene from aqueous solution. High-molecular-weight PAHs (HMW PAHs) such as chrysene and benzo[*a*]pyrene are hard to be biodegraded (Massie *et al*., 1985; Heitkamp and Cerniglia, 1987; Yamada *et al*., 2003). However a number of bacterial species have been noted to degrade chrysene *Rhodococcus* sp.Strain UW1 (Walter *et al*., 1991), *Sphingomonas yanoikuyae* which oxidized

chrysene (Boyd *et al*., 1999) and *Pseudomonas fluorescens* that utilise chrysene and benz[a]anthracene as sole carbon sources (Caldini *et al*., 1995). Thus the efficiency in which PAH is biodegraded in different environment differs from another. Chrysene oxidation occurs by incorporation of an oxygen molecule in an aromatic ring. This is catalyzed by dioxygenase to a *cis*-dihydrodiol intermediate, which undergoes further metabolism via pyridine nucleotide dependent dehydrogenation reaction to produce catechols (Hinchee *et al*., 1994). In the reports of (Laor *et al*., 1999), the biodegradation of PAHs are reduced by sorption of the PAHs to sediments. Because PAHs are highly lipophilic, they tended to sorb tightly limiting their availability to microorganisms. Our bacterial strains have shown abilities to utilize chrysene, fluoranthene and pyrene significantly at low concentration. This further confirms the influence of toxicity, hydrophobicity to the biodegradation rate of PAHs. Obviously, the capacity of the bacterial strains OC-1, OC-2, OC-3 and OC-4 to utilize both low and high molecular weight PAHs is an indication of their possession of the ring fission enzymes (Ilori and Amund, 2000; Amund *et al*., 2006). Thus the isolation and phylogenetic characterization of our bacterial strains have provided a valuable resource for detailed examination of the PAH catabolic potential and identification of novel functional genes associated with the PAH degradation.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the financial support of Institute of International Education, administrators of the Fulbright Scholarship which supported O. Nwinyi and the School of Public and Environmental Affairs Indiana University, Bloomington IN, USA, for additional support.

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Received: Dec 6, 2012; Revised: Feb 26, 2013;
Accepted: March 1, 2013.