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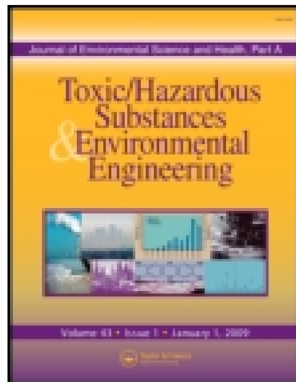

Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/lesa20

Biodegradation of soil-applied polycyclic aromatic hydrocarbons by sulfate-reducing bacterial consortium

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Published online: 27 Mar 2012.

To cite this article: Mathava Kumar , Pei-Chi Wu , Jen-Chieh Tsai & Jih-Gaw Lin (2009) Biodegradation of soil-applied polycyclic aromatic hydrocarbons by sulfate-reducing bacterial consortium, Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering, 44:1, 12-20, DOI: 10.1080/10934520802515178

To link to this article: http://dx.doi.org/10.1080/10934520802515178

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Journal of Environmental Science and Health Part A (2009) **44**, 12–20
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ISSN: 1093-4529 (Print); 1532-4117 (Online)
DOI: 10.1080/10934520802515178

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Biodegradation of soil-applied polycyclic aromatic hydrocarbons by sulfate-reducing bacterial consortium

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In the present study, the potential of polycyclic aromatic hydrocarbons (PAHs) biodegradation by sulfate-reducing bacterial consortium enriched from piggery wastewater was investigated. The batch experiments of soil-applied PAH biodegradation were conducted with a mixture of PAHs, i.e., naphthalene, fluorene, phenanthrene, fluoranthene and pyrene, at a concentration of 50 μg of each PAH per g of soil. A central composite design (CCD) was applied to determine the experimental conditions of each batch assay. The pH, biomass and ethanol concentrations were selected as independent variables and the PAH removal percentage was considered as a dependent variable. The optimal conditions for PAH biodegradation were found to be a pH between 4 and 6.5, an ethanol concentration less than 35 mg/L and a biomass concentration greater than 65 mg/L. Bench scale experiments were carried out at the optimal conditions. At the end of experiment (27 d), total PAH removals by biodegradation and volatilization were around 74% and 20%, respectively. The order of PAH removal was naphthalene, phenanthrene, fluorene, fluoranthene, and pyrene. Throughout the study, PAH biodegradation was in good correlation with sulfate reduction. Results of the kinetics study indicated a competitive inhibition between PAHs investigated.

Keywords: Polycyclic aromatic hydrocarbon, biodegradation, sulfate-reducing bacteria, kinetics study.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous as natural constituents and combustion products of fossil fuels,[1] which exist as compounds with two to seven condensed rings.[2] The low molecular weight PAHs are readily degradable in nature whereas the high molecular weight PAHs are regarded as recalcitrant and genotoxic.[3] PAHs are nonpolar hydrophobic compounds with low volatility and high soil organic partition coefficient (Log K_{oc} from 2.81 to 5.74). Therefore, PAH contamination is not readily amenable to remediation by simple soil-washing techniques. On the other hand, biological treatment of PAHs is considered as a suitable detoxification method.[4] Bioavailability of PAHs is often limited by their sorption on natural organic matter, which is an important factor affecting the microbial degradation rates in soils and sediments.[2,5]

Several studies confirmed the aerobic and anaerobic biodegradation of PAHs.[6–13] Numerous varieties of microorganisms including algae, fungi, cyanobacteria and heterotrophic bacteria were successfully enriched and applied for PAH degradation[1,14,15] under nitrate-reducing,[16–19] sulfate-reducing,[6,12,20,21] methanogenic[22] and Fe(III)-reducing[23] conditions. Among these conditions investigated, PAH biodegradation was predominant under sulfate-reducing environment.[2,24,25] Many researchers carried out PAHs biodegradation experiments using both pure[26] and mixed sulfate-reducing cultures.[2,12,21,27] However, no researcher intended to investigate the biodegradation of soil-applied PAHs neither with anaerobic sulfate-reducing consortium nor with pure sulfate-reducing culture.

PAH biodegradation can be influenced by pH, bioavailability of organic substrates, PAH concentration and temperature. Therefore, it is essential to quantify the optimal conditions to design an efficient PAH biodegradation system. In the present study, the degradation of a mixture of soil-applied PAHs including naphthalene, fluorene, phenanthrene, fluoranthene and pyrene by sulfate-reducing bacterial consortium was investigated under a range of pH, biomass and co-substrate concentrations.

Materials and methods

Chemicals

Naphthalene (purity >99%) was supplied by Aldrich (Milwaukee, WI, USA), and all other PAHs (fluorene,

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Received May 7, 2008.

phenanthrene, fluoranthene and pyrene) were by Fluka Chemical Co. (Switzerland). The solvents dichloromethane (99.9%) and acetone (99.8%) and the surfactant (Triton X-100) were purchased from local market. The other reagents used for the chemical analysis were of HPLC grade. All glassware used in the experiments was cleaned with distilled water and dried at 110°C before each experiment.

Bacterial source

Initially, an anaerobic sludge was collected from piggery wastewater treatment plant in Hsinchu, Taiwan. From the anaerobic sludge, a mesophilic sulfate-reducing bacterial consortium was enriched using modified Postgate's C medium (composition in g/L: 0.25 $NaHCO_3$, 0.25 KH_2PO_4, 1 NH_4Cl, 0.06 $CaCl_2 \cdot 6H_2O$, 2.5 Na_2SO_4, 0.04 $FeCl_3 \cdot 7H_2O$, 3.5 mL lactate and 0.1 yeast extract) for 4 years.[28] The presence and relative abundance of sulfate-reducing bacteria (SRB) in the inoculum were determined by fluorescence in-situ hybridization (FISH) with probes EUB338 (for eubacteria), SRB385 (for *δ-Proteobacteria*) and SRB385Db (for *Desulfobacteriaceae*).[29,30] FISH procedures including fixation, hybridization and staining were followed as reported by Amann et al.[31] The sum of SRB detected by probes SRB385 and SRB385Db was regarded as the total SRB population.[30,32] FISH results indicated that 87% of bacterial consortium belongs to SRB.

Prior to the biodegradation experiments, SRB consortium was grown in dark to a mid-log growth phase using modified Postgate's C medium. Further, the culture was harvested and concentrated by centrifugation at 6000 rpm for 10 min.

Soil samples

A surface soil previously unexposed to PAHs was collected from the premises of the National Chiao Tung University campus in Hsinchu, Taiwan. The soil used for biodegradation experiment was sieved through a U.S sieve No.40 (420 μm), oven dried at 105°C for 24 h and stored in a plastic container. The pH of the soil specimen was around 7 and the moisture content was 1.2%. The volatile solids content was 4.7%, and the carbon, nitrogen and hydrogen contents were 1.5%, 0.5% and 0.9%, respectively. The soil specimen was spiked with five PAHs (naphthalene, fluorene, phenanthrene, fluoranthene and pyrene) to the designed concentrations and the experiments were duplicated.

Experimental methods

The present study was executed in three phases. Initially, soil-applied PAHs batch biodegradation experiments were carried out. The batch experiments were designed using a central composite design (CCD) with three independent variables, i.e., pH, biomass and ethanol concentrations. The optimal pH, biomass and ethanol concentrations were determined using the response surface methodology (RSM). Subsequently, bench-scale soil-applied PAHs biodegradation experiments were carried out under these identified optimal conditions. Finally, batch kinetics studies were carried out at different PAH and biomass concentrations.

Batch experiments

The total number of batch PAHs biodegradation experiments (N) required for three independent variables was determined as per Gunawan et al. ($N = 2^K + 2K + n$; where, K is the number of independent variables and n is the number of center points).[33] The batch experiments were designed using MINITAB® 14 (Minitab Inc., USA) and the experimental design is shown in Table 1.

An oven-dried soil specimen weighing exactly 10 g was spiked with 50 μg/g of each PAH, 100 mL of modified Postgate's C medium and 130 mg/L of Triton X-100. The contents were homogenized in a rotary shaker at 300 rpm for 15 min and the mixture was transferred to a 250 mL serum bottle. Subsequently, biomass and ethanol (as per Table 1) were added into the serum bottle under a nitrogen atmosphere and the pH was adjusted to the required level by the addition of NaOH and/or HCl. The headspace of the serum bottle was purged with nitrogen gas and immediately sealed with thick butyl rubber stopper. Finally, the serum bottle was kept in a temperature-controlled (35°C) shaker at 150 rpm for 30 d.[11] An inhibition-control study (with 4000 mg/L of Na_2MoO_4 and 52 mg/L of biomass) and a control study (with 10,000 mg/L of NaN_3 and without biomass)

Table 1. Experimental framework and experimental results of batch PAH biodegradation experiments by CCD.

Run order	*pH*	*Biomass (mg/L)*	*Ethanol (mg/L)*	*PAH removal[1] (%)*
1	4.0	52	70	82.6
2	7.5	52	110	78.5
3	5.4	38	46	66.5
4	7.5	52	30	86.7
5[2]	7.5	52	70	89.6
6	9.6	38	46	77.3
7	7.5	76	70	75.2
8	9.6	67	94	53.3
9	9.6	67	46	73.4
10[2]	7.5	52	70	68.6
11	5.4	38	94	78.4
12	5.4	67	94	53.7
13	11.0	52	70	50.9
14[2]	7.5	52	70	70.5
15	5.4	67	46	88.2
16	9.6	38	94	77.0
17	7.5	29	70	57.7

[1]Total removal of four PAH compounds (exclusive of naphthalene).
[2]Center point in triplicate.

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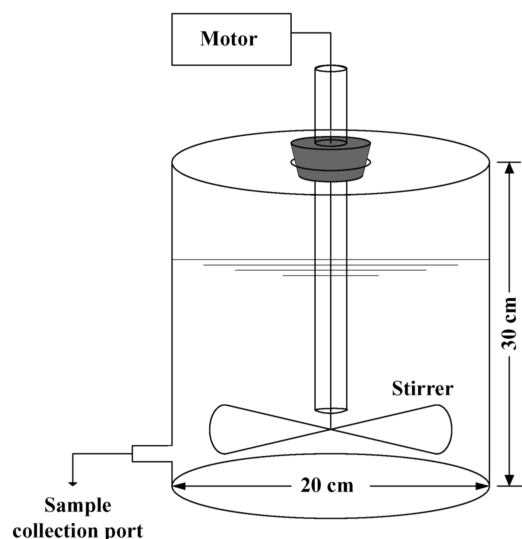

Fig. 1. Schematic diagram of bench-scale soil-slurry reactor.

were also conducted under similar conditions. At the initial (0 d) and final (30 d) of the experiments, 2 mL of soil slurry was collected from the serum bottles and analyzed for PAHs concentration.

Bench-scale experiments

For bench-scale experiments, a 6 L flask with a working volume of 4 L provided with a mechanical mixer was used as a bioreactor (BR) (Fig. 1). Exactly 400 g of soil specimen was spiked with 50 μg/g of each PAH and placed in the bioreactor. The working volume of the reactor was adjusted to 4 L using modified Postgate's C medium and subsequently spiked with 130 mg/L of Triton X-100, 66.5 mg/L of biomass and 30 mg/L of ethanol. The reactor contents were homogenized and the pH was adjusted to 5. Finally, the headspace of the reactor was purged with nitrogen gas, sealed completely and incubated at 35°C. Simultaneously, a control reactor (CR) study without biomass was carried out at similar operating conditions. Duplicate samples (36 mL each) were collected from BR and CR at different time intervals and analyzed for PAH, sulfate and biomass concentrations.

Kinetics study

Batch PAH biodegradation experiments were carried out using 250 mL of serum bottles at different concentrations of PAH (10, 50, and 100 μg/g) and biomass (7.7, 38.5 and 61.6 mg/L). Twenty grams of soil specimen spiked with PAH and biomass was placed in a serum bottle and supplemented with 100 mL of modified Postgate's C medium. The reactor contents were mixed well and the pH was adjusted to 7. The headspace of the serum bottle was purged with nitrogen gas, immediately sealed with butyl rubber stopper and incubated at 35°C with agitation at 150 rpm. At the end of 0, 0.5, 1, 2, 3, 4, 6, 8, 12 and 14 d, 2 mL of soil slurry was collected from each serum bottle and analyzed for PAHs concentration.

Analytical methods

PAHs

Extraction

PAHs were extracted from soil by the microwave-assisted extraction (MAE) method (U.S. EPA SW-846 Method 3546). The soil slurry collected from the bioreactor was centrifuged at 3000 rpm for 10 min and the resulting soil residue was mixed with 20 g of anhydrous Na_2SO_4 to remove the moisture.[13] Further, the moisture free soil residue was placed inside a Teflon-lined extraction vessel and added with 36 mL of dichloromethane/acetone mixture (1:1, v/v). Initially, the extraction temperature was set at 96°C for 17 min, and then the vessel was cooled down to room temperature. Finally, the extract was centrifuged at 3000 rpm for 10 min and concentrated to 3 mL by a rotary vacuum evaporator.

Quantification

PAH concentration was determined by the Hewlett-Packard (HP) 5890 series II gas chromatography (GC) equipped with a flame ionization detector (FID) and DB-5 capillary column (30 m × 0.52 mm i.d., with 1.5 μm film thickness). The initial oven temperature was maintained at 100°C for 1 min, then increased to 280°C at a rate of 6°C/min, and held at this temperature for 10 min. Both, the injector and detector temperatures were maintained at 280°C. Helium was used as a carrier and a make-up gas at rates of 3.5 and 30 mL/min, respectively. Hydrogen gas and airflow rates were maintained at 30 mL/min and 300 mL/ min, respectively. Exactly 1 μL of extracted sample was injected manually under split-less mode. Under these conditions, the retention times of naphthalene, fluorene, phenanthrene, fluoranthene and pyrene were 9.4, 18.4, 22.5, 27.7 and 28.7 min, respectively. The recovery efficiencies of naphthalene, fluorene, phenanthrene, fluoranthene and pyrene from soil by the MAE method were 62%, 93%, 100%, 88% and 125%, respectively. The method detection limit (MDL) was 0.8 μg/L for naphthalene, 0.7 μg/L for fluorene, 0.6 μg/L for phenanthrene, 0.5 μg/L for fluoranthene, and 1.1 μg/L for pyrene.

Bacterial quantification and sulfate measurement

The volatile suspended solids (VSS) concentration in the reactor was used to reflect the biomass concentration. Exactly 50 mL of well-mixed soil slurry was collected from the reactor, and filtered through a 0.45 μm glass-fibre filter paper.

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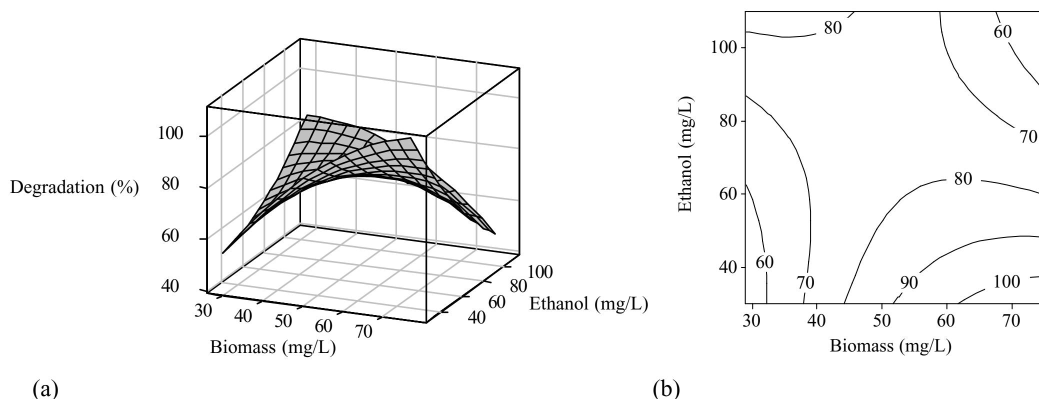

Fig. 2. The response surface plot (a) and contour plot (b) of PAH removal at different biomass and ethanol concentrations.

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The residue was dried at 105°C for 3 h and weighed (W_1). Further, the dried residue was ignited to constant weight at 550°C, cooled to room temperature and weighed again (W_2). The difference in weight ($W_1 - W_2$) was noted as the weight of VSS.

To measure sulfate concentration, the slurry samples were centrifuged at 3000 rpm for 10 min and the supernatant was diluted with deionized water. The sulfate concentration in the diluted sample was measured spectrophotometrically using a UV/VIS spectrophotometer at a wave length of 420 nm (OD_{420}).[34]

Results and discussion

Batch experiments

Samples collected from the batch experiments at the end of 0 d and 30 d were analyzed for PAHs concentration. The total PAHs removal percentage in each batch biodegradation experiment is shown in Table 1. The highest removal of total PAHs was observed in both Runs 5 and 15 (around 89%) and the lowest observed in Run 13 (around 51%). The molybdate-inhibited and blank control experiments showed 10% and 5% of total PAHs losses from the experiments, respectively by volatilization (Table 2). In the biotic, molybdate-inhibited and blank control experiments, the naphthalene concentration was below the MDL at the end of 1 d. The reason for such a drastic decrease in naphthalene concentration was unknown. The order of PAH removals in the batch experiments was phenanthrene followed by fluorene, pyrene and fluoranthene. The highest phenanthrene removal was observed in Run 5 (>98%). The lower removal percentages of fluoranthene and pyrene in all runs can be attributed to their recalcitrant toxicity and higher molecular weight.

Table 2. Results of molybdate-inhibited and blank controls.

	PAH loss (%)	
Compound	*Molybdate-inhibited control*	*Blank control*
Naphthalene[1]	—	—
Fluorene	14.08	8.95
Phenanthrene	11.47	4.48
Fluoranthene	8.23	2.65
Pyrene	7.20	2.63
Total[2]	10.33	4.63

[1]The concentration of naphthalene was lower than MDL value (0.8 μg/L) on d 1.
[2]The total PAH loss was calculated exclusive of naphthalene.

The response surface (RS) and contour plot (CP) corresponding to the total PAHs removal percentage under varying biomass and ethanol concentrations are shown in Figures 2a and 2b, respectively. Figure 2 reflects that PAHs removal was more influenced by the change in the biomass concentration than by the change in the ethanol concentration. Moreover, it can be envisaged that more than 90% of total PAHs removal can be achieved when the initial biomass and ethanol concentrations are maintained around 65 mg/L and below 40 mg/L, respectively. An increase in total PAHs removal was observed with proportionate decrease in pH of the medium (Figs. 3 and 4).

The total PAHs removal remains unchanged when ethanol was added in the system under both acidic and alkaline conditions (Fig. 3a). This finding agrees with Lei et al.[13] From Figure 3, it can be projected that nearly 100% PAHs removal can be achieved when the ethanol concentration and pH are maintained under 40 mg/L and between 4 and 6.5, respectively. The results of total PAHs removal under different initial pH and biomass concentrations are shown in Figures 4a and 4b. A significant increase in total PAHs removal was observed with increase in biomass concentration in both acidic and alkaline conditions (Fig. 4). This observation may be due to the enhanced microbial growth rate and activity resulting from the high biomass seeding at initial stage of the experiments, i.e., 0 d. However, the increase in total PAHs removal was found to be higher in acidic conditions compared to alkaline conditions.

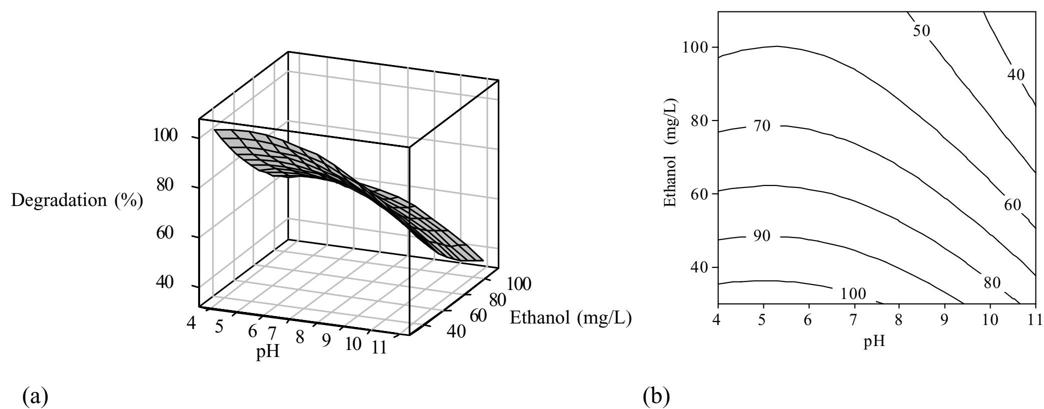

Fig. 3. The response surface plot (a) and contour plot (b) of PAH removal at different pH and ethanol concentrations.

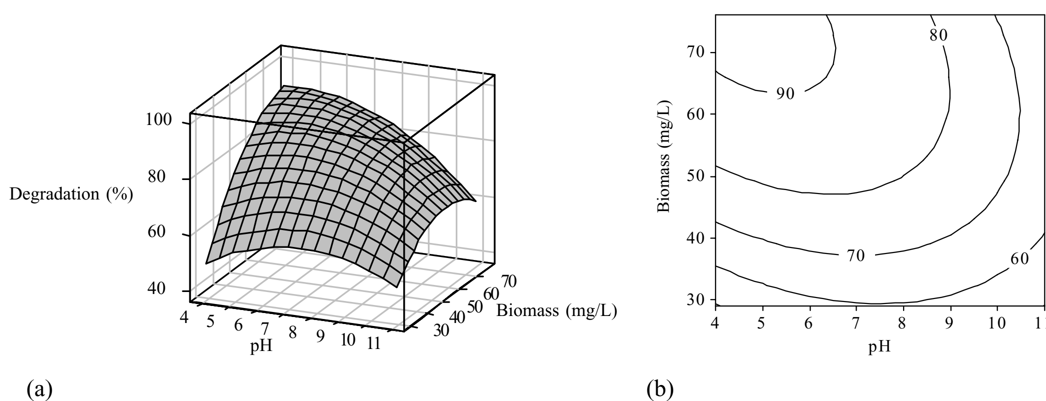

Fig. 4. The response surface plot (a) and contour plot (b) of PAH removal at different pH and biomass concentrations.

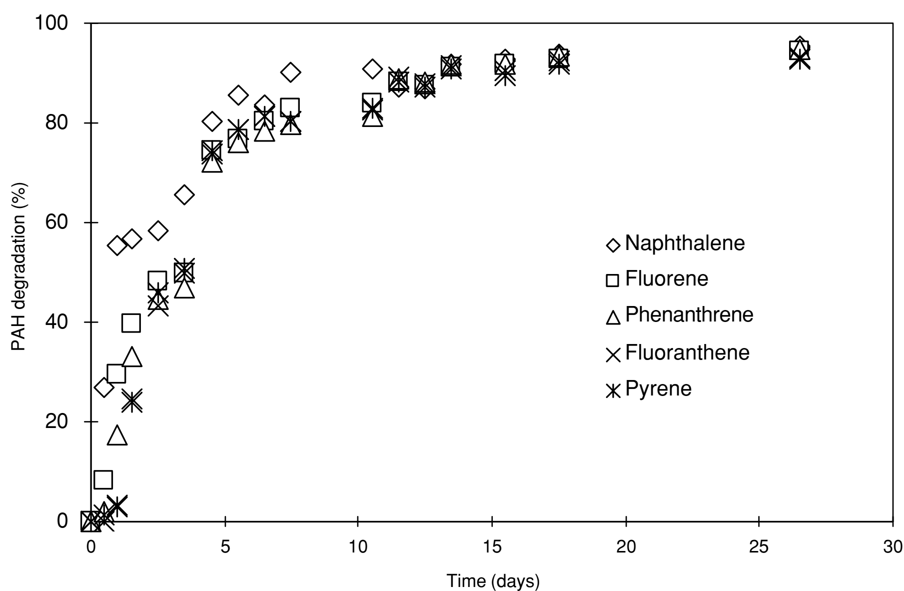

Fig. 5. PAH biodegradation profile in bench-scale soil-slurry reactor.

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Table 3. The optimal operating conditions for PAH biodegradation.

PAH compound	*Optimal conditions* *pH*	*Biomass (mg/L)*	*Ethanol (mg/L)*	*PAH removal (%)*
Naphthalene	—	—	—	—
Fluorene	4.5–8.5	>55	<50	>90
Phenanthrene	5.5–7.5	>60	<35	>95
Fluoranthene	4.0–6.5	>60	< 40	>90
Pyrene	4.0–7.0	>65	<35	>90
Mixture of PAHs	4.0–6.5	>65	<35	>90

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It can be observed from Figure 4a that the maximum total PAHs removal is achieved when the system is operated under acidic conditions (pH < 7) and with biomass concentrations greater than 60 mg/L.

The optimal operating conditions for maximum total PAHs removal were predicted using Figures 2 to 4 and the values are listed in Table 3. As shown in Equation 1, a second-degree quadratic model fitted well with the batch PAHs removal data.

$$Y = 78.0 + 0.07X_1 - 5.57X_2 - 3.39X_3 + 1.22X_1^2 - 5.44X_2^2 + 0.43X_3^2 - 4.05X_1X_2 + 3.19X_1X_3 - 15.7X_2X_3 \quad (R^2 = 0.76) \quad (1)$$

where Y is predicted response of total PAHs removal in%, and X_1, X_2 and X_3 are the input variables of pH, biomass and ethanol concentrations, respectively. The analysis of the batch experimental data was performed using the analysis of variance (ANOVA) and subsequently by the regression analysis (data not shown). The probability P-value was used to indicate the significance of the model. The smallest P-value of interaction factor (0.034) denoted the best correlation to the model ($P < 0.05$). The regression analysis showed that the interaction between the biomass (X_2) and ethanol (X_3) concentrations had greatest effect in PAHs removal. The model also shows an insignificant lack-of-fit, as is evident from the lower computed F value (7.82).[33] These observations show that the second-degree quadratic model is highly significant and sufficient to represent the effect of independent variables on PAHs removal.

Bench-scale experiments

Degradation profiles of naphthalene, fluorene, phenanthrene, fluoranthene and pyrene in the BR are shown in Figure 5, and the total PAHs degradation profile is shown in Figure 6. In the initial phase (0 to 1 d), the total PAHs degradation was insignificant, which can be attributed to the lag phase of SRB in degrading PAHs. However, the PAHs degradation increased rapidly from 1 to 4.5 d, and the total PAHs removal percentage at the end of this period was around 75%. After 13.5 d, the total PAHs degradation remained unchanged. The observed maximum PAH degradation percentages were 96%, 95%, 95%, 93% and 93% for naphthalene, fluorene, phenanthrene, fluoranthene and pyrene, respectively at the end of 27 d (Fig. 5) and the corresponding total PAHs degradation was 94% (Fig. 6). Initially (at the end of 2 d), the percentage removals of both fluoranthene and pyrene were nearly 3%. Thereafter, the percentage removals were increased rapidly and they were close to the percentage removals of naphthalene and fluorene at the end of the experiment. This reflects the ability of enriched SRB culture in the degradation of both low and high molecular weight PAHs. From CR data, it was

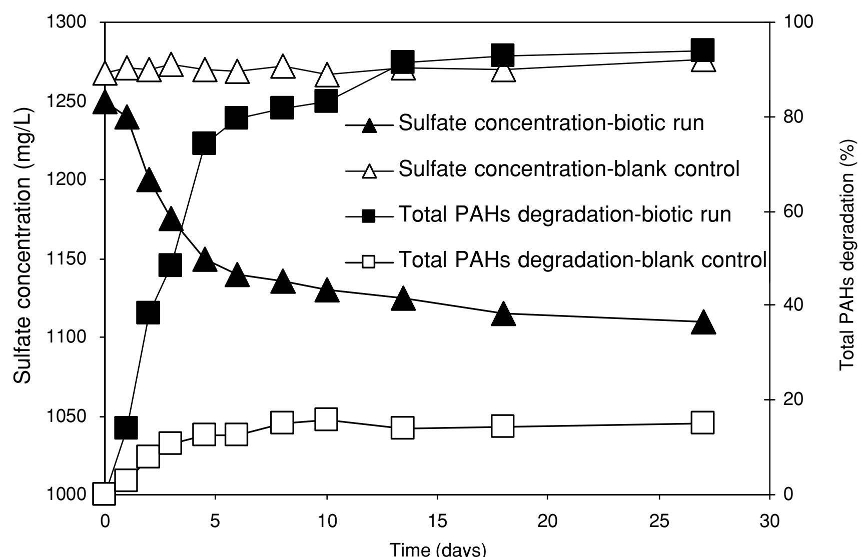

Fig. 6. Profiles of sulfate concentration and total PAH degradation in bench-scale soil-slurry reactor.

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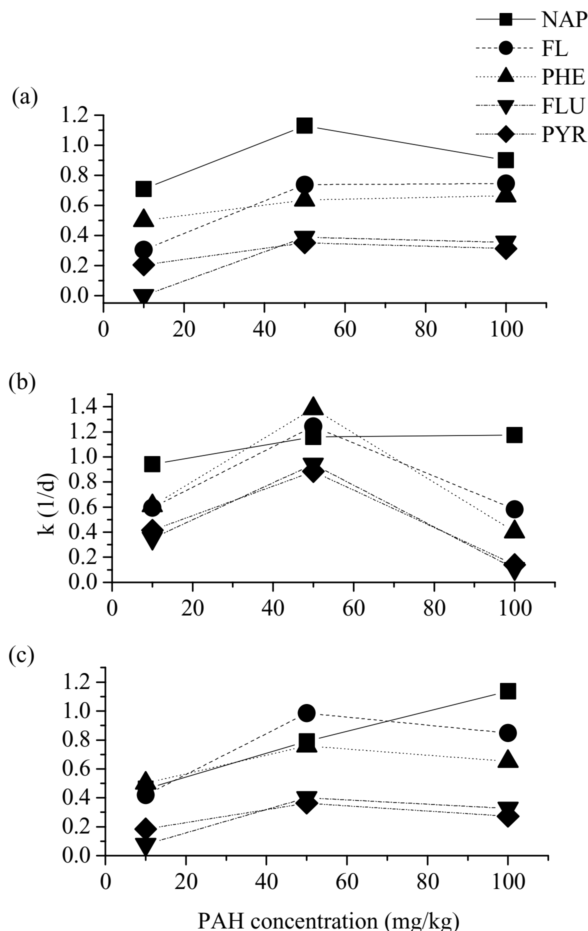

Fig. 7. First-order PAH degradation kinetic coefficients at different initial biomass concentrations of (a) 7.7 mg/L (b) 38.5 mg/L and (c) 61.6 mg/L.

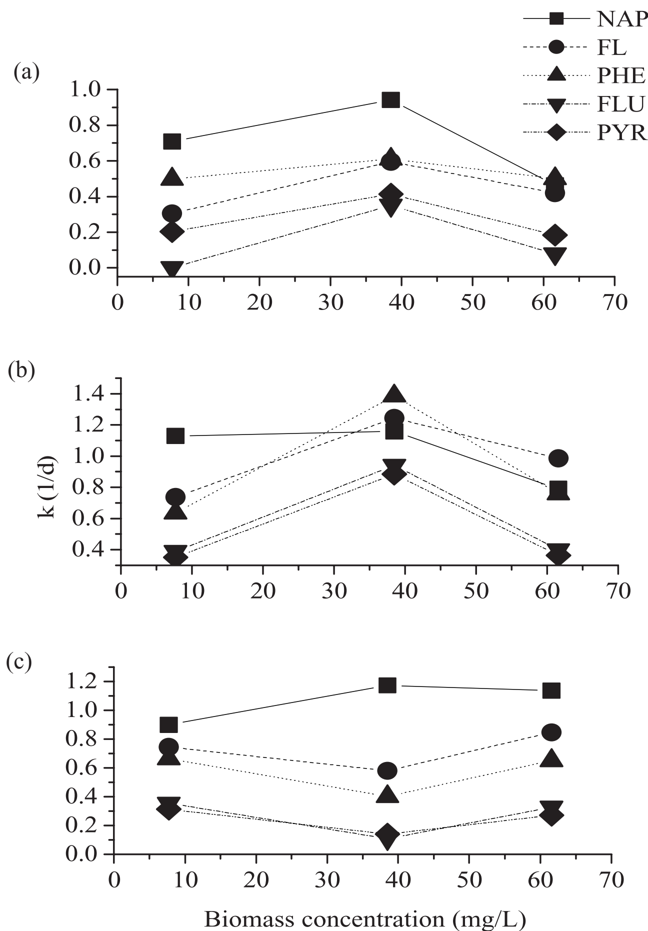

Fig. 8. First-order PAH degradation kinetic coefficients at different initial PAH concentrations of (a) 10 μg/g (b) 50 μg/g and (c) 100 μg/g.

observed that 53%, 17%, 19%, 12% and 12% of naphthalene, fluorene, phenanthrene, fluoranthene and pyrene, respectively were removed by volatilization at the end of 27 d, which corresponds to a total PAHs removal of 23%. Therefore, percentages of naphthalene, fluorene, phenanthrene, fluoranthene and pyrene removed by biodegradation were 43%, 78%, 76%, 81% and 81%, respectively, corresponding to a total PAHs biodegradation of 72% at the end of 27 d.

In the present study, sulfate is utilized as an electron acceptor and consumed by SRB for its growth. In both bench scale BR and CR experiments, the initial sulfate concentration was 1250 mg/L. At the end of experiment, the sulfate concentration was reduced to 1100 mg/L in BR whereas no significant change in the sulfate concentration was observed in CR (Fig. 6). The sulfate reduction in BR was in good correlation with the total PAHs removal (Fig. 6). Similar finding was reported while conducting PAH degradation studies under sulfate-reducing conditions.[13] A rapid decrease in sulfate concentration was observed in BR between 1 d and 4.5 d. During this period, the percentage removals of fluoranthene and pyrene were increased from 3% to 75% (Fig. 5). The decrease in sulfate concentration was slowed down between 4.5 d and 10 d, and thereafter, remained almost a constant. These observations indicate that PAHs biodegradation is significantly related to the sulfate reduction, which confirm the observations of Coates et al.[24] and Rothermich et al.[25]

Kinetics study

In order to obtain the PAHs biodegradation kinetics, batch experiments were conducted at various initial PAH and biomass concentrations. The first-order kinetics model as shown in Equation 2 was used to model the experimental data.[2]

$$dC/dt = -K_d C \qquad (2)$$

Where, C is substrate concentration [mg/L] and K_d is first-order rate coefficient [h^{-1}]. The first-order coefficient of PAHs biodegradation at different initial biomass and PAHs concentrations are shown in Figures 7 and 8, respectively. From the experimental data, it was observed that the ratio

of PAHs biodegradation increased in the range of 10 to 50 μg PAHs/g of soil (irrespective of the initial biomass concentration). In contrast, a decrease in PAHs biodegradation ratio was observed in the range of 50 to 100 μg PAHs/g of soil. Rothermich et al.[25] and Guha et al.[35] reported that the increase in PAH concentration beyond a certain extent can reduce the degradation coefficient of PAH. Although the initial PAHs concentration varied widely, the degradation of similar molecular weight PAHs in the present study (i.e. fluoranthene and pyrene) remained as a constant. Similar trends were observed by many researchers.[35,36] Degradation profiles of naphthalene, fluorene, phenanthrene, fluoranthene and pyrene were similar at the initial PAHs concentration of 10 and 50 μg PAHs/g of soil (Figs. 8a and 8b), whereas decreases in PAHs removal were observed at the elevated initial PAHs concentration, i.e., 100 μg PAHs/g of soil (Fig. 8c). These findings reflect that the PAHs degradation ratio depends on the initial PAH concentration to a certain extent irrespective of the biomass concentration.

Conclusion

The enriched SRB consortium successfully degraded PAHs and the biodegradation followed the first-order kinetics. PAHs biodegradation was the highest under acidic conditions. The addition of carbon source has not increased the PAHs biodegradation efficiency. Further investigations on the mechanisms, pathways of PAHs biodegradation and the detailed characterization of enriched SRB are required before employing the culture for the remediation of PAH-contaminated real world systems.

Acknowledgments

This research was supported by funds from the National Science Council, Taiwan, Republic of China (NSC 94-2211-E-009-008).

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