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Biodegradation of pyrene by a mixed culture isolated from hydrocarbon-polluted soil

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This paper presents the biodegradation of pyrene ($C_{16}H_{10}$), a four-benzene-ring polycyclic aromatic hydrocarbon (PAHs) using a mixed culture of bacteria. The mixed culture was enriched from hydrocarbon-polluted soil sample, using a plate incubated with a thin film of pyrene. The isolates were identified biochemically as *Bacillus cereus* and *Enterobacter aerogenes*. The mixed culture bacteria degraded approximately 90% of the pyrene in aqueous medium within six days of incubation. The assessment of other environmental factors necessary for the activity of the mixed culture revealed that the mixed culture grows well between pH of 5.0-7.0 and temperatures of between 20-30°C. From the results of this trial therefore, it is suggested that the mixed culture has good potential for bioremediation applications.

Key words: Pyrene, biodegradation, mixed culture, polycyclic aromatic hydrocarbon (PAHs), environmental factors.

INTRODUCTION

Pyrene (Pyr) is a polycyclic aromatic hydrocarbon (PAHs) containing four benzene rings fused together ($C_{16}H_{10}$) with a molecular weight of 202 g/mol. It has relatively lower vapor pressure and higher partition coefficient than other low molecular weight PAHs, poorly soluble in aqueous solutions, but is easily volatilized into the atmosphere. It is known as an unwanted by-product of gasification and other incomplete combustion processes (Juhasz and Naidu, 2000; Mrozik et al., 2003). However, its aromatic structure contributes to its high chemical stability, as is also the case in the localization of electrons between adjacent conjugate bonds in high molecular

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weight PAHs (Schwurzenbach and Gschwend, 2003). The persistence of Pyr in the environment is attributed to its chemical stability, low water solubility and high absorption capacity to soil matrix; as a result, it is low biodegradable (Yan et al., 2004). Although not as carcinogenic as benzo[a]pyrene (BaP), it has nonetheless, been reported among other carcinogenic PAH molecules. Pyr could impair the skin or other sensory organs and has a long-lasting effect on terrestrial and marine organisms (Peng et al., 2008). Consequently, Pyr is listed among the priority environmental pollutants by the United States Environmental Protection Agency (US EPA) (Yan et al., 2004).

Microbial degradation is among the numerous factors that determine the fate of Pyr in the environment. Although recalcitrant, some studies have confirmed the ability of microbes to degrade Pyr.: Bacilus species (Samanta et al., 2002), Burkholderia species (Deng et al., 2012), Cycloclasticus species (Samanta et al., 2002), Mycobacterium species PYR-1, Mycobacterium flavescens (Ho et al., 2000), and Sphingomonas species (Daugulis and McCracken, 2003). These pure cultures of bacteria usually have difficulties of survival especially when introduced to contaminated sites, due to stiff competition and the lack of adaptability leading to poor survival in the newly introduced environment (Goldstein et al., 1985). This is particularly important when considering in-situ remediation of soils contaminated with PAHs.

Even though variety of pure bacterial isolates can degrade Pyr in the laboratory culture, inoculating pure culture to the environment for bioremediation purposes still remains a great challenge and may not be feasible, because of the aforementioned inoculum limitations. Previously, studies have used bacteria in the form of mixed consortium (Luo et al., 2009; Yu et al., 2005), as well as an indigenous form isolated directly from soil (Goldstein et al., 1985) that are capable of degrading PAHs. Presumably, the use of mixed cultures as a solution may be worth evaluating: to utilize a synergistic approach in degrading the recalcitrant compound. During mineralization, a parent compound may be transformed through different series of metabolic process by members of the mixed culture and has been reported that the first metabolic product of biodegradation could be a carbon source for another strain within the culture consortium (Luan et al., 2006). In the process, the parent compound could be biodegraded to its benign stage. Consequently, the application of a mixed culture or consortium of bacteria may be a more effective alternative to the use of pure cultures. The objective of this work was to isolate a mixed culture of bacteria degrading Pyr from used-oil-dump-soil from a local automobile workshop. This was because petroleum contaminated soils or sediments harbor varieties of hydrocarbon-degrading micro-organisms; therefore, isolating a mixed culture with diversity of metabolic capabilities would be used to solve the inability of pure culture in remediating contaminated sites.

MATERIALS AND METHODS

Chemicals

Chemicals such as Pyr 98% purity, analytical reagent standard of methanol, acetone, hexane, dichloromethane and acetonitrile are of high performance liquid chromatography (HPLC) standard purchased from Fisher Scientific Inc. (Malaysia). Other PAHs such as napthalene (Nap) with 99% purity, phenanthrene (Phn) with 97% purity and flouranthene (Fla) with 98% purity were purchased from Fisher Scientific Inc (Malaysia). All the glass wares, flasks, test tubes, sample bottles used in this study were washed with detergent, sonicated and washed three times with methanol, acetone and hexane in succession in ensuring removal of any organic contaminant that could interfere with the experiments. Because organic chemical such as PAHs tend to stick to the glass body and thereby interfere with the quality of the work, stock solutions for PAHs were prepared in acetone solvent.

Collection of soil samples

A polluted soil sample was obtained at a dumping site of an automobile workshop (near Bangi, Selangor, Malaysia). The site has been used for over fifteen years and made up of a one-side-opened drum where all oil and grease washed out, used crankcase oil and condemned engine oil were dumped. The drum was filled and overflowed on the surrounding soils causing the soil beneath to be highly contaminated with hydrocarbons. These soils were chosen as a potential source for indigenous microorganisms with hydrocarbon tolerant. A sample of soil smelling with hydrocarbon was scooped with hand trowel and poured carefully into a zip-lock bag, placed in a cooler and transported to the laboratory prior enrichment and isolation steps.

Preparation of culture media

Chemicals of analytical grade purchased from Fisher Scientific (Malaysia) were used in the media preparation. A mineral salt medium (MSM), which was used for culture media has the following compositions and this formulation is according to Tao et al. (2007). 5.0 mL of phosphate buffer solution per litre, containing: 566.5×10^{-3} mM $K_2HPO_4{\cdot}H_2O$, 312.5×10^{-3} mM KH_2PO_4, 466.5×10^{-3} mM $Na_2HPO_4{\cdot}12\ H_2O$, 471.5×10^{-3} mM NH_4Cl); mineral salts solution (561×10^{-3} mM $MgSO_4$, 1.539×10^{-3} mM $FeCl_3$, 327.9×10^{-3} mM $CaCl_2$); trace elements solution (236.1×10^{-3} mM $MnSO_4$, 265.18×10^{-3} mM $ZnSO_4$ and 28.08×10^{-3} mM $(NH_4)_6Mo_7O_{24}{\cdot}\ 4H_2O$) were weighed, dissolved according to their individual composition and stored at 4°C until used. This (MSM) was used throughout this work.

Enrichment and isolation of Pyr degraders

A sterile nutrient broth (NB, 100 mL) was prepared in 250-mL conical flask with 2 g of the polluted soil sample added into it followed by incubation at 180 rpm and 30°C for 24 h in order to harvest enough Pyr-degraders. Thereafter, 2 mL of the 24 h old growth culture was inoculated aseptically into a 100-mL mineral salt medium (MSM) and Pyr (15 ppm) dissolved in acetone was poured into the flask. The sample was incubated at 30°C with 180 rpm agitation for 2 weeks. Further, 1 mL of culture was transferred to

MSM media supplemented with 15 ppm Pyr for further enrichment step. This step was repeated until the culture is considered acclimatized with Pyr. This culture was used as a stock culture for Pyr degradation and subsequent isolation step. Isolation of Pyr-degraders was performed by streaking plate method. Briefly, several MSM-agar plates supplemented with Pyr were prepared and streaked. Colony showing a clear zone on MSM agar supplemented with Pyr was scored as positive, subsequently picked and repeatedly streaked several times and finally stored using enriched nutrient agar medium.

Preparation of mixed culture inoculum

Several isolated strains were individually incubated using a NB media supplemented with 0.15 ppm of Pyr at 30°C under 180 rpm agitation in the dark. After reaching the late exponential phase using optical density (OD) at 600 nm (OD_{600}=0.8-0.9), the cells were harvested by centrifugation at 3500 rpm for 20 min. Then, the cells were resuspended in 50 mL MSM medium and remixed until the suspension was homogeneous. From each culture, 100 µL was added to a mixed culture of fourteen different isolates. This mixed culture was stored with 20% glycerol and kept at -80°C until further use.

Determination of cell concentration

All the glassware including flask, measuring cylinders and pipettes were washed thoroughly and dried in an oven at 60°C for 24 h. All the glasswares were autoclaved at 121°C for 15 min in an Autoclave SX-700, Tokyo, Japan. A mineral salt medium (MSM) supplemented with 1000 ppm of yeast extract was prepared and autoclaved (121°C for 15 min). A 500-mL conical flask was filled with 400 mL of (MSM+yeast extract) and the pH adjusted to 7.2 using 2 M phosphate buffer. Thereafter, 1 mL inoculum was introduced into the 500-mL flask containing 400 mL MSM-yeast extract. The flask was then incubated in an orbital shaker at 30°C (at 180 rpm agitation) for 24 h.

After 24 h, the culture was divided into an equal volume of 200 mL each. The first portion was centrifuged at 6000 g at 5°C for 5 min. Then the supernatant was removed, and the cell pellet dried for 24 h at 65°C to achieve a constant cell dry weight. From the other remaining 200 mL portion, 1 mL of sample was measured for turbidity with distilled water as diluent (where necessary) and measured at 600 nm. Series of dilution were plotted against turbidity/absorption at 600 nm. A correlation curve was obtained using the cells dry weight of the mixed microbial culture. This procedure was conducted according to Alfermann et al. (1994).

Identification of Pyr-degraders

All the samples were identified biochemically in the Department of Bacteriology, Faculty of Veterinary Medicine, Universiti Putra Malaysia. The Pyr-degrading consortium was routinely cultured on Pyr-supplemnted MSM liquid medium as earlier described and was inoculated into growth tubes containing the standard diagnostic substance and reactants listed in Table 1. In addition, the cells were Gram stained. The consortium grew well on Pyr-supplemented MSM medium in the laboratory, and after extensively subculturing, maintained their growth rate and Pyr-degrading activity (Quinn et al., 1994).

Biodegradation study on Pyr

The inoculum was developed using a stock culture of enriched mixed bacterial consortium by growing in a sterilized MSM medium containing 1.5 ppm Pyr for a week, or until the growth reaches late lag phase. 1 mL of inoculum was further subcultured into 100 mL of MSM at pH 7.2 with an initial concentration of 1.5 ppm and 3.0 ppm of Pyr respectively. A flask without any bacterial inoculum was also prepared with 1.5 ppm of Pyr as the control for abiotic loss during degradation studies. Then, an inoculum size of 1% (v/v) was inoculated aseptically into culture flasks. The cultures were incubated in an oven shaker (180 rpm agitation) at 30°C in the dark. The growth and Pyr degradation were monitored over a period of 12 days by taking triplicate flasks of each concentration and abiotic samples every 24 h. The aqueous samples were monitored with a spectrophotometer (HACH, DR/2500) at 600 nm and converted to cell dry weight using the correlation curve as previously described. The biomass growth in the aqueous samples was quantified and reported in microgram per litre dry weight. Other PAHs compounds: Naphthalene (Nap), Phenanthrene (Phn) and Floranthene (Fla) were also tested with the mixed culture using the same technique. The residual of Pyr and other PAHs tested were quantified using HPLC method described in "Extraction and Analysis Pyr". Biodegradation percent in all the experiments were calculated according to Equation 1:

$$Biodeg = \frac{(Pyr_{res} - Pyr_{fls})}{Pyr_{res}} \times 100 \quad (1)$$

Where Biodeg= % degradation, Pyr_{res} is residual Pyr concentration in the control, Pyr_{fls} is residual Pyr concentration in degradation flask. To meet quality assurance and quality control (QA/QC) on Pyr determination, all the analytical procedures were checked by a known concentration of surrogate internal standard m-typhenyl. Recovery of Pyr was back-calculated using the surrogate. Also, procedure blank was performed on every batch of sample analysed. In case of GC-MS, auto-tuning was done every time of sample injection.

Extraction and analysis of Pyr

1 mL of aqueous sample was extracted with dichloromethane three times in equal volume. Aqueous sample was filtered with pre-dried anhydrous sodium sulphate (dried at 200°C for 2 h) through a funnel lined with glass-wool bed filter. The filtrate was collected and subjected to concentration step using a rotary evaporator at reduced pressure and concentrated to 1 ml under slow nitrogen purging. The quantification of Pyr was conducted using a high-performance liquid chromatograph (Shimadzu HPLC, Japan) equipped with UV detector. Phenomenex Synergi 4 m Max-RP80A column (250 × 4.6 mm) was used with a water-acetonitrile mobile phase in a gradient mode at a fixed flow rate of 1.5 mL/min. The column was equilibrated with 35% acetonitrile in distilled water for 2 min and its concentration was increased linearly at rate of 2.7% per minute to 100% in 24 min, and then held up for another 10 min. The temperature of column was set at ambient condition, and column eluent was detected at 254 nm. The unknown samples were quantified using standard Pyr calibration curves. However, other PAHs (Nap, Phn and Fla) were quantified using separate correlation curves developed with DCM as carrier solvent and detected with a spectrophotometer.

Identification of metabolite of Pyr

Sample from the culture medium between the lag phase and exponential phase were submitted to Analytical Laboratory, Department of Chemistry, Faculty of Science, Universiti Putra Malaysia. The samples were analyzed using GC (GC-17A Ver.3)

Table 1. Biochemical characteristics of Pyrene-degrading pure isolates.

Test	Isolates	
	Group 1 (12 samples)	**Group 2 (2 samples)**
Growth at room temperature 37°C	Aerobic	Aerobic
Description of colony	Greenish large rod	Grayish large rod
Gram stain	+	-
Hemolysis	+	
Catalase	+	
Blood broth	+	
Trahalsoe Lactus	+	
SIM (sulphide/indole mobility)	+	
Glucose	+	
Sorbitol/Sucrose	+	
Dextrose nitrate reduction	+	
Urea	NA	-
TSI		
Citrate		+

Group 1= *B. cereus*; Group 2: *E. aerogenes*.

with a column of ZEBRON ZB-FFAP 30 m × 0.25 mm I.D x 0.25 µm film thickness. The sampling time was 2.0 min while initial injection temperature and interface temperature were set at 250°C, with an OCI/PTV Fan temperature set at 50°C, and finally the control mode was set at splitless. The GC-MS model, Shimadzu QP5050A with initial injection and interface temperature of GC-MS was set up at 70 and 250°C, respectively. The column oven temperature will rise up 5°C/min up to 250°C and will be maintained at 250°C for 15 min. Helium gas was used as a carrier gas with the flow rate at 1.0 ml min^{-1}.

RESULTS

Biochemical characteristics and identification of PAH-degraders

A soil sample taken from aged petroleum dumping site was used as a potential re-source for PAH-degraders. Strains were enriched and acclimatized to Pyr condition for approximately two months, and selected colonies with Pyr-degrading potential were biochemically characterized and identified. It was a common practice to use higher concentration for enrichment stage. For example, Yu et al. (2005) and Tam et al. (2003) used 10 ppm of PAH. In this study, 15 ppm Pyr was adopted as a primary instigation on a condition of no alternative carbon source other than Pyr as a strategy to encourage fast acclimatization of the selected consortium. All the isolated strains were characterized as aerobic bacteria and grew well at a temperature of 37°C. 12 Gram-positive with code numbers of WT1, WT2, WT3 (1), WT3 (2), WT5, WT7 (1), WT7 (2), WT7 (3), WT7 (40, WT8 (1), WT8 (2) and WT9 were biochemically characterized (Table 1). These isolates appeared as a large greenish rod on blood agar plates while the other 2 Gram-negative bacteria with code numbers of WT10 (1) and WT10 (2) appeared as a grayish large rod. They grew rapidly within 24 h of incubation at 37°C except for the two Gram-negative strains, which were grouped as slow growing degraders.

To encourage the growth of slow growing bacteria, the media was supplemented with 0.75 ppm of Pyr during inoculum preparation; also, Pyr may encourage the slow growing ones to grow together with fast growing degraders. All the 12 Gram-positives indicated relatively positive reaction to almost all the biochemical test such as: hemolysis, catalase, blood broth, trehalose/lactose, SIM (sulphide/indole mobility) glucose, sorbitol/sucrose, dextrose nitrite reduction, urea, oxidase and triple sugar iron agar (TSL). In contrast, the 2 Gram-negative reacted only with citrate. The 12 Gram-positive and the 2 Gram-negative isolates were identified as *Bacillus cereus* and *Enterobacter aerogenes* respectively according to Jang et al. (1988).

Effect of environmental factors on degradation of Pyr

The ability of this mixed culture to grow well under different temperatures indicates that their growth and degradation of Pyr were affected by temperature changes after series of Pyr degradation test under different incubation temperatures of 20, 30 and 40°C, and at varying pH of 5, 6.5, 7 and 8. At incubation temperature of 20°C, the percent degradation of Pyr was the highest at pH 7 (92%) and the lowest was at pH 8 (37%). However, the percent degradation was about 85% at pH 6.5 under the same incubation temperature of 20°C as shown in Figure 1.

In contrast, at 30°C incubation temperature, there was

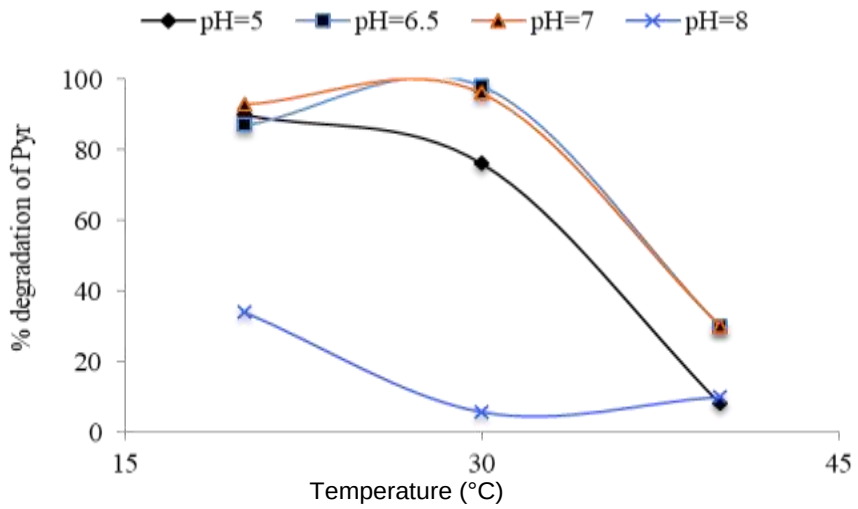

Figure 1. Effect of pH on the percentage degradation of Pyr on different temperature.

highest percent degradation of Pyr at both pH of 6.5 and 7, which was over 92% respectively, while at pH 5, the percent degradation was about 78% with the lowest percent degradation of 9% at pH 8 (Figure 1). The effect of temperature on percent degradation of Pyr was more pronounced when the incubation temperature was 40°C for all the pH range tested. The percent degradation was 34% at pH 7 but below 20% at either pH 5, 6.5 or 8 (Figure 1).

The result of the effect of pH on the percent degradation of Pyr at different incubation temperatures show that at pH 5, the percent degradation of Pyr were 92, 78 and 9% at 20, 30 and 40°C, respectively, whereas at pH of 6.5, the percent degradation of Pyr were 88, 98 and 37% at a temperature of 20, 30 and 40°C, respectively. Conversely, the percent degradations of Pyr at pH 7 were 95, 95 and 37% at 20, 30 and 40°C, respectively. The lowest percent degradations were recorded at pH of 8, with 37, 8 and 38% for 20, 30 and 40°C respectively (Figure 1).

Figure 2 shows the effect of pH on the concentration of biomass which indicated that at pH 4 there was long lag phase at about 3 days; and the biomass growth which was about 50 µg/mL as well as the growth which reached its stationary phase and lasted up to 3 days, subsequently dropped. At pH 5, the effect on the biomass growth equally shows long lag phase, but with a relatively higher biomass concentration of 78 µg/mL than pH 4. On the other hand, the effect of pH 6 on biomass production includes the growth having shorter lag phase than either pH 4 or 5, whereas the log phase started on 3rd day and reached its maximum on the 6th day. The biomass concentration of 60 µg/mL was obtained and was found to be less than that at pH 5; however, its biomass began to drop on the 8th day and continued till below 45 µg/mL on the 10th day. In contrast, biomass growth at pH 7 has a very short lag phase of 24 h only. It reached its highest biomass concentration on the 4th day of about 100 µg/mL, which was higher than all other pH range tested. pH 7 was the best for biomass growth having shorter lag phase and higher total biomass at the end of log phase (Figure 2). Since microbial groups have different pH preferences, drop or increase in pH beyond preference level may disrupt the plasma membrane or inhibit the activities of enzyme and membrane transport proteins. Therefore, a neutral pH of 7 might be preferential of this mixed culture; hence, it favors the higher growth rate and subsequently reducing the period of acclimatization.

However, it is significantly reduced to approximately similar values as other pHs on the 10th day (prolonged generation), a situation where bacterial cell uses a strategy to grow under a limited substrate concentration (Bren et al., 2013). This form of the growth strategy is very different from normal growth, where substrate concentration and nutrient supply were not limited. At pH of 8, there was a very long lag phase than all other pH. Its maximum biomass growth was reached on the 9th day and reduced on the 10th day. The highest biomass concentration at this pH was 60 µg/mL. Biomass concentration at alkaline pH of 8 was inferior to acidic; however, acidic condition at pH 5 is also considerable. This is also corroborated by estimated maximum specific growth rate μ_{max} obtained for all the pHs (Table 2).

pH 7 has highest specific growth rate (0.147 and 0.144 (h^{-1})) at 1.5 and 3.0 ppm, respectively, and shortest lag phase (2 and 3 days) than the other pH tested. pH 4 and pH 8 both have lowest maximum specific growth rates (0.044 and 0.047 (h^{-1})) respectively, however, pH 8 has the longest lag phase (8 days). External pH of 4 or below may lead to acid shock protein, prevent the denaturation of proteins and thereby retard the growth of the mixed culture. This may be the possible reason at pH 4 where the mixed culture growth rate was very low (0.044 (h^{-1})) (Willey, 2008).

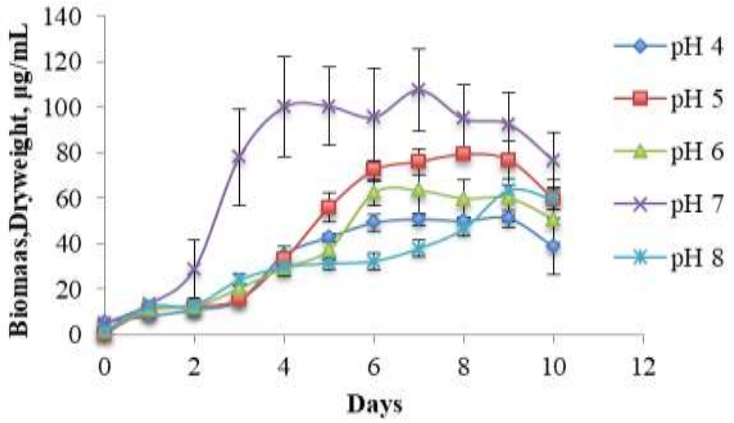

Figure 2. Effect of pH on the biomass growth (1.5 ppm of Pyr at 30°C and 180 rpm). Each data point represents the mean±standard error of three replicates.

Table 2. Effect of pH on specific growth rate (μ_{max}).

pH	μ_{max} (h^{-1})	Pyr Concentration, ppm	Days
4	0.044	1.5	3
5	0.074	1.5	4
6	0.060	1.5	5
7	0.130	1.5	3
8	0.047	1.5	8
7	0.147	1.5	2
7	0.144	3	3

Biodegradation of Pyr

The mixed culture was tested on Pyr degradation in an aqueous medium containing MSM with Pyr concentration of 1.5 and 3.0 ppm as a sole carbon source. The growth was measured by OD at 600 nm and converted to dry weight using correlation curve in Figure 3, with r^2 of 0.989 which has been described by Alfermann et al. (1994) with little modification (using different carbon source) from the one reported by Alfermann et al. (1994).

The biomass growth and Pyr degradation occurred within six days of incubation. The lag period was very short indicating faster acclimatization and followed by a log phase from Day 2 to 6 with specific growth rate, µ at 0.147 and 0.144 h^{-1}, respectively (Figure 4).

Also, at 3.0 ppm there is better biomass growth while 1.5 ppm resulted in shorter lag phase, probably due to low concentration and assimilated easily, but with lower biomass harvest. Perhaps, the lower the concentration the lower the growth of biomass. Meanwhile, more than 80% of Pyr was degraded during this log phase growth period and can thus be classified as -growth associated-degradation kinetics, indicating the overall growth of the mixed culture could be due to substrate consumption. This means that substrate consumption and degradation of Pyr followed a Monod-type growth pattern, which indicates that the mixed culture grew due to the consumption of Pyr as a source of carbon and energy. The growth curve and the percent degradation rate are given in Figures 4 and 5, respectively.

From Figure 5, there is a marked relationship with the rate at which Pyr was degraded by the mixed culture. The concentrations of Pyr dropped sharply at the initial stage in the 1.5 ppm concentration than from the 3.0 ppm. However, both concentrations were linearly reduced by 80% on the sixth day. In contrast, the degradation in the 3.0 ppm at 6 and 8 days slowed down but reached nearly 90% on the 10th day. From Figure 6, the abiotic control dropped from 1.5 ppm to 1.348 ppm and remained constant, accounting for about 10.1% loss. This could be attributed to volatilization or sticking to the wall of the glass.

Utilisation of Pyr-degraders on other PAHs

Other PAHs such as Nap, Phn and Fla were tested using the previous experimental setup as described earlier

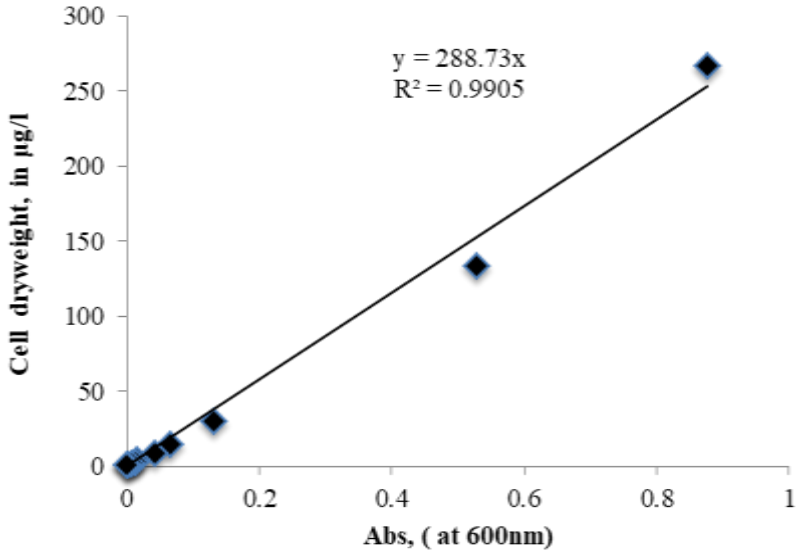

Figure 3. Correlation curves for optical density against dry weight of mixed culture.

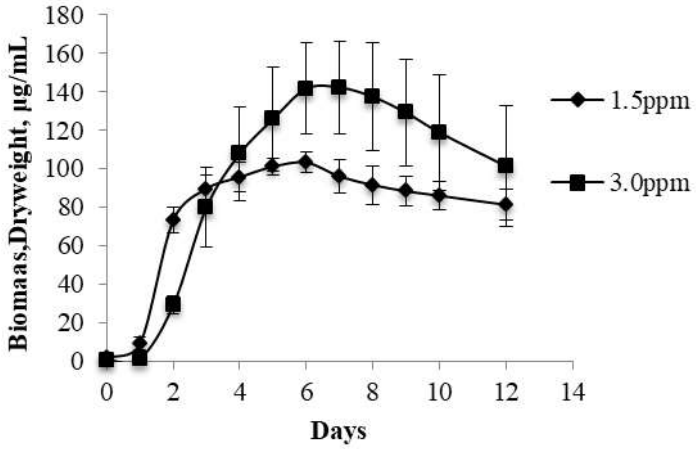

Figure 4. Percentage of residual Pyr remaining over time in the culture media during incubation in the dark with 3.0 and 0.15 ppm of pyrene at 30°C, pH 7.0 and180 rpm). Each data point represents the mean± standard error of three replicates.

under “Methodology”. These PAHs were incubated as sole source carbon with the concentration of Nap, Phn and Fla of 0.86, 1 and 1.38 ppm respectively. They were incubated at 50 mL of MSM with pH 7.0, at 30°C, and 180 rpm in the dark and samples were taken at the end of two weeks. The result of degradation experiments for other PAHs, Nap, Phn and Fla (as source of carbon) in Figure 7 indicates that more than 90% of the three compounds were degraded within three weeks of incubation. In fact, the degradation of Fla is about 98%, and maybe because Fla is also a four-ring PAHs like Pyr, or the mixed culture may have metabolic capacity to degrade Fla. But, for the remaining two (Nap and Phn), they are LMW and are not recalcitrant to bacterial degradation, therefore easily degraded by the mixed culture. It has been proposed by Tam et al. (2003) that PAH-degrading bacteria enriched with any particular PAH compound as sole source carbon and energy could have the ability to metabolize varieties of alternative PAHs. The utilization of another PHA by a mixed culture has been reported (Yu et al., 2005; Yuan et al., 2000).

Metabolites of Pyr

To possibly identify any metabolite, a degradation culture

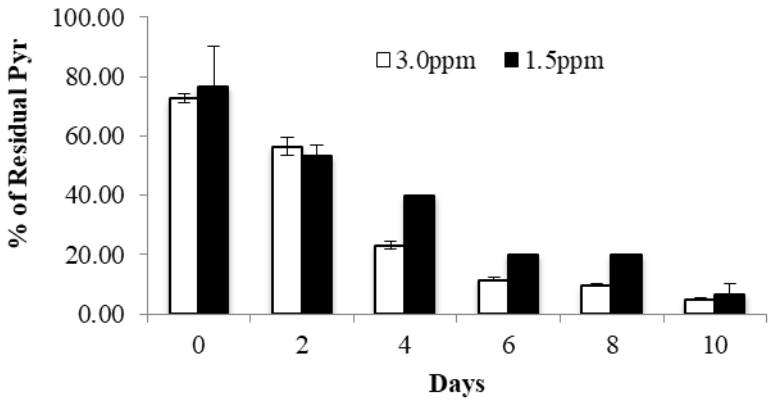

Figure 5. Percentage of residual Pyr remaining over time in the culture media during incubation in the dark with 3.0 and 1.5 ppm of Pyr at 30°C, pH 7.0 and at 180 rpm). Each data point represents the mean± standard error of three replicates.

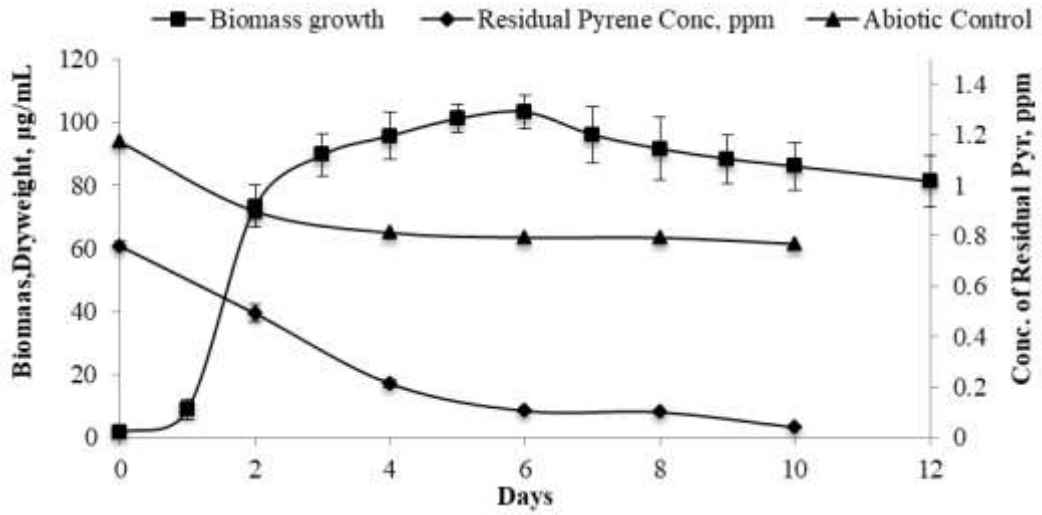

Figure 6. Concentration of residual Pyr and biomass growth by bacterial mixed culture isolated from the automobile workshop refuse dump (1.5 ppm of Pyr, at 30°**C**, pH 7.0 and 180 rpm) incubated in the dark. Each data point represents the mean± standard error of three replicates.

sample was extracted using DCM three times. The sample was dried using anhydrous sodium sulphate and the extracts recombined and concentrated as previously described under “Methodology”. These samples were taken to the Chemistry Department, Universiti Putra Malaysia for GC-MS analysis. The results were interpreted based on the library similarity index provided by the software. Table 3 show some of the probable metabolites from the culture sample as revealed by the GC-MS chromatograph. There are many metabolites on Day 6, and benzoic acid, which is also one of the catabolic product of PAH degradation appeared on Day 4, 6, 8 and 10. Di-n-octyl phthalate appeared on Day 0, 2, 4 and 10.

DISCUSSION

The ability of *B. cereus* to transform Pyr into cis-4,5-dihydrodiol-4,5 dihydroxypyrene in a single culture have been reported (Kazunga and Aitken, 2000); also, *E. aerogenes* in degrading Pyr has been reported in other works (Bastiaens, et al., 2000). The dominant member of the mixed culture is *B. cereus* from the soil exposed to hydrocarbon pollution. The enrichment was approached with a pure Pyr of about 98% purity as a carbon source

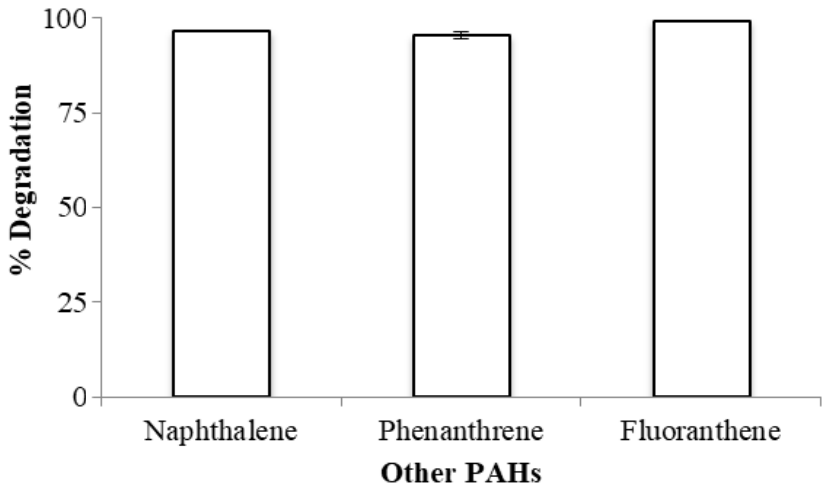

Figure 7. Percentage degradation of naphthalene, phenanthrene and fluoranthene at a concentrations of 0.86, 1 and 1.38 ppm, respectively. Each data point represents the mean of two replicates.

Table 3. Probable metabolites of Pyr degradation.

No	RT GC	MW	Probable metabolites	Time Pyr Degrades (day)						Reference
				0	2	4	6	8	10	
1	9.43	156	1,8-Dimethylnappthalene	-	-	-	+	-	-	Cerniglia (1993)
2	9.65	204	Benzenepropanal	-	-	-	+	-	-	-
3	9.78	314	1,4-Benzenediol	-	-	-	+	-	-	-
4	10.30	194	Benzoic acid	-	-	+	+	+	+	Kanaly and Harayama (2000)
5	10.80	184	2(3H)-Furanone	-	-	-	+	-	-	Zeng et al. (2000)
6	10.45	178	Ethanone	-	-	-	+	-	-	
7	13.25	278	Dibutyl phthalate	-	-	-	+	-	-	Peng Hua et al. (2012)
8	15.52	390	Di-n-octyl phthalate	-	+	+	-	-	+	Zeng et al. (2000)
9	3.82	156	1,2-Dioxane	-	-	-	+	-	-	-
10	7.43	198	6-Octen-1-ol	-	-	-	+	-	-	-
11	8.43	154	1,6-Octadien-3-ol	-	-	-	+	-	-	-
12	11.25	215	2-(phenlmethylene)	-	-	-	+	-	-	(Zeng Hong et al. (2000)

solely. Therefore, the bacteria isolated were not as a result of other organic sources since the media used was mineral salt medium only. Although, from the isolation process, few members of *E. aerogenes* were isolated, it could be concluded that this strain prefers particular metabolite to the parent compounds. Despite the unavailability of experiments in this study to prove this, physical observations indicated that *B. cereus* colonies appeared first on agar plate during plate counting. The reason is that, at the early stage of incubation in an aqueous medium, only *B. cereus* were seen on the agar plates. Not until after six days before the *E. aerogenes* would begin to appear on the plates during plate counting for monitoring growth. Especially, during monitoring of growth in soil slurry batch reactor, colonies of *E. aerogenes* appear only on the six days.

For successful bioremediation of any organic contaminant, sufficient information on the microorganisms capable of mineralizing or degrading the contaminant to a benign and non-toxic end product is necessary. Several studies have been carried out on the isolation of bacteria, fungi, and algae that degrade varieties of aromatic hydrocarbons and other organic contaminants in the environment. Microbial isolation is done using the same method of enrichment and PAHs as the source of carbon and energy. For example, a polluted soil sample is serially diluted and plated on minimal basal salt (MBS) incorporated in agar, together with PAHs, or minimal salt medium (MSM) incorporated in agar and PAHs spread on the surface of the agar plate and incubated at certain temperature. This method was used by Kiyohara and Nagao (1978) for isolating bacteria degrading

phenanthrene through identification of the clear zones surrounding the bacterial colony of interest. Heitkamp and Cerniglia (1989) also applied this method with little modification, using composite sediment and water samples exposed to radio-labeled [4-^{14}C]Pyr and non-labeled Pyr. The Pyr-degrading bacteria were recognized as colonies surrounded by clear zones due to Pyr utilization. Due to these successes, many workers have applied this method either directly or with little modification; including Laehy et al. (2003), Yu et al. (2005) and Zhang et al. (2009) for PAHs isolation. In another method, PAHs crystals in liquid medium were also degraded with several subcultures in a fresh medium, as demonstrated by Bastiaens et al. (2000) and bacteria were isolated from sediment samples. Gaskin and Bentham (2005) compared the ability of different enrichment methods to select diverse, abundant bacteria population from contaminated soil samples; although, they found out that using Pyr alone as a source of carbon has lower ability to select diverse isolates; nonetheless, this study confirmed the suitability of Pyr as a carbon source.

Generally, to conduct biodegradation Pyr at 40°C was inferior compared to either 20 or 30°C; and is similar to the effect of temperature reported by Kim et al. (2005) and Kim and Freeman (2005). Temperature usually affects microbial degradation of hydrocarbon metabolism, and there is an optimum temperature beyond which biological activities often decreases (Riser-Roberts, 1998). In this study, therefore, the optimum temperature for the highest percent degradation of Pyr is between 20 and 30°C. Also, this result suggest that mixed culture could endure different temperature ranges from 20 to 30°C and thus have potentials for bioremediation application. Nevertheless, normal microbial metabolism would be usually affected by either too low or too high a temperature, thereby affecting the degradation of Pyr. Consequently, a temperature of 30°C was adopted for degrading Pyr in this study. This optimal growth condition was also reported by Zhang et al. (2009).

The ability of some bacteria to grow well under acidifying and neural condition was also reported in the past by Wong et al. (2002). Biodegradation is also pH influenced; the percent degradation was highest around neutral (pH 6.5 - 7.0) and neural pH was found to be the most ideal. This optimal pH and temperature values has been previously reported by Zhang et al. (2009) on biodegradation of PAH by bacteria. Temperature and pH are two important environmental factors that could determine the suitability of the environmental conditions to metabolic functions.

The hydrocarbon-polluted soil or sediment has been associated with diversities of pollutant-degraders with the ability to remove or degrade many recalcitrant organic contaminants (Yu et al., 2005). In a comprehensive bacterial diversity study of PAH-contaminated sites, Mueller et al. (1997) pointed out that PAH degradation capabilities of bacteria might not be unconnected with phylogenetic related genera; and are not restricted to a particular location. They stressed that uncontaminated aquatic sites might naturally harbor bacteria that can degrade PAH. However, it is challenging to obtain PAH-degrading organisms from uncontaminated soil sites. Several works reported that isolation of bacteria capable of degrading PAH could be easily obtained from the hydrocarbon-contaminated soil than otherwise (Tam and Wong, 2008; Tian et al., 2008). In this study, 14 Pyr-degrading bacterial strains were isolated from the hydrocarbon polluted soils, belonging to the genera of *B. cereus* and *E. aerogenes*.

Several reports indicated the suitability of a mixed culture than single strains. This is primarily due to their synergic response and better performance when compared to single pure culture (Mikesková et al., 2012). In the employment of defined mixed culture or co-culture of fungi and bacteria, synergic degradation capacity as compared to single strength was observed. Because of the synergic interaction among members or between members, one strain may attack the parent compound, or another may use the first metabolite as a carbon source (González et al., 2011). Although individual strength in a mixed culture has not been fully understood, detection of different strains in hydrocarbon polluted soils indicates individual strength in the transformation process (Ghazali et al., 2004).

It has been proposed by Tam et al. (2003) that PAH-degrading bacteria enriched with any particular PAH compound as sole source of carbon and energy could have the ability to metabolize varieties of alternative PAHs. The utilization of another PAH by a mixed culture has been reported by Yu et al. (2005). Apparently, some of the metabolites like Di-n-octyl phthalate were reported as metabolic product of initial ring fission of benzo[a]pyrene whereas others are from combined ozonation and bacterial degradation of Pyr (Zeng et al., 2000). Another aspect of this result is that some of the metabolites had higher molecular mass than Pyr, and were adjudged to be surfactants produced during degradation. This is so because, from physical observation of the culture medium during incubation as from Day 4 to 6, there were bobbles and thin films covering the surface of the medium. This could be due to productions of some surfactant; therefore, the mixed culture could have transformed some of the metabolites into some sort of surfactants to enable them dissolve the Pyr. The assumption is that Pyr has been completely degraded by the mixed culture; although there are differences between these metabolites and those reported due to pure culture. For example, Seo et al. (2010) found some metabolites that are similar to one found in this study.

Conclusion

Therefore, in this study, a mixed culture of *B. cereus* and

E. aerogenes was successfully enriched from a hydrocarbon- polluted soil using Pyr as sole source carbon and energy. The mixed culture degraded Pyr within a very short period as environmental factors such as pH and temperature affect the metabolic function of a microorganism. The result indicated that the culture performs well under a range of pH between 5-7 and a temperature range of 20 − 30°C. This isolated mixed culture could be a potential inoculum to ex-situ bioremediation of PAHs contaminated soils.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

Alfermann AW, Dombrowski K, Petersen M, Schmauder H-P, Schweizer M (1994). Basic Scientific techniques for biotechnology In: Schmauder H-P (ed) Methods in Biotechnology Taylor and Francis pp.12-14.

Bastiaens L, Springael D, Wattiau P, Harms HDE, Wachter R, Verachtert H, Diels L (2000). Isolation of adherent polycyclic aromatic hydrocarbon (PAH) degrading bacteria using PAH-sorbing carriers Applied and Environmental Microbiology 66(5):1834-1843.

Bren A, Hart Y, Dekel E, Koster D, Alon U (2013). The last generation of bacterial growth in limiting nutrient BMC Systematic Biology 7(1):27.

Cerniglia CE (1993). Biodegrdadtion of polycyclic aromatic hydrocarbons. Current Opinion in Biotechnology 4:331-338.

Daugulis AJ, McCracken CM (2003). Microbial degradation of high and low molecular weight polyaromatic hydrocarbons in a two-phase partitioning bioreactor by two strains of *Sphingomonas sp.* Biotechnology Letters (17):1441-1444.

Deng L, Ren Y, Wei C (2012). Pyrene degradation by Pseudomonas sp and Burkholderia sp enriched from coking wastewater sludge J Environ Sci Health Part A 47:1984-1991.

Gaskin S, Bentham R (2005). Comparison of enrichment methods for the isolation of pyrene-degrading bacteria. International Biodeterioration & Biodegradation 56(2):80-85.

Ghazali FM, Rahman RN, Salleh AB, Basri M (2004). Biodegradation of hydrocarbons in soil by microbial consortium. International Biodeterioration & Biodegradation 54(1):61-67.

Goldstein RM, Mallory LM, Alexander M (1985) Reasons for possible failure of inoculation to enhance biodegradation. Applied and Environmental Microbiology 50:977-983.

González N Simarro R Molina M Bautista L Delgado L Villa J (2011). Effect of surfactants on PAH biodegradation by a bacterial consortium and on the dynamics of the bacterial community during the process. Bioresource Technology 102(20):9438-9446.

Heitkamp MA, Cerniglia CE (1989). Polycyclic aromatic hydrocarbon degradation by a Mycobacterium sp in microcosms containing sediment and water from a Pristine Ecosystem. Applied and Environmental Microbiology 55(8):1968-1973.

Jang S, Biberstein EL, Hirsh D (1988). A diagnostic manual of veterinary clinical bacteriology and mycology. http://agris.fao.org/agris-search/search.do?recordID=US19900045714

Juhasz AL, Naidu R (2000). Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo[a]pyrene. International Biodeterioration & Biodegradation 45(1-2):57-88.

Kanaly RA, Harayama S (2000). Biodegrdation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. Journal of Bacteriology 184(8):2059-2067.

Kazunga C, Aitken M (2000). Products from the incomplete metabolism of pyrene by polycyclic aromatic hydrocarbon-degrading bacteria. Applied and Environmental Microbiology 66:1917-1922.

Kim J-D, Shim S-H, Lee C-G (2005). Degradation of phenanthrene by bacterial strains isolated from soil in oil refinery fields in Journal of Microbiology and Biotechnology 15(2):337-345

Kim YH, Freeman JP (2005). Effects of pH on the degradation of phenanthrene and pyrene by Mycobacterium vanbaalenii PYR-1. Applied Microbiology and Biotechnology 67:275-285.

Kiyohara H, Nagao K (1978). The catabolism of phenanthrene and naphthalene by bacteria. Journal of General Microbiology 105:69-75.

Laehy JG, Tracy KD, Eley MH (2003). Degradation of mixtures of aromatic chloroaliphatic hydrocarbons by aromatic hydrocarbon-degrading bacteria FEMS. Microbial Ecology 43:271-276.

Luan TG, Yu KSH, Zhong Y, Zhou HW, Lan CY, Tam NFY (2006). Study of metabolites from the degradation of polycyclic aromatic hydrocarbons (PAHs) by bacterial consortium enriched from mangrove sediments Chemosphere 65:2289-2296.

Luo YR, Tian Y, Huang X, Yan CL, Hong HS, Lin GH, Zheng TL (2009). Analysis of community structure of a microbial consortium capable of degrading benzo(a)pyrene by DGGE. Marine Pollution Bulletin 58(8):1159-1163.

Mikesková H, Novotný Č, Svobodová K (2012). Interspecific interactions in mixed microbial cultures in a biodegradation perspective. Applied Microbiology and Biotechnology 95:861-870.

Mrozik A, Piotrowska-Seget Z, Labuzek S (2003). Bacterial degradation and bioremediation of polycyclic aromatic hydrocarbons. Polish Journal of Environmental Studies 12(1):15-25.

Mueller J, Devereux R, Santavy D, Lantz S, Willis S, Pritchard P (1997). Phylogenetic and physiological comparisons of PAH-degrading bacteria from geographically diverse soils Antonie van Leeuwenhoek 71(4):329-343.

Peng H, Hua Y, Jun D, Jin-Shao Y, Shuo-Na C, Bao-Yan H, Na Z (2012). Biodegradation of Benzo[a]pyrene by Arthrobacter oxydans \B4\ Pedosphere 2012 22(4):554-561.

Peng R-H Xiong A-S Xue Y Fu X-Y Gao F Tian Y-S Yao Q-H (2008) Microbial biodegradation of polycyclic aromatic hydrocarbons FEMS Microbial Ecology 32:927-955

Quinn P, Carter M, Markey B, Carter G (1994). Primary identification of bacteria 42-55 Grafos SA Arte Sobre Papel Spain: Wolfe Publishing.

Riser-Roberts E (1998). Remediation of petroleum contaminated soils: biological physical and chemical processes Levis publishers CRC Press LCC.

Samanta SK, Singh OVRK J (2002). Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation Trends in Biotechnology 20(6):243-248.

Schwurzenbach RP, Gschwend PM (2003). Imboden DM (eds) Environmental Organic Chemistry John Wiley & Sons Inc New York.

Seo J-S, Keum Y-S, Kim K, Li Q (2010). Degradation of pyrene by Mycobacterium aromativorans strain JS19b1. Journal of the Korean Society for Applied Biological Chemistry 53(3):323-329.

Tam NFY, Guo CL ,Yau LK, Wong YS (2003). Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by microbial consortia enrinched from mangrove sediments. Water Science and Technology 48(8):177-183.

Tam NFY, Wong YS (2008). Effectiveness of bacterial inoculum and mangrove plants on remediation of sediment contaminated with polycyclic aromatic hydrocarbons. Marine Pollution Bulletin 57:716-726.

Tao X, Lu G, Dang Z, Yi X, Yang C (2007). Isolation of phenanthrene degrading bacteria and characterisation of phenanthrene metabolites. World Journal of Microbiology and Biotechnology 23:647-654.

Tian Y, Liu HJ, Zheng TL, Kwon KK, Kim SJ, Yan CL (2008). PAHS contamination and bacterial communities in mangrove surface sediments of the Jiulong River Estuary China. Marine Pollution

Bulletin 57:707-715.
Willey J (2008). Prescott Harley and Klein's Microbiology-7th international ed/Joanne M Willey Linda M Sherwood Christopher J Woolverton New York [etc]: McGraw-Hill Higher Education.
Wong JWC, Lai KM, Wan CK, Ma KK, Fang M (2002). Isolation and optimization of PAH-degradative bacteria from contaminated soil for PAHs bioremediation. Water, Air, and Soil Pollution 139:1-13.
Yan J, Wang L, Fu P, Yu H (2004). Photomutagenicity of 16 polycyclic aromatic hydrocarbons from the US EPA priority pollutant list Mutation Research.
Yu SH, Ke L, Wong YS, Tam NFY (2005). Degradation of polycyclic aromatic hydrocarbons (PAHs) by a bacterial consortium enriched from mangrove sediments Environment International 31:149-154.
Yuan S, Wei S, Chang B (2000). Biodegradation of polycyclic aromatic hydrocarbons by a mixed culture Chemosphere 41(9):1463-1468.
Zeng Y, Hong PKA, Wavrek DA (2000). Chemical biological treatment of pyrene. Water Research 34(4):1157-1172.
Zhang G-Y, Ling J-Y, Sun H-B, Luo J, Fan Y-Y, Cui Z-J (2009). Isolation and characterization of a newly isolated polycyclic aromatic hydrocarbons-degrading Janibacter anophelis strain JY11. Journal of Hazardous Materials 172:580-586.