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Mixture of High Molecular Weight Polycyclic Aromatic Hydrocarbons Biodegradation by Tropical Bacteria and via Co-Metabolism with Phenanthrene

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Abstract: The experiment was conducted to evaluate the potential of tropical bacterial isolates and its consortium to biodegrade mixture of high molecular weight polycyclic aromatic hydrocarbons (chrysene, fluoranthene and pyrene). The effect of phenanthrene in the degradation was also investigated. The bacterial consortium was made up of *Sphingomonas* sp., *Pseudomonas* sp. and *P. putida* and biodegradation set up for 8 days with initial 100 mg L^{-1} substrate concentration. Degradation by *Sphingomonas* sp., *Pseudomonas* sp. and *P. putida*, respectively after 8 days gave higher residual chrysene of 40.2±1.4, 40.3±2.2 and 27.4±1.8 mg L^{-1}, fluoranthene of 32.5±1.3, 35.4±1.2 and 10.1±2.5 mg L^{-1} and pyrene of 37.5±1.2, 34.2±2.4 and 32.0±1.2 mg L^{-1} compared to 11.5±1.4 (chrysene), 6.2±1.3 (fluoranthene) and 6.0±1.8 (pyrene) mg L^{-1} obtained using the bacterial consortium. When the media was supplemented with 100 mg L^{-1} phenanthrene, after 8 days of degradation by bacterial consortium residual chrysene, fluoranthene and pyrene was 0.45±0.25, 0.02±0.02 and 0.20±0.14 mg L^{-1}, respectively while phenanthrene was undetectable. No statistical significant ($p < 0.05$) difference was obtained between degradation by bacterial consortium and consortium via co-metabolism with phenanthrene rather they had a strong correlation of $r = 0.99$. The results suggest that bacterial consortium may be useful for the decontamination of sites polluted with high molecular weight polycyclic aromatic hydrocarbons due to synergistic effect.

Key words: Bacterial consortium, biodegradation, chrysene, fluoranthene, pyrene

INTRODUCTION

Anthropogenic activities such as crude oil refining, fossil fuel combustion, waste incineration and automobile usage deposit Polycyclic Aromatic Hydrocarbons (PAHs) into the environment (Christensen and Bzdusek, 2005; Nganje *et al*., 2007; Wilcke, 2007). This has raised environmental concern about PAHs because of their biological toxicity which includes cytotoxic, carcinogenic, genotoxic and/or mutagenicity (Castorena-Torres *et al*., 2008; Topinka *et al*., 2008). PAHs polluted sites tend to pose contamination concerns to sources of drinking water and agricultural produce thereby constituting a significant health hazards to current and future generations (Tao *et al*., 2004; Meudec *et al*., 2006; Anyakora and Coker, 2007).

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Bacterial bioremediation of PAHs-contaminated sites has been found to be a promising alternative remedial strategy (Samanta *et al*., 2002; Mrozik *et al*., 2003). Studies have shown that the low molecular weight PAHs (LMW PAHs) containing less than four benzene rings such as naphthalene, anthracene and phenanthrene are readily biodegraded (Santos *et al*., 2008; Seo *et al*., 2009). Whereas, the High Molecular Weight PAHs (HMW PAHs) containing four or more benzene rings such as chrysene, fluoranthene and pyrene are generally recalcitrant to microbial attack and this contributes to their persistence in the environment (Jonathan *et al*., 2003; Johnsen *et al*., 2005). In addition, the success of most PAHs bioremediation projects has been shown to be limited by failure to remove the HMW PAHs (Wilson and Jones, 1993). This is further compounded by the existence of PAHs in complex mixture (Leblond *et al*., 2001; Lotfabad and Gray, 2002). Earlier studies have found that interactions between PAHs in the environment are possible which can alter the rate and extent of their biodegradation within a mixture (Beckles *et al*., 1998; Dean-Ross *et al*., 2002). The study have also shown that HMW PAHs may be biodegraded via co-metabolism using LMW PAHs or degradation pathway intermediates as carbon and energy source (Chen and Aitken, 1999; Supaka *et al*., 2001). However, some other studies indicated that the presence of LMW PAHs such as anthracene and phenanthrene in biodegradation of a HMW PAH may cause delay in the HMW PAH biodegradation (Dean-Ross *et al*., 2002; Igwo-Ezikpe *et al*., 2010). This has been attributed to the preference utilization of the LMW PAH (Tadros and Hughes, 1997; Lotfabad and Gray, 2002).

Therefore, for bioremediation to be an effective tool for the clean-up of HMW PAHs contaminated soils, greater understanding of the degradation of mixture of HMW PAHs and the contribution of consortium of isolates are required. The present study aims to investigate (1) the degradation ability of mixture of HMW PAHs by bacterial isolates and consortium; and (2) co-metabolism effect of phenanthrene on biodegradation of mixture of HMW PAHs.

MATERIALS AND METHODS

Chemicals and Media

All chemicals and solvents used were of analytical grade. Chrysene, fluoranthene, pyrene, phenanthrene, ethyl acetate, acetonitrile and salts of Mineral Salt Media (MSM) were sourced from Sigma Chemical Co. (Germany). All PAHs had purity = 96%. Nutrient agar and nutrient broth were from Fluka (Germany).

Microorganisms and Culture Conditions

Sphingomonas sp., *Pseudomonas* sp. and *Pseudomonas putida* used in this study were previously isolated from PAH-contaminated soils in our laboratory (Igwo-Ezikpe *et al*., 2010). They were maintained freeze-dried and stored at - 4°C. The frozen stock was used to prepare seed cultures for all experiments. For inoculation, the frozen stock was cultivated on nutrient broth for three days, bacterial cells were harvested by centrifugation (4000x g, 4°C, 15 min) and washed twice with phosphate buffer (50 mM, pH 7.2). The cells were resuspended in same buffer and used as the starter culture for biodegradation experiments. The Mineral Salt Medium (MSM) for biodegradation experiments contained per liter (pH 7.2): NH_4NO_3, 4.0 g; Na_2HPO_4, 2.0 g; KH_2PO_4, 0.53 g; K_2SO_4, 0.17 g, $MgSO_4.7H_2O$, 0.10 g and trace elements solution (1 ml L^{-1}), sterilized by autoclaving at 121°C for 20 min as described by Ilori and Amund (2000).

Biodegradation Studies

This research was carried out between June, 2007 and 2008 in Biochemistry Department, College of Medicine, University of Lagos, Nigeria.

Biodegradation of Mixture of HMW PAHs by Bacterial Isolates

The bacterial isolates were used to evaluate their ability to degrade mixture of the HMW PAHs (Chrysene, fluoranthene and pyrene) using them as sole carbon and energy sources. MSM (20 mL, pH 7.2) were put into different 250 mL Erlenmeyer flasks containing mixture of the HMW PAHs to a final concentration of 100 mg L^{-1} each. The media were sterilized and inoculated with 3 mL (10^4 cells) of the starter culture. The culture flasks were wrapped with aluminum foil and incubated aerobically in the dark at 30±2.0°C with agitation at 150 rpm. This set up was designated experiment (E).

Two controls (C1 and C2) were also included; C1 consisted of the same materials present in E but without HMW PAHs while C2 contained all the materials in E with no test isolate inoculated. The experiment was set up in duplicate for 8 days, samples taken at 48 h intervals. Biodegradation of the HMW PAHs was assayed by determining the residual HMW PAHs using HPLC after extracting twice with ethyl acetate, microbial population density measured spectrophometerically as optical density at wavelength 600 nm ($OD_{600\ nm}$), growth of the bacterial isolates in the media estimated by the Most Probable Number (MPN) technique using nutrient broth as the growth media (Gonzalez, 1996) and pH of the media. The HPLC analyses were performed with VYDAC RP C18 reverse phase column (250×0.4 mm). Separation was achieved by gradient elution in acetonitrile: water (60, 50, 40, 30, 20, 10 and 0% water), temperature 25°C, with a flow rate of 0.8 mL min^{-1} and UV absorbance detector set at 254 nm.

Biodegradation of Mixture of HMW PAHs by Bacterial Consortium

Sphingomonas sp., *Pseudomonas* sp. and *P. putida* were used together as a consortium to evaluate ability to degrade mixture of the HMW PAH (100 mg L^{-1} of each HMW PAH) in 20 mL MSM. The controls C1 and C2 were included in the set-up. The experiments were carried out in duplicate for 8 days, incubated aerobically in the dark at 30±2.0°C with agitation at 150 rpm and biodegradation analyzed as earlier stated.

Biodegradation of Mixture of HMW PAHs by Bacterial Consortium via Co-Metabolism with Phenanthrene

Bacterial consortium was used to degrade mixture of the HMW PAHs (100 mg L^{-1} each of HMW PAH) in 20 mL MSM supplemented with 100 mg L^{-1} phenanthrene as co-substrate. The experiments were set up in duplicate for 8 days, incubated aerobically in the dark at 30±2.0°C with agitation at 150 rpm and biodegradation analyzed as earlier stated.

Data Analysis

The experimental assays were done in triplicates, unless otherwise stated. Statistically significant difference ($p<0.05$) was determined using Analysis of Variance (ANOVA). Results are expressed as Mean±SEM (Standard Error of Mean). Correlation test between biodegradation of mixture of the HMW PAHs by bacterial consortium and consortium via co-metabolism with phenanthrene were compared using Pearson-product-moment correlation. These statistical analyses were done using Statistical Package for the Social Science15.0 for windows (SPSS 15.0).

RESULTS

Residual HMW PAH During Degradation of Mixture of HMW PAHs by Bacterial Isolates

Sphingomonas sp., *Pseudomonas* sp. and *P. putida* degraded mixture (100 mg L^{-1} each) of chrysene, fluoranthene and pyrene (Fig. 1a-c). However, a lag degradation of the HMW PAHs was observed such that at day 4, residual chrysene was 89.2±0.9, 90.1±2.5 and

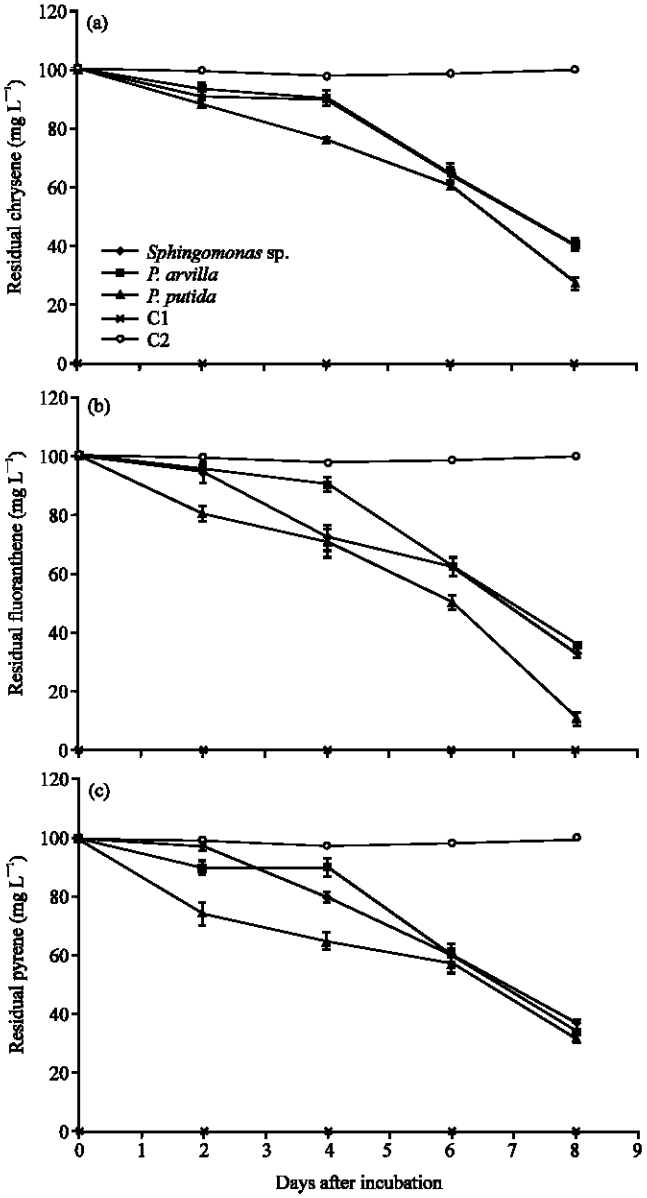

Fig. 1: Residual HMW PAH during degradation of mixture of HMW PAHs by bacterial isolates (a) Chrysene, (b) Fluoranthene and (c) Pyrerne

76.0± 0.9 mg L^{-1}, fluoranthene was 72.1±4.5, 90.1±2.1 and 70.4±5.3 mg L^{-1} and pyrene was 80.1±2.1, 90.2±3.4 and 65.2±2.8 mg L^{-1} for *Sphingomonas* sp., *Pseudomonas* sp. and *P. putida*, respectively. There were also no significant ($p<0.05$) differences in degradation between the isolates unlike with control C2. In addition, no significant ($p<0.05$) changes in HMW PAHs concentration of all controls C2 was observed during the experimental period.

Growth Profile of Bacterial Isolates During Degradation of Mixture of HMW PAHs

Growth profile of the isolates during degradation of mixture of the HMW PAHs determined by MPN, optical densities ($OD_{600\ nm}$) and pH of the media is shown in Fig. 2. Highest cell densities of 3.5×10^4, 3.6×10^4 and 4.7×10^4 cfu mL^{-1}, respectively were obtained for *Sphingomonas* sp., *Pseudomonas* sp. and *P. putida*. No significant ($p<0.05$) microbial cell increase was observed in all controls C1 rather cell count of C1 decreased during the experimental period and there was also no significant ($p<0.05$) appearance of microbial growth in all the controls C2. Population density ($OD_{600\ nm}$) of E increased as degradation proceeded when compared to the controls. The pH of E for all the isolates dropped from 7.2±0.0 to 6.9±0.1 at day 8 of experiment while there were no observable change in pH of the controls.

Residual HMW PAH During Degradation of Mixture of HMW PAHs by Bacterial Consortium

When consortium of the isolates was cultivated on MSM containing mixture of 100 mg L^{-1} each of chrysene, fluoranthene and pyrene as the sole carbon and energy sources, degradation resulted in a decrease to 11.5±1.4, 6.2±1.3 and 6.0±1.8 mg L^{-1} for chrysene, fluoranthene and pyrene respectively after 8 days (Fig. 3). There was rapid degradation of mixture of the HMW PAHs using consortium compared to use of bacterial isolates such that residual chrysene, fluoranthene and pyrene were 52.4±3.3, 50.6±3.2 and 45.1±2.1 mg L^{-1}, respectively at day 4 and the three HMW PAHs were concomitantly degraded.

Growth Profile of Bacterial Consortium During Degradation of Mixture of HMW PAHs

Mixture of the HMW PAHs did not inhibit microbial growth compared to C1 controls where there was gradual decrease in cell count (Fig. 4). Population density ($OD_{600\ nm}$) of consortium in E increased as degradation proceeded compared to controls. The pH of E increased from 7.2±0.0 to 7.5±0.1 compared to controls where there were no changes during the experiment.

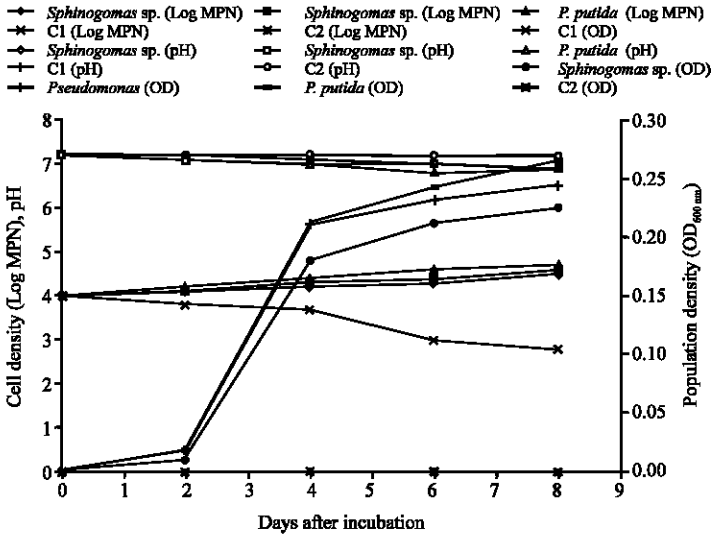

Fig. 2: Growth profile of bacterial isolates during degradation of mixture of HMW PAHs

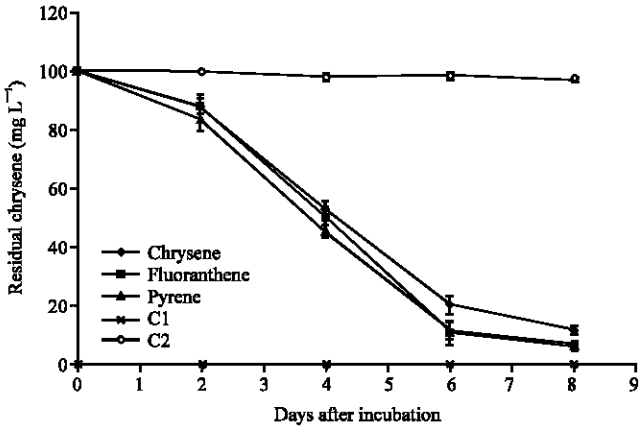

Fig. 3: Residual HMW PAH during degradation of mixture of HMW PAHs by bacterial consortium

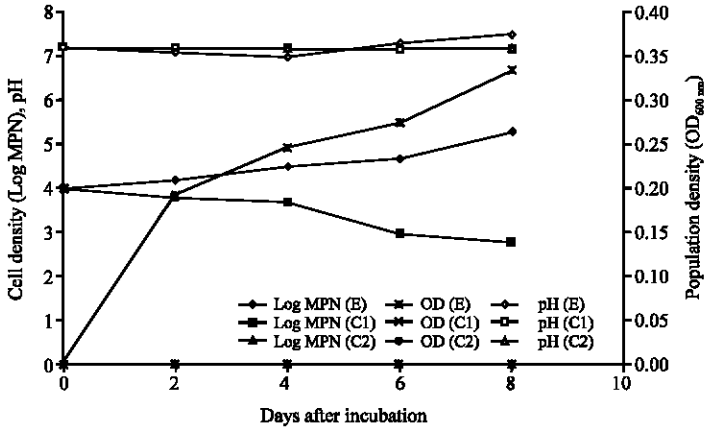

Fig. 4: Growth profile of bacterial consortium during degradation of mixture of HMW PAHs

Residual HMW PAH During Degradation of Mixture of HMW PAHs by Bacterial Consortium via Co-Metabolism with Phenanthrene

Residual chrysene, fluoranthene, pyrene and phenanthrene after 8 days were 0.45±0.25, 0.02±0.02, 0.20±0.14 and 0.000±0.00 mg L^{-1}, respectively (Fig. 5). At day 4 of degradation, residual chrysene, fluoranthene, pyrene and phenanthrene were 50.1±0.5, 40.5±2.5, 42.2±3.6 and 0.82±0.5 mg L^{-1}, respectively. Phenanthrene rapidly degraded to 20.2±0.2 mg L^{-1} at day 2 and was not detectable after day 6. Likewise the HMW PAHs degraded at a rapid rate and their degradation was concomitant with phenanthrene degradation. No statistical significant ($p<0.05$) difference was obtained in mixture of the HMW PAHs degradation between using bacterial consortium alone or via co-metabolism with phenanthrene, rather they had a strong correlation of r = 0.998, 0.997 and 0.995 for chrysene, fluoranthene and pyrene, respectively.

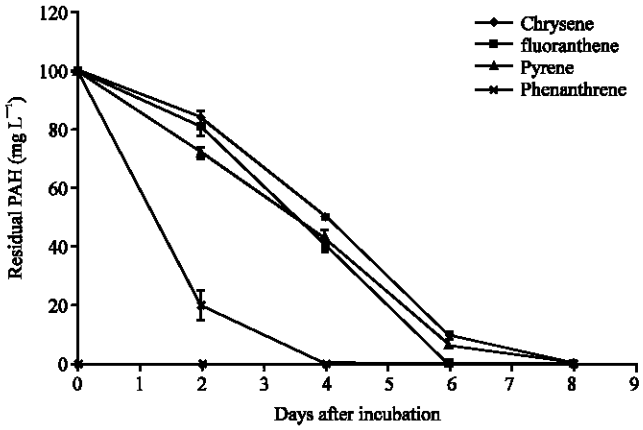

Fig. 5: Residual PAH during degradation of mixture of HMW PAHs by bacterial consortium in the presence of phenanthrene

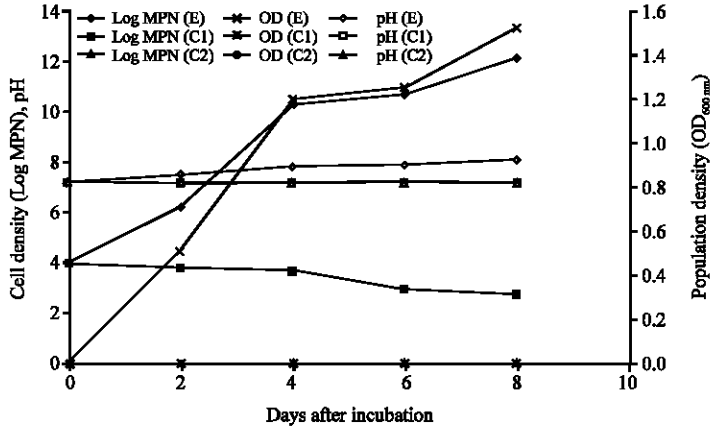

Fig. 6: Growth profile of bacterial consortium during degradation of mixture of HMW PAHs in the presence of phenanthrene

Growth Profile of Bacterial Consortium During Degradation of Mixture of HMW PAHs via Co-Metabolism with Phenanthrene

Highest cell density of 12.4×10^{12} cfu mL^{-1} was obtained when consortium degraded mixture of the HMW PAHs in the presence of phenanthrene. There was rapid increase in cell densities at day 2 from Log MPN of 4.0±0.0 to 6.2±0.3 (Fig. 6). Control C1 did not show significant ($p<0.05$) growth increase but there was gradual decrease in cell density. Control C2 also showed no significant ($p<0.05$) microbial presence during the experimental period. A rapid increase in population density ($OD_{600\ nm}$) of consortium in the experimental media was observed. The pH of E increased from 7.2±0.0 to 8.1±0.1 as degradation proceeded compared to the controls where there was no change.

DISCUSSION

In this study, *Sphingomonas* sp., *Pseudomonas* sp. and *P. putida* and their consortium were observed to concomitantly degrade mixture of chrysene, fluoranthene and pyrene.

However, the observed relatively higher residual HMW PAHs during individual isolates degradation of mixture of the HMW PAHs when compared to bacterial consortium may be as a result of the constitutive toxicity of the HMW PAHs mixture to the individual isolates affecting their degradation. This may account for the persistence of HMW PAHs in the environment especially in areas colonized by specific bacteria consortia. The collective metabolism by bacterial consortium may have resulted in the enhanced mixture of HMW PAHs degradation since intermediary biotransformation products from one isolate may serve as substrate for catabolism and growth by others. This corroborates suggestion of Johnsen *et al.* (2005). Bouchez *et al.* (1995) also suggested that degradation of PAH-mixture may be as a result of a co-operative process involving a consortium of strains with complementary capacities. The possible reason for the complementary degradation capacities of the bacterial consortium could be that they have varied genetic make-up and degradation enzyme activity working in synergy.

The comparable residual HMW PAHs obtained during degradation by bacterial consortium and via co-metabolism with phenanthrene buttressed the potential of the bacterial consortium in degrading mixture of the HMW PAHs. This showed that interaction amongst the PAHs did not inhibit their degradation using bacterial consortium.

The increase in cell count of the isolates during degradation was an indication that the HMW PAHs supported microbial biomass production even as sole source of carbon and energy. This is similar to previous findings that some HMW PAHs may serve as sole carbon and energy source for growth to some bacteria (Guo *et al*., 2005; Yu *et al*., 2005; Igwo-Ezikpe *et al*., 2010). The presence of phenanthrene which greatly increased biomass production may suggest its microbial growth supporting role. This is in accordance with previous research (Igwo-Ezikpe *et al*., 2010). Likewise, fluoranthene better support of biomass generation than the other HMW PAHs may reflect its better degradation which may also be as a result of its physio-chemical properties. In addition, the lowered biomass generation obtained during degradation by bacterial isolates compared to the bacterial consortium may further indicate the toxicity of mixture of HMW PAHs to the individual isolates and may also account for HMW PAHs persistence in the environment.

Researchers have shown that population density increase in media was a reflection of degradation process and proliferation of cell mass (Maskaoui *et al*., 2004; Na *et al*., 2005). As such, the increase in population density of the isolates in the experimental media compared to the controls as degradation proceeded was an indication of metabolic activity reflected in the increased cell count. Moreover, the enhanced population density of the isolates during degradation via co-metabolism with phenanthrene also indicated phenanthrene biomass growth supporting role.

The pH changes of the experimental media may signify metabolic activity leading to production of acidic or alkaline metabolites during breakdown of HMW PAHs. Kim *et al.* (2005) observed that acidic pH conditions promote uptake of PAHs for degradation in *Mycobacteria vanbaalenii*. It can therefore be suggested that monitoring pH of media may be used to check the progress of HMW PAHs degradation. This corroborates previous research where the dynamics of pH changes in cultures were consistent with that of PAH concentration change (Lin and Cai, 2008).

CONCLUSION

This study demonstrates the potential of bacterial consortium made up of *Sphingomonas* sp., *Pseudomonas* sp. and *P. putida* to simultaneously degrade a mixture of HMW PAHs; chrysene, fluoranthene and pyrene. Residual HMW PAHs obtained during degradation using bacterial consortium were similar to when degradation occurred using

bacterial consortium via co-metabolism with phenanthrene. The bacterial consortium was able to utilize the HMW PAHs as sole carbon and energy sources. We hereby suggest that the use of bacterial consortium may be ideal in environmental application for bioremediation of mixture of HMW PAHs polluted sites due to synergistic effect.

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