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High Molecular Weight Polycyclic Aromatic Hydrocarbons Biodegradation by Bacteria Isolated from Contaminated Soils in Nigeria

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Abstract: Bacteria isolated from various contaminated soils in Nigeria were investigated for their potential to utilize and biodegrade high molecular weight polycyclic aromatic hydrocarbons which include chrysene, fluoranthene and pyrene. Biochemical and morphological studies identified the isolates as *Sphingomonas* sp., *Pseudomonas* sp. and *Pseudomonas putida*. Biodegradation studies showed that *Sphingomonas* sp., *Pseudomonas* sp. and *P. putida* degraded 100 mg L⁻¹ chrysene to 30.5±0.3, 40.6±0.7 and 17.2±0.2 mg L⁻¹, respectively after 8 days of incubation. Similarly, fluoranthene was degraded to 2.0±0.1, 2.0±0.4 and 0.12±0.1 mg L⁻¹ while pyrene to 0.16±0.2, 6.5±0.3 and 6.6±0.4 mg L⁻¹ correspondingly. Consortium of the isolates degraded 100 mg L⁻¹ chrysene, fluoranthene and pyrene, respectively to 21.3±0.9, 2.2±0.8 and 10.6±0.8 mg L⁻¹. In the presence of phenanthrene as co-substrate, chrysene, fluoranthene and pyrene were, respectively degraded by consortium to 12.4±0.5, 0.2±0.3 and 0.7±0.2 mg L⁻¹ while phenanthrene was undetectable. This study showed that there was delayed degradation of chrysene and fluoranthene in the presence of phenanthrene, this may account for the persistence of these compounds in polycyclic aromatic hydrocarbons polluted sites. This is the first report on the potential of these isolates simultaneous utilization and biodegradation of chrysene, fluoranthene and pyrene when used as sole carbon and energy source.

Key words: Bacteria, biodegradation, chrysene, fluoranthene, pyrene

INTRODUCTION

Polycyclic Aromatic Hydrocarbons (PAHs) refer to a group of hydrocarbons containing two or more fused aromatic rings in linear, angular or clustered arrangements. They are produced during fossil fuel combustion, waste incineration or as by-products of industrial processes, such as coal gasification, production of aluminum, iron, steel and petroleum refining, component of crude oil, wood preservatives, smoke houses, wood stoves and also found in emissions from power generators and motor vehicles (Christensen and Bzdusek, 2005; Willeke, 2007).

The PAHs with two to three fused aromatic rings are considered as low molecular weight (LMW) PAHs; this include naphthalene, anthracene and phenanthrene, while those with four and more fused rings are High Molecular Weight (HMW) PAHs, which include chrysene,

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fluoranthene and pyrene. They have been detected in numerous aquatic and terrestrial ecosystems at concentrations high enough to warrant concern about their bioaccumulation (Masih and Taneja, 2006; Anyakora and Coker, 2007). The HMW PAHs are of principal concern due to their recalcitrance, persistence, bioaccumulation, carcinogenicity, genotoxicity and mutagenicity (Xue and Warshawsky, 2005; Castorena-Torres *et al.*, 2008). There is therefore need to study ways of remediating HMW PAHs polluted sites.

Studies have shown that microbial biotransformation is a major environmental process affecting the fate of PAHs in both terrestrial and aquatic ecosystems (Samanta *et al.*, 2002). Several bacterial strains have been observed to utilize and degrade LMW PAHs (Ilori and Amund, 2000; Woo *et al.*, 2004; Santos *et al.*, 2008). However, previous research have reported HMW PAHs biodegradation mostly via co-metabolism using LMW PAHs as the growth substrate (Foght and Westlake, 1988; Weissenfels *et al.*, 1991; Supaka *et al.*, 2001).

The aims of this study include isolating bacterial isolates from PAHs contaminated soils in Nigeria capable of utilizing chrysene, fluoranthene and pyrene as sole carbon and energy source and evaluating their biodegradation potential. In addition, the effect of phenanthrene as co-substrate in degradation of the HMW PAHs was evaluated.

MATERIALS AND METHODS

Chemicals and Media

All solvents used had purity = 99.9%. 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).

Soil Sampling and Analysis

Soil samples were collected from various PAHs-contaminated sites located in three Nigerian cities. These are Refinery site, PortHarcourt and Coal mining site, Enugu, both located on the Eastern part of Nigeria, a Wood processing site and Diesel engine-power-generator site located in Oshodi and idiaraba areas of Lagos city, respectively, in the Western part of Nigeria. Soil samples were collected using sterile spatula at a tillage depth of about 2 cm randomly from 20 core points and analyzed for PAHs content using high performance liquid chromatography (HPLC). One gram of soil sample was extracted with 5 mL ethyl acetate twice and filtered through a 0.45 μm membrane. The HPLC analyses were performed with VYDAC RP C18 reverse phase column (250 \times 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.

Isolation of HMW PAHs Degrading Bacteria

HMW PAHs degrading bacteria were isolated from the soil samples by enrichment culture technique on Mineral Salt Media (MSM) as described by Kästner *et al.* (1994) using chrysene, fluoranthene and pyrene, respectively as the sole carbon and energy source. The MSM composed per liter (pH 7.2): NH₄NO₃, 4.0 g; Na₂HPO₄, 2.0 g; KH₂PO₄, 0.53 g; K₂SO₄, 0.17 g, MgSO₄.7H₂O, 0.10 g and trace elements solution (1 mL), sterilized by autoclaving at 121°C for 20 min as described by Ilori and Amund (2000).

Chrysene, fluoranthene and pyrene were, respectively dissolved in 250 mL Erlenmeyer flasks containing 5 mL ethyl acetate, evaporated and wrapped with aluminum foil to prevent photolysis. MSM (20 mL, pH 7.2) were added to the culture flasks to a final concentration of

100 mg L⁻¹ HMW PAH, sterilized by autoclaving at 121 °C for 20 min. A 10-fold serial dilution of respective soil samples were inoculated into MSM HMW PAH and incubated in the dark aerobically at 30±2.0°C for 7 days with agitation at 150 rpm. Control MSM inoculated with the diluted soil samples without the test HMW PAH was included to check the increase in turbidity of the experimental media. On day 7, growth were visualized, 5 mL of the enrichment cultures were transferred to a fresh MSM with the respective HMW PAH and incubated under the same conditions. At the end of this adaptation period, the cells were aseptically cold harvested by centrifugation (4,000x g, 4°C, 20 min), washed twice in 10 mL phosphate buffer (50 mM, 4°C, pH 7.2) and purified by sub-culturing on nutrient agar plate by streaking.

Screening and Selection of Best HMW PAHs Degrading Bacteria

In order to screen the isolates for the best HMW PAHs degrading bacteria, purified isolates were cultured on solid MSM agar plates containing chrysene, fluoranthene and pyrene, respectively as the sole carbon and energy source by spray-plate technique (Survery *et al.*, 2004). Culture plates were wrapped with aluminum foil and black polyethylene bag and incubated in the dark at 30±2.0°C for 14 days. Bacterial colonies which form cleared zones on the HMW PAH coated plates were selected and further screened by subjecting the selected isolates to growth on respective MSM HMW PAH broths and compared with control MSM broth without HMW PAH (Supaka *et al.*, 2001).

Identification of the Selected Best HMW PAHs Degrading Bacterial Isolate

Morphological and biochemical studies were undertaken to identify the selected HMW PAHs degrading bacteria (Holt *et al.*, 1994; Barrow and Feltham, 2004). The following test were carried out: Gram stain, morphology, catalase, oxidase, colony motility, methyl red, Voges-Proskauer test, indole, nitrate reduction, gelatin hydrolysis, spore test, starch hydrolysis, coagulase test, citrate and sugar utilization.

Biodegradation Studies

This study was conducted between March, 2007 and 2008 in Biochemistry Department, College of Medicine, University of Lagos, Nigeria.

Preparation of Starter Culture

Bacterial isolates selected as best HMW PAHs degraders were inoculated into sterilized 10 mL nutrient broth and incubated for 48 h at 30±2.0°C. Growth cultures from nutrient broth were readapted on the HMW PAHs by pouring into respective sterilized 20 mL MSM HMW PAH broths and incubated for 7 days at 30±2.0°C. The cells were harvested, washed twice by centrifugation (4000x g, 4°C, 15 min) and suspended in 10 mL phosphate buffer (50 mM, pH 7.2). The harvested cells were used for the HMW PAHs biodegradation experiment.

Biodegradation of HMW PAH by Bacterial Isolate

The bacterial isolates were used individually to biodegrade 100 mg L⁻¹ each of the HMW PAHs (chrysene, fluoranthene and pyrene) using it as sole carbon and energy source. MSM (20 mL, pH 7.2) were put into different 250 mL Erlenmeyer flasks containing each of the HMW PAHs to a final concentration of 100 mg L⁻¹. The media were sterilized and inoculated with 3 mL (10⁴ cells) of the starter culture. These 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 PAH while C2 contained all the materials in E with no test inoculum added.

The experiment was set up in duplicate for 8 days, samples taken at 48 h intervals. Biodegradation was assayed by determining the residual HMW PAHs using HPLC after extracting twice with equal volume of ethyl acetate and the growth of the HMW PAH degrading bacteria in the media estimated by the Most Probable Number (MPN) technique using nutrient broth as the growth media (Gonzalez, 1996). The MPN tubes were incubated at room temperature ($30 \pm 2.0^\circ\text{C}$) for 48 h.

Biodegradation of HMW PAH by Consortium of the Isolates

The isolates were used together as a consortium to biodegrade 100 mg L^{-1} each of the HMW PAHs (chrysene, fluoranthene and pyrene) using it as sole carbon and energy. The controls C1 and C2 were included in the set-up. The experiment was set up in duplicate for 8 days and biodegradation analyzed as earlier stated.

Biodegradation of HMW PAH by Consortium in the Presence of Phenanthrene

The isolates were used as a consortium to biodegrade the individual HMW PAHs in the presence of a co-substrate; phenanthrene. Phenanthrene (100 mg L^{-1}) was added to the experimental media containing the respective HMW PAHs (100 mg L^{-1}). The controls C1 and C2 were included in the set-up. The experiment was set up and 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 \pm SEM (Standard Error of Mean). These statistical analyses were done using Statistical Package for the Social Science 15.0 for windows (SPSS 15.0).

RESULTS AND DISCUSSION

PAHs Composition of the Soil Samples

Soil samples from locations of potential sources of PAHs seeded for HMW PAHs degrading bacteria were found to contain both high and low molecular weight PAHs (Table 1).

Isolation, Selection and Identification of the Best HMW PAHs Degrading Bacteria

Twenty seven isolates were obtained as HMW PAHs degrading bacteria based on utilization of chrysene, fluoranthene or pyrene as sole carbon and energy source for growth. Out of these, three isolates were selected as the best concomitant HMW PAHs utilizing bacteria compared to other isolates based on clearing of zones on MSM HMW PAH agar plates and growth on the respective MSM HMW PAH broths. The three isolates were common amongst the various locations sampled. The results obtained from the morphological and biochemical characteristics of the organisms identified the isolates as *Sphingomonas* sp., *Pseudomonas* sp. and *Pseudomonas putida* (Table 2).

Table 1: Polycyclic aromatic hydrocarbons concentration in soils samples

Soil sample site	Polycyclic aromatic hydrocarbons ($\mu\text{g g}^{-1}$)			
	Chrysene	Fluoranthene	Pyrene	Phenanthrene
Refinery	45.210 \pm 0.67	57.01 \pm 0.93	46.35 \pm 0.22	50.37 \pm 0.45
Coal mining	157.21 \pm 0.25	10.83 \pm 0.21	0.000 \pm 0.00	0.000 \pm 0.00
Wood processing	58.230 \pm 0.38	15.82 \pm 0.11	7.570 \pm 0.35	14.01 \pm 0.15
Diesel-power-generator	70.970 \pm 0.41	96.78 \pm 0.45	50.29 \pm 0.20	43.69 \pm 0.20
Sample Mean \pm SEM				

Table 2: Morphological and biochemical properties of the selected HMW PAHs degrading bacteria

Properties	Colony morphology		
	<i>Sphingomonas</i> sp.	<i>Pseudomonas</i> sp.	<i>Pseudomonas putida</i>
Gram reaction	-	-	-
Cellular morphology	Rods	Rods	Rods
Pigmentation	Yellow	-	-
Spore test	-	-	-
Motility	+	+	+
Biochemical test			
Catalase	+	+	+
Oxidase	+	+	+
Citrate	-	+	+
Urease	-	-	-
Methyl red	-	-	-
Voges-proskauer	-	-	-
Starch hydrolysis	-	-	-
Gelatin hydrolysis	-	-	-
Oxidative O-F medium	-	+	-
Coagulase test	-	-	-
NO ₃ reduction	-	+	-
Acid production from			
Mannitol	-	-	-
Ethanol	+	-	-
Fructose	+	-	-
Glucose	+	+	+
Xylose	+	+	+
Lactose	+	+	-
Sucrose	+	-	-
Raffinose	-	-	-
Arabinose	+	+	+
Maltose	+	+	-
Cellobiose	+	+	-
Salicin	-	-	-

-: Negative reaction, +: Positive reaction

Residual HMW PAH and Bacterial Growth During Degradation of Respective HMW PAHs by Bacterial Isolates

The degradation of 100 mg L⁻¹ chrysene by *Sphingomonas* sp., *Pseudomonas* sp. and *P. putida*, respectively resulted in a decrease to 30.5±0.3, 40.6±0.7 and 17.2±0.2 mg L⁻¹ after 8 days incubation (Fig. 1a).

Residual fluoranthene after 8 days degradation of 100 mg L⁻¹ fluoranthene by *Sphingomonas* sp., *Pseudomonas* sp. and *P. putida*, respectively was 2.0±0.1, 2.0±0.4 and 0.12±0.7 mg L⁻¹ (Fig. 1b). On the other hand, residual pyrene after 8 days degradation of 100 mg L⁻¹ pyrene by *Sphingomonas* sp., *Pseudomonas* sp. and *P. putida*, respectively was 0.16±0.2, 6.5±0.3 and 6.6±0.4 mg L⁻¹ (Fig. 1c). In all the C1 controls, no significant HMW PAH was detected and there were no significant (p<0.05) change in HMW PAH concentration in all C2 controls.

Growth profile revealed that the MPN (cfu mL⁻¹) of the isolates increased in the experimental media during degradation of the HMW PAHs compared to the controls. Highest cell density of 1.3×10⁶, 6.3×10⁵ and 3.2×10⁶ cfu mL⁻¹, respectively were obtained for *Sphingomonas* sp., *Pseudomonas* sp. and *P. putida* after 8 days of chrysene degradation (Fig. 1a). Growth of *P. putida* was better supported during fluoranthene degradation with peak cell density of 7.9×10⁹ cfu mL⁻¹ while *Sphingomonas* sp. and *Pseudomonas* sp., respectively had cell densities of 2.5×10⁹ and 2.5×10⁸ cfu mL⁻¹ (Fig. 1b). Highest cell densities of 8.9×10⁴, 4.7×10⁴ and 7.0×10⁴ cfu mL⁻¹ were obtained for *Sphingomonas* sp., *Pseudomonas* sp. and *Ps. putida*, respectively after 8 days of pyrene degradation (Fig. 1c). Controls C1 did

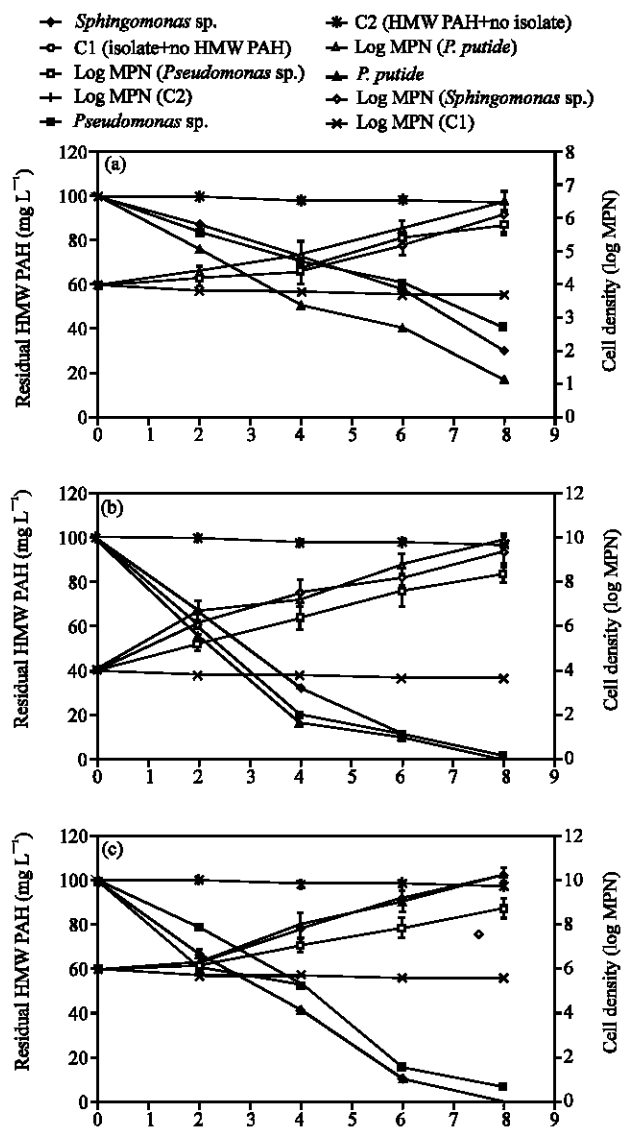


Fig. 1: Residual HMW PAH and growth profile during degradation by bacterial isolates. (a) chrysene, (b) fluoranthene and (c) pyrene

not show significant ($p < 0.05$) microbial growth increase but there was gradual decrease in cell mass. Controls C2 also showed no significant ($p < 0.05$) microbial presence during the experimental period.

Residual HMW PAH and Bacteria Growth During Degradation of Respective HMW PAH by Bacterial Consortium

The bacterial consortium cultivated on MSM containing 100 mg L⁻¹ of chrysene, fluoranthene and pyrene, respectively as sole carbon and energy source, resulted to decrease in concentration to 21.3±0.9 (chrysene), 2.2±0.8 (fluoranthene) and

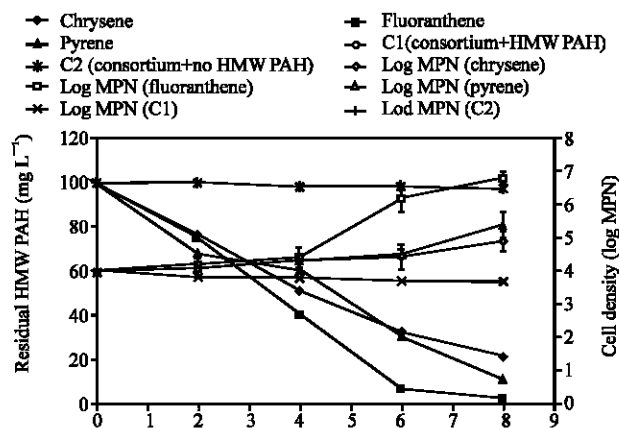


Fig. 2: Residual HMW PAH and growth profile during bacterial consortium degradation of chrysene, fluoranthene and pyrene

10.6±0.8 (pyrene) mg L⁻¹ after 8 days. Fluoranthene was better degraded than the other HMW PAHs with 97.8% degradation while 78.7% chrysene and 89.4% pyrene were degraded (Fig. 2). No significant ($p < 0.05$) loss of HMW PAH was observed in all C2. Highest cell densities of 8.9×10^4 , 2.3×10^6 and 2.5×10^5 cfu mL⁻¹, respectively were obtained when the bacterial consortium degraded chrysene, fluoranthene and pyrene (Fig. 2).

Residual HMWPAH and Bacteria Growth during Degradation of Respective HMW PAH by Bacterial Consortium in the Presence of Phenanthrene

In degradation of the chrysene, fluoranthene and pyrene, respectively in the presence of phenanthrene by bacterial consortium, it was observed that chrysene degradation was delayed until 80.7% of phenanthrene had been utilized on day 4 with chrysene being degraded to 12.4 ± 0.5 mg L⁻¹ after 8 days of incubation (Fig. 3a). Fluoranthene degradation was also delayed until 63.8% of phenanthrene had been utilized on day 2 with fluoranthene being degraded to 0.2 ± 0.3 mg L⁻¹ at 8 days (Fig. 3b). Within day 4, phenanthrene was not detected by HPLC analysis during degradation of chrysene and fluoranthene, respectively. In the case of pyrene degradation in the presences of phenanthrene, their degradation proceeded simultaneously. Pyrene and phenanthrene were degraded from 100 mg L⁻¹ to 40.3 ± 0.5 and 25.3 ± 0.6 mg L⁻¹, respectively on day 4, phenanthrene was undetected after day 6 while 0.7 ± 0.2 mg L⁻¹ of pyrene was obtained at day 8 (Fig. 3c). In all C2 controls, no significant ($p < 0.05$) loss in the PAHs was observed. In the presence of phenanthrene, there was increased cell densities of the consortium compared to the controls. Highest cell densities of 2.3×10^6 , 2.3×10^{11} and 8.9×10^9 cfu mL⁻¹, respectively were obtained from chrysene, fluoranthene and pyrene media (Fig. 3).

Bacterial strains have been isolated from PAHs-contaminated sites and have degradability potential (Yu *et al.*, 2005; Igwo-Ezikpe *et al.*, 2006). It has also been reported that bacterial cells tend to detect temporal changes in the concentrations of specific chemicals and respond behaviorally to these changes, thereby adapting to the new concentration of the chemical stimuli through chemotaxis (Guerin, 1999). This explains the presence of selective microbial consortia in a specific pollutant ecosystem (Bhushan *et al.*, 2000). Studies have also shown improved microbial degradation of pollutants by isolates cultivated from samples sourced from sites of pollution (Yu *et al.*, 2005; Johnsen *et al.*, 2007).

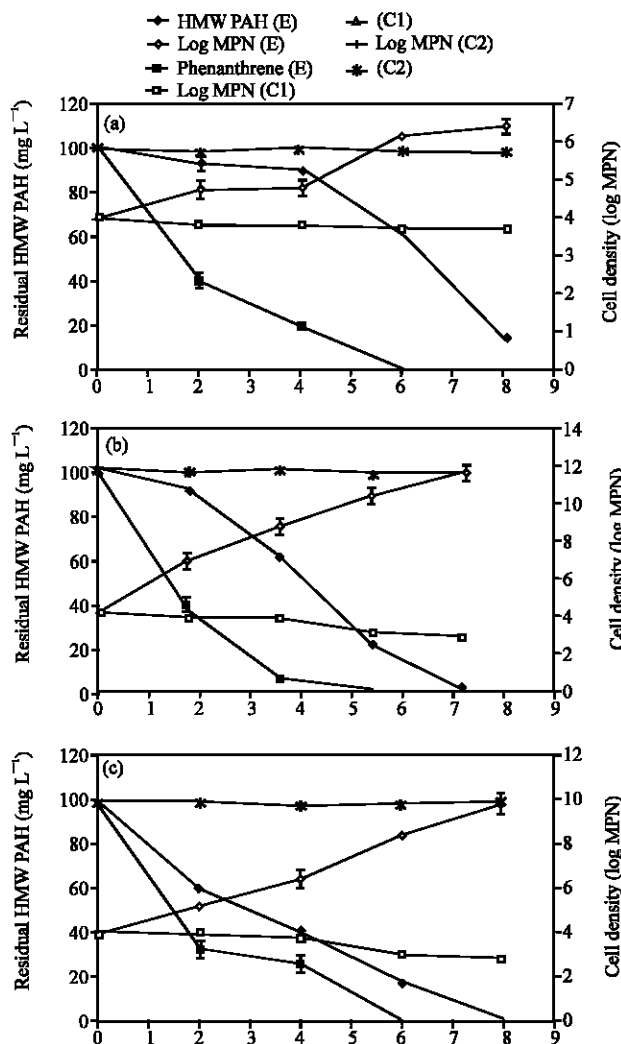


Fig. 3: Residual PAH and growth profile during bacterial consortium degradation of HMW PAH in the presence of phenanthrene. (a) Chrysene+phenanthrene, (b) Fluoranthene+phenanthrene and (c) Pyrene+phenanthrene

Therefore, microbial seeding from PAHs-contaminated soils was essential for isolating HMW PAHs degrading bacteria. The protocol of isolation and selection employed in this study ensured that the isolates; *Sphingomonas* sp., *Pseudomonas* sp. and *P. putida* could concomitantly utilize chrysene, fluoranthene and pyrene which are HMW PAHs as sole carbon and energy source and this was hitherto unreported. The isolates also showed potential for application in both solid and liquid phase HMW PAHs decontamination. Only a few number of bacteria genera have been isolated that can grow in pure cultures on HMW PAHs which include *Pseudomonas* sp., *Mycobacterium* sp., *Sphingomonas* sp. and *Rhodococcus* sp. (Walter *et al.*, 1991; Kanaly and Harayama, 2000; Samanta *et al.*, 2002).

In this study, the no significant change in HMW PAH concentration observed in all the C2 controls showed the potential of the isolates in degrading the HMW PAHs in the

experimental media. The relatively lower residual HMW PAH when the organisms were used as a consortium compared to when they were used as individual isolates to degrade the respective HMW PAH may be as a result of the constitutive degradation potential of the consortium. Furthermore, the delayed degradation of chrysene and fluoranthene, respectively in the presence of phenanthrene may be as a result of preferential utilization of phenanthrene as carbon and energy source and may account for the persistence of HMW PAHs in the environment. This is in accordance with previous research that microbial strains could degrade several PAHs but preferred one (Tadros and Hughes, 1997; Lotfabad and Gray, 2002).

In addition, the observed growth of the organisms in the experimental media compared to the controls and the gradual decrease of microbial cells in C1 controls due to lack of carbon source to support the microbial growth both implied that the isolates were able to utilize the HMW PAH as sole carbon and energy source. The utilization and degradation of HMW PAHs potentials of the isolates may be as a result of their phenotypic characteristics such as plasmid mediation and enzyme activity. Previous studies have shown the inability of some organisms to utilize HMW PAHs as sole carbon and energy source as such their degradation of HMW PAHs were via co-metabolism (Supaka *et al.*, 2001; Dean-Ross *et al.*, 2002). This situation has been considered to contribute to the recalcitrance and persistence of PAHs in the environment and has lead to the search for strains that could utilize and degrade HMW PAHs (Mrozik *et al.*, 2003; Van Hamme *et al.*, 2003).

CONCLUSION

The enrichment culture and selection protocol employed in this study ensured the isolation of HMW PAHs degrading bacterial isolates belonging to *Sphingomonas* and *Pseudomonas* genera. The isolates were able to utilize and biodegrade chrysene, fluoranthene and pyrene using it as sole carbon and energy source, this prospects their possible application in bioremediation of high molecular weight polycyclic aromatic hydrocarbons polluted site. There is need to further studies on biodegradation of mixture of HMW PAHs by bacterial isolates and consortium which would have implication in development of efficient bioremediation strategy for HMW PAHs-contaminated sites.

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