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Determination of optimal phenanthrene, sulfate and biomass concentrations for anaerobic biodegradation of phenanthrene by sulfate-reducing bacteria and elucidation of metabolic pathway

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ABSTRACT

Anaerobic biodegradation of phenanthrene (PHE) was investigated using an enrichment culture consists predominantly of sulfate-reducing bacteria (87 ± 6%). Aqueous biodegradation experiments were designed using the rotatable central composite design with five levels. The designed concentrations were 2–50 mg L^{-1} for PHE, 480–3360 mg L^{-1} for sulfate, and 5–50 mg L^{-1} for initial biomass. Experimental results indicated that the biomass concentration was the most significant variable, followed by the sulfate and PHE concentrations. The desirability functions methodology (DFM) was applied to find out the maximum specific PHE removal rate (R_s). The maximum R_s of 9.0 mg g^{-1} VSS d^{-1} within the designed ranges was obtained when the initial PHE, sulfate and biomass concentrations were 18.5, 841 and 50 mg L^{-1}, respectively. The R_s observed in the present study was higher than the values reported in the previous studies. Subsequently, a confirmation study was performed under the optimal conditions, and the results matched well with the R_s estimated using DFM. Samples collected during PHE biodegradation experiments inferred the formation of two novel metabolic intermediates, 2-methyl-5-hydroxybenzaldehyde and 1-propenyl-benzene, and subsequently degraded to *p*-cresol, phenol and hydrocarbons.

1. Introduction

Phenanthrene (PHE) is a polycyclic aromatic hydrocarbon (PAH) composed of three fused benzene rings and has been identified as priority pollutant by the United States Environmental Protection Agency (USEPA) [1]. The major sources of PHE include incomplete combustion of fossil fuels, vehicular emissions, municipal incinerators and coke plants [2–4]. PHE is widely distributed throughout the environment and is one of the most frequent and abundant PAHs found at contaminated sites [5,6]. Anaerobic biodegradation/biotransformation is an important removal mechanism for PHE in contaminated sites because of the scarcity of oxygen [7]. Evidences have shown the anaerobic degradation of PHE under sulfate-reducing conditions, and sulfate-reducing bacteria (SRB) are the major group of microorganisms involved in the degradation of PHE [8–13].

The extent and rate of degradation of PHE depend on the complex interactions between the contaminants, microorganisms and environmental conditions [14]. The treatment efficiency is influenced by numerous physico-chemical and microbial factors such as pH, temperature, contaminant concentration, level of electron acceptor, biomass concentration and acclimation of microorganisms [15,16]. Previously, effects of pH and temperature on PAH biodegradation under anaerobic conditions have been investigated [12,17]. Results of these reports demonstrated that the biodegradation of 2-, 3-, and 4-ring PAHs was the best at 30 °C and pH 7–8. In addition, the contaminant concentration can influence the efficiency of biodegradation. A decrease in the degradation rate of PHE under sulfate-reducing conditions was observed with the increase in PHE concentration beyond a certain level due to its inhibitory effect or toxicity to microorganisms. Moreover, biodegradation of PHE was substantially influenced by the addition of sulfate [17]. The high sulfate concentration reduced the PHE degradation through the inhibition of microorganism enzyme synthesis. Therefore, it is very important to evaluate both the range of PAH concentration that can be successfully biodegraded by SRB and the optimal dosage of sulfate. On the other hand, the inoculation of sufficient PHE-degrading bacteria may speed up the degradation process. Nevertheless, the effect of biomass concentration on PAH biodegradation has not been well understood.

Several reaction mechanisms including hydroxylation [18], hydrogenation [19], carboxylation [9,20] and methylation [21] have been proposed for naphthalene biodegradation under strict anaerobic sulfate-reducing conditions. However, the metabolic

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breakdown of PHE by SRB under anaerobic conditions was not enumerated completely. The only published metabolite from PHE under sulfate-reducing conditions was phenanthrenecarboxylic acid, and carboxylation was proposed as the initial metabolic reaction [9]. In our previous study, identification of metabolites by GC–MS revealed that PHE was biotransformed by a sequence of hydration and hydrolysis reactions followed by decarboxylation with the formation of *p*-cresol and phenol. Thus, a novel biotransformation pathway of PHE was proposed [22]. However, further studies are required to identify more metabolites and to detail the complete biodegradation pathway of PHE.

In summary, the PHE, sulfate and biomass concentrations are significant factors in the biodegradation of PHE. A thorough understanding of the effect of these factors on PHE biodegradation is required. The rotatable central composite design (RCCD) and response surface methodology (RSM) have been widely used in multifactor experiments to interpret the relationship between the independent and dependent variables [23–25]. This experimental strategy allows a large number of factors to be screened simultaneously and can reduce the number of experiments. In addition, the desirability functions methodology (DFM) is a mathematical optimization method. This method converts each response into a desirability value ranging between 0 (for an unacceptable response value) and 1 (for a completely desirable one). The overall desirability is calculated by applying the geometric mean of the individual desirability values. An algorithm is then applied to the desirability function in order to determine the set of variable values that maximize it [26]. The RSM along with DFM has been proven to be a useful statistical tool to optimize one or several responses [24]. Therefore, in the present study, a series of statistically designed batch PHE biodegradation experiments based on the RCCD were performed in an aqueous system. The general objective was to apply the RSM and DFM to determine an optimal set of operational conditions for PHE biodegradation. This study was also focused on the identification of metabolic intermediates of PHE.

2. Materials and methods

2.1. Chemicals and reagents

The PHE used in this study was obtained from Fluka (Neu-Ulm, Germany) with a specified purity of greater than 97%. Both dichloromethane (DCM) and N,N-dimethylformamide (DMF) were purchased from Sigma–Aldrich (MO, USA) with 99.9% and 100% purities, respectively. The stock solution of PHE was prepared by dissolving different amounts of PHE in DMF. The other reagents used for medium preparation and chemical analysis were of HPLC grade.

2.2. Enrichment of SRB

The mesophilic SRB culture, initially obtained from the anaerobic sludge of swine wastewater, was enriched in a specially designed 1.2 L serum bottle, i.e. incubation reactor (Schott, Germany) fitted with pH (Suntex, Taiwan) and oxidation–reduction potential (ORP) probes (Orion, Japan). The enrichment procedures and the medium used for this study have been described previously [22]. The presence and relative abundance of SRB in the culture system were determined by the fluorescence in situ hybridization technique [22]. At the end of the fifth sub-cultivation, up to $87 \pm 6\%$ ($n = 3$) of SRB population was observed in the enrichment culture.

2.3. Biodegradation of PHE

The biodegradation study was conducted with four series of independent experiments including a biotic, an inhibition-control, an abiotic control (cell-free reactor) and a bacterial control. Experiments were carried out in 1.2 L bioreactors with a working volume of 1 L. Before the experiments were started, the sulfate-reducing enrichment culture was grown to the mid-log growth phase (0.2 optical density (OD_{660}) = 100.2 mg L^{-1}), harvested, concentrated by centrifugation at 6000 rpm for 10 min and washed thoroughly with sterilized medium to remove the residual PHE. In the biotic experiments, the concentrated culture suspension was introduced into the bioreactors containing sterilized medium to obtain the desired concentrations. To ensure the absence of oxygen in the medium, bioreactors were purged with filter-sterilized high purity nitrogen gas (99.99%) for 10 min at a flow rate of 10 L min^{-1}. PHE was then added into the bioreactors at the designed concentrations (Table 1). The headspace of the bioreactors was replaced with pure nitrogen gas and sealed with a Teflon-lined cap. Bioreactors were incubated for 21 d in dark without shaking in a temperature-controlled chamber (Advantec C1-612, Taiwan) at 30 °C. Inhibition-control runs were prepared in the same manner as the biotic experiments except the addition of molybdate (4 g L^{-1}) as the inhibitor for SRB. Abiotic controls were prepared with sterilized medium, PHE and sodium azide (NaN_3, 10 g L^{-1}). Bacterial controls were treated similar to the cultures with the addition of DMF but without PHE. Samples were collected under strictly anaerobic condition at time zero and at the end of 3, 6, 12 and 21 d to measure the pH, bacterial cell density and concentrations of sulfate and PHE. At the end of the experimental period, ORP of the systems was measured.

2.4. Experimental design and statistical analysis

To investigate the effect of independent variables (i.e. PHE, sulfate and biomass concentrations) on the dependent variable (i.e. maximum specific PHE removal rate, R_s), the RCCD with five levels was applied and results were evaluated by the RSM with DFM. The experimental design consisted of six star points, eight factorial points and six center points, resulting in twenty randomized runs (Table 1). The center point was repeated six times to assess the repeatability of experiments. The experimental range was chosen based on the results of previous PAH biodegradation studies [10,12,27,28]. The lowest and highest values given to each variable were 2 and 50 mg L^{-1} for the PHE concentration, 480 and 3360 mg L^{-1} for the sulfate concentration, and 5 and 50 mg L^{-1} for the initial biomass concentration.

The statistical software, Design-Expert® 7.1.3 (Stat-Ease Inc., MN, USA), was employed to perform the experimental design, regression analysis and ANOVA, as well as to generate the contour plot. To interpret the relationship between the independent variables and their responses, an empirical second-order polynomial model was developed by multiple regression analysis. The ANOVA was used to estimate the statistical significance of variables and the model developed. The model adequacy was evaluated using the correlation coefficient (r^2), prediction error sum of square (PRESS) and adequacy precision [24]. Contour plots were plotted as a function of two variables, while holding the other variable at the optimal level as predicted. Numerical optimization was also conducted by the DFM to predict the exact optimal levels of the independent variables leading to the desired response goal, i.e. maximum R_s.

2.5. Analytical methodology

2.5.1. Extraction and quantification of PHE

The extraction and analysis of PHE were performed as previously described [22]. For measuring the concentrations of remaining PHE in the aqueous solution and adsorbed PHE in the SRB biomass, the cell suspension was centrifuged at 6000 rpm for 10 min to separate

Table 1
Experimental conditions and results of the RCCD for the anaerobic PHE biodegradation.

Run	Experimental condition			Experimental result			
	PHE ($mg\,L^{-1}$)	Sulfate ($mg\,L^{-1}$)	Biomass ($mg\,L^{-1}$)	Final biomass ($mg\,L^{-1}$)	PHE removal (%)	PHE removal rate[b] ($mg\,L^{-1}\,d^{-1}$)	R_s ($mg\,g^{-1}\,VSS\,d^{-1}$)[c]
1	26.0	1920	5	119	26	0.34	3.0
2[a]	26.0	1920	28	141	39	0.52	4.6
3[a]	26.0	1920	28	115	34	0.44	5.1
4	40.3	1064	41	158	22	0.52	4.4
5	40.3	2776	14	133	26	0.54	4.6
6	11.7	1064	14	131	57	0.31	2.7
7[a]	26.0	1920	28	149	41	0.53	4.4
8	40.3	1064	14	135	19	0.38	3.2
9	40.3	2776	41	160	22	0.45	3.7
10[a]	26.0	1920	28	139	38	0.49	4.4
11	11.7	1064	41	116	58	0.57	7.6
12	26.0	480	28	117	28	0.32	3.5
13	50.0	1920	28	120	10	0.25	2.7
14[a]	26.0	1920	28	125	35	0.50	5.1
15	26.0	3360	28	113	37	0.51	6.0
16	26.0	1920	50	135	42	0.63	7.4
17	2.0	1920	28	109	77	0.19	2.4
18	11.7	2776	41	128	63	0.49	5.6
19	11.7	2776	14	96	51	0.29	3.6
20[a]	26.0	1920	28	127	37	0.51	5.2

[a] Center point ($R_s = 4.8 \pm 0.4\,mg\,g^{-1}\,VSS\,d^{-1}$).
[b] Data from the zero-order period.
[c] R_s = PHE removal rate ($mg\,L^{-1}\,d^{-1}$)/(final biomass concentration − initial biomass concentration) ($mg\,L^{-1}$) × 10^3.

the biomass as a pellet. Subsequently, the supernatant and pellet were harvested and separately extracted with DCM. The sum of dissolved PHE in aqueous solution and adsorbed PHE in the SRB biomass was regarded as the total residual concentration of PHE in the cell suspension. After the extraction process, the gas chromatograph (Agilent 7890A, USA) equipped with a flame ionization detector (GC-FID) and HP-5 fused capillary column (30 m length, 0.53 mm inner diameter and 1.5 μm film thickness) was applied to determine the PHE concentration. The recovery of PHE was 95.3 ± 7.5%. The method detection limit (MDL) was $1.5 \pm 0.2\,\mu g\,L^{-1}$. The reported degradation in the present study was determined based on the total concentrations of PHE (PHE remaining in the aqueous solution and that sorbed onto the biomass) before and after the experiment.

2.5.2. Identification of metabolites of PHE

For the detection of metabolites, measurements were performed with a HP-6890 GC equipped with a HP-5973 mass spectrometry detector (GC–MSD) [22]. One microlitre of the sample was injected in a HP-5ms fused silica capillary column (30 m length, 0.25 mm inner diameter and 0.25 μm film thickness). Metabolites were identified by comparing their mass spectra with the reference mass spectra from the National Institute of Standards and Technology library and those of authentic standard compounds.

2.5.3. Quantification of metabolites

Phenol and *p*-cresol concentrations were quantified by using high performance liquid chromatography (HPLC). Prior to analysis, the particulates in the aqueous samples were removed by centrifugation at 10,000 rpm for 3 min. The cell-free supernatants were analyzed on HPLC with a RP-18 reverse-phase C_{18} column (250 mm length, 4.6 mm inner diameter and 5 μm particle size). The mobile phase consisted of a mixture of methanol and water (1:1, v:v) at a flow rate of $1\,mL\,min^{-1}$. Both phenol and *p*-cresol were detected by the measurement of UV absorbance at 254 nm using a single-wavelength UV–VIS detector (Hitachi L-2420, Japan) [29,30]. The retention times of phenol and *p*-cresol were 5.8 and 8.8 min, respectively. The recoveries of *p*-cresol and phenol were 101.7 ± 8.9% and 103.9 ± 7.0%, respectively. The MDLs of *p*-cresol and phenol were 25.5 and 36.8 $\mu g\,L^{-1}$, respectively.

2.5.4. Measurement of bacterial cell density, pH, ORP and sulfate concentration

The bacterial cell density measured either gravimetrically or spectrophotometrically was used as an indicator to represent the bacterial growth [22,31]. The mixed liquor volatile suspended solids (MLVSS) measurement was made gravimetrically, according to the Standard Methods (Method 10200 I-5) [32]. For spectrophotometric measurement, the bacterial pellets were obtained by centrifuging the cell suspensions at 6000 rpm for 10 min, resuspended and diluted in a series of dilution with 0.8% NaCl solution. The OD of the diluted samples was determined using a spectrophotometer (Hitachi U-3010, Japan) at a wavelength of 660 nm [33]. The OD_{660} values were correlated with respective MLVSS concentrations in a culture suspension. A calibration graph with r^2 of 0.99 ($n = 3$) was developed between the MLVSS and absorbance and the bacterial cell density was calculated by the determined conversion factor ($0.1\ OD_{660} = 50.1\,mg\,L^{-1}$).

pH and ORP meters were used to measure the pH and ORP of the homogenized samples, respectively. For measuring the sulfate concentration, samples were filtered through 0.2 μm nylon membrane filters (Advantec No. A020A047A, Japan) and concentrations were measured using the turbidimetric method (Method 4500-SO_4^{2-}-E) as described in the Standard Methods [32].

3. Results and discussion

3.1. Biodegradation study

3.1.1. Results of RCCD experiments

Table 1 summarizes the results of RCCD experiments. The optimal conditions were estimated based on the R_s. The maximum R_s of $7.6\,mg\,g^{-1}\,VSS\,d^{-1}$ was obtained under a PHE concentration of $11.7\,mg\,L^{-1}$, a sulfate concentration of $1064\,mg\,L^{-1}$ and an initial biomass concentration of $41\,mg\,L^{-1}$. A minimum R_s of $2.4\,mg\,g^{-1}\,VSS\,d^{-1}$ was observed in run 17 with the lowest PHE concentration of $2\,mg\,L^{-1}$. The low standard deviation of R_s ($4.8 \pm 0.4\,mg\,g^{-1}\,VSS\,d^{-1}$) of center point runs (runs 2, 3, 7, 10, 14 and 20) indicates a good repeatability of these experiments. In order to obtain an adequate model fitted by RSM, the analysis of the sum of sequential squares of the model was carried out (data not shown). Results showed that the experimental data were best represented

by a quadratic model. An empirical second-order polynomial model was developed and given in Eq. (1):

$$R_s = -4.524 + 0.322A + 7.66 \times 10^{-4}B + 0.215C + 1.75 \times 10^{-5}AB - 4.25 \times 10^{-3}AC - 5.40 \times 10^{-5}BC - 4.56 \times 10^{-3}A^2 + 1.56 \times 10^{-7}B^2 + 1.46 \times 10^{-3}C^2 \quad (1)$$

where A is PHE concentration (mg L^{-1}), B is sulfate concentration (mg L^{-1}) and C is biomass concentration (mg L^{-1}).

The F-test for the independent variables shows that the biomass concentration is the significant parameter influencing R_s followed by the sulfate and PHE concentrations (data not shown). Contour plots were prepared to observe the relationship between the variable levels and responses, as well as to determine the optimal level of each variable. The Fig. 1a exhibits the interactive effect of sulfate and PHE concentrations on R_s. As seen in this figure, there is a greater R_s at lower levels of sulfate concentration (<1000 mg L^{-1}) and at low–medium levels of PHE concentration (5–30 mg L^{-1}). The PHE concentration has both positive and negative effects on R_s depending on the concentration of PHE. For example, regardless of the sulfate concentration applied, it is observed that the R_s increases from 4.7 to 6.2 mg g^{-1} VSS d^{-1} as the PHE concentration increases from 2 to 26 mg L^{-1}, whereas it decreases to 2.9 mg g^{-1} VSS d^{-1} at 50 mg L^{-1} PHE. The decrease in R_s for the PHE concentration higher than 26 mg L^{-1} indicates possible inhibitory effects on the microbial activity. The toxicity of PAH to microorganisms has been demonstrated to cause a decrease in the biodegradation rate [17]. Furthermore, a lower R_s is observed when sulfate is supplied at higher levels than at lower levels. This observation can be explained by the inhibition of microorganism enzyme synthesis due to the high sulfate concentration [17]. The highest R_s of 6.8 mg g^{-1} VSS d^{-1} is predicted for the combination of a PHE concentration between 5 and 28 mg L^{-1} and a sulfate concentration between 480 and 1000 mg L^{-1}. The variation of R_s as a function of biomass and sulfate concentrations is illustrated in Fig. 1b. In the contour plot, it is observed that the R_s is affected more by the biomass concentration than the sulfate concentration. The effect of biomass concentration on R_s produces an expected result, i.e. the increase in biomass leads to an increase in the R_s. The highest R_s of 7.8 mg g^{-1} VSS d^{-1} is observed when the biomass and sulfate concentrations are found to be in the range of 45–50 mg L^{-1} and 480–1500 mg L^{-1}, respectively (Fig. 1b). Similar R_s is obtained when the biomass and PHE concentrations are in the range of 45–50 mg L^{-1} and 2–30 mg L^{-1}, respectively (Fig. 1c).

3.1.2. Optimization of PHE biodegradation

In order to determine the optimal conditions for PHE biodegradation, the DFM was applied and fifteen solutions were obtained (Table 2). The solution 7 has shown the highest predicted R_s of 9.0 mg g^{-1} VSS d^{-1}. The obtained natural values corresponding to the optimal conditions were as follows: 18.5 mg L^{-1} for PHE concentration, 841 mg L^{-1} for sulfate concentration and 50 mg L^{-1} for biomass concentration. Table 3 compares the obtained R_s values with the reported data [10,34,35]. It is noticed that the R_s values vary considerably across the literatures, which can be attributed to the differences in initial PHE concentrations, electron acceptors, and bacterial genus/species. The direct comparison of the R_s values in Table 3 is unsuitable to identify the best biodegradation efficiency between these studies. Roughly, the high R_s values in this study (2.4–7.6 mg g^{-1} VSS d^{-1}) demonstrate the finer potential of the enriched SRB culture used in this study.

3.2. Confirmation study

The verification of the predicted R_s from the model was assessed by conducting two confirmation experiments in duplicate from

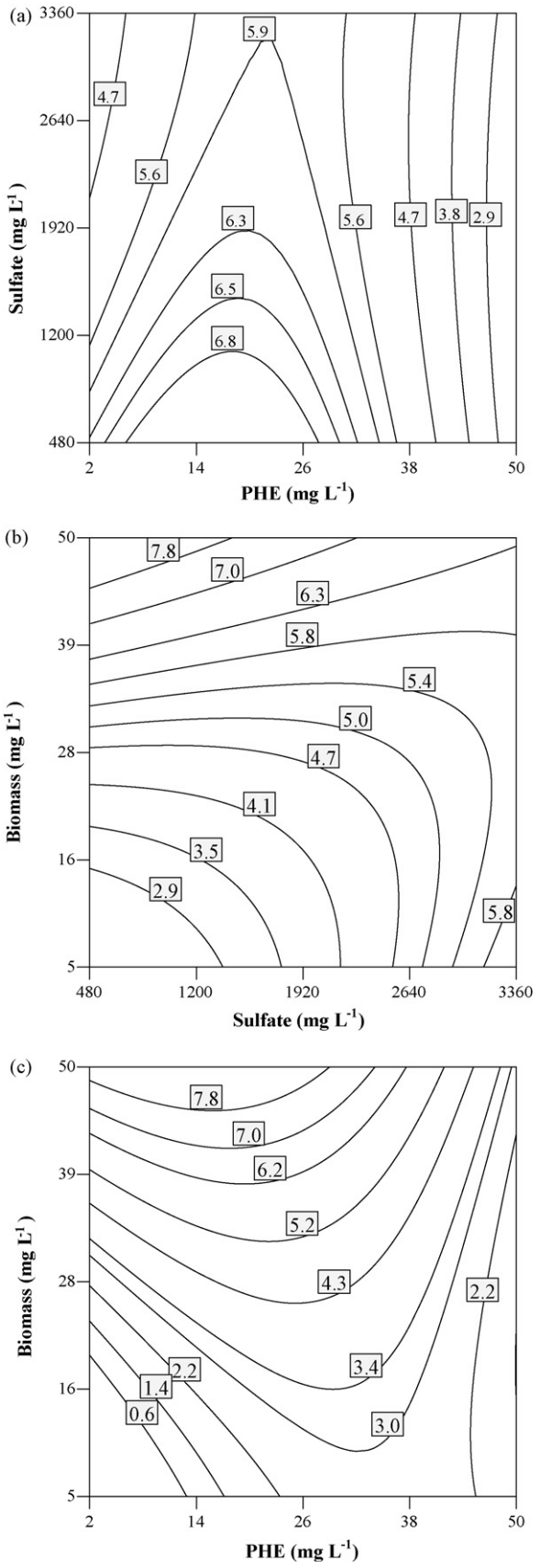

Fig. 1. Contour plots for R_s vs. sulfate and PHE concentrations at an initial biomass concentration = 41 mg L^{-1} (a), R_s vs. biomass and sulfate concentrations at an initial PHE concentration = 26 mg L^{-1} (b), and R_s vs. biomass and PHE concentrations at an initial sulfate concentration = 1064 mg L^{-1} (c).

Table 2
Optimal conditions for the anaerobic PHE biodegradation applying the desirability functions methodology.

Solution	PHE (mg L^{-1})	Sulfate (mg L^{-1})	Biomass (mg L^{-1})	R_s (mg g^{-1} VSS d^{-1})	Desirability
1	17.5	1293	49	8.4	1
2	26.3	694	49	8.4	1
3	19.1	1344	50	8.4	1
4	19.2	963	48	8.4	1
5	5.0	504	48	8.8	1
6	12.6	1167	49	8.5	1
7[a]	18.5	841	50	9.0	1
8	24.8	836	49	8.4	1
9	16.3	984	47	8.3	1
10	21.7	489	47	8.5	1
11	5.1	969	49	8.4	1
12	16.5	909	48	8.5	1
13	25.4	555	49	8.6	1
14	24.2	847	49	8.5	1
15	12.5	1440	50	8.3	1

[a] Optimal run.

solutions 7 (biotic run 1) and 13 (biotic run 2) along with abiotic and bacterial controls. Results of the confirmation experiments showed a similar R_s value as predicted by the model. The R_s observed in biotic run 1 (8.1 ± 0.1 mg g^{-1} VSS d^{-1}) was 9.9% less (average) compared to the R_s (9.0 mg g^{-1} VSS d^{-1}) predicted by the model. A similar decrease of 9.7% (average) was found in the biotic run 2 (R_s of 7.8 ± 0.1 mg g^{-1} VSS d^{-1}).

The pH (ranged from 7.1 to 7.3) and ORP (decreased quickly from −100 to −380 mV) variations in the biotic reactors indicated that systems were under the favorable growth conditions for SRB throughout the study [36]. Variations of PHE concentration, sulfate concentration and bacterial cell density over the incubation period are plotted in Fig. 2a–c. As seen in Fig. 2a, PHE concentrations in biotic runs 1 and 2 decreased considerably, while only slight PHE was lost in the abiotic control reactors. From Fig. 2b, the sulfate concentrations in the biotic reactors consumed without a lag time, whereas sulfate concentrations in the abiotic systems maintained at the same levels. In order to support the coupling of PHE oxidation with sulfate reduction, the theoretical stoichiometry was calculated assuming a complete oxidation of PHE to carbon dioxide. The oxidation half-reaction for PHE, written on one electron equivalent basis, is

$$\frac{1}{66}C_{14}H_{10} + \frac{28}{66}H_2O = \frac{14}{66}CO_2 + H^+ + e^- \quad (2)$$

The reduction half-reaction for sulfate, also written as one electron equivalent, is

$$\frac{1}{8}SO_4^{2-} + \frac{19}{16}H^+ + e^- = \frac{1}{16}H_2S + \frac{1}{16}HS^- + \frac{1}{2}H_2O \quad (3)$$

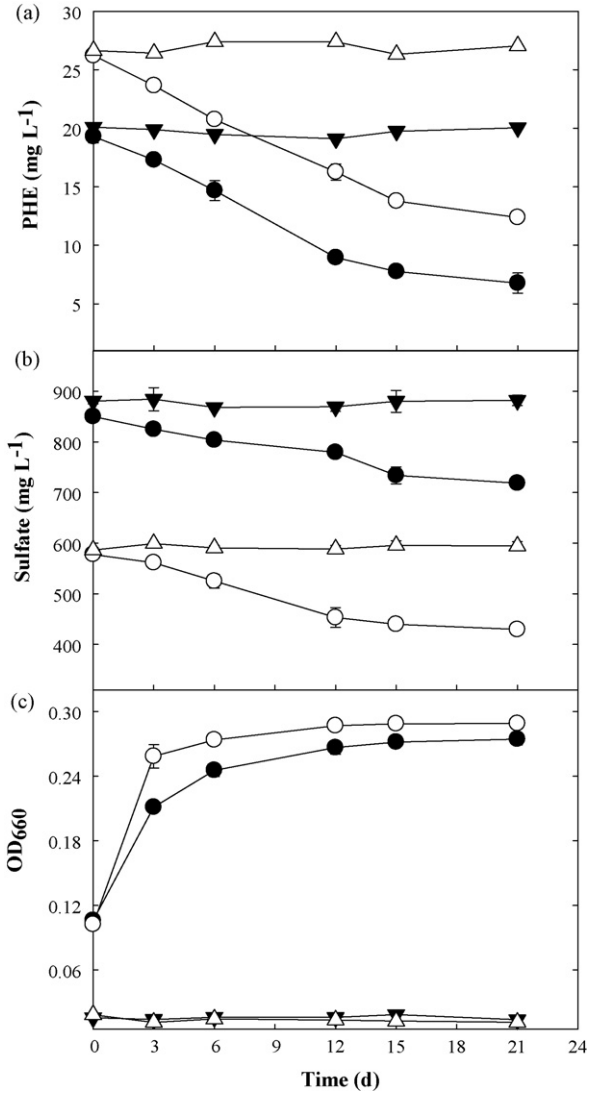

Fig. 2. Variations of PHE concentration (a), sulfate concentration (b) and bacterial cell density (c) during the biodegradation process. Symbols: biotic run 1 (PHE = 18.5 mg L^{-1}, sulfate = 841 mg L^{-1}, biomass = 50 mg L^{-1}), ●; biotic run 2 (PHE = 25.4 mg L^{-1}, sulfate = 555 mg L^{-1}, biomass = 49 mg L^{-1}), ○; abiotic control 1, ▼; abiotic control 2, △. The presented data of biotic runs are mean values of duplicate incubations.

Summing Eqs. (1) and (2) gives the overall balanced reaction, in which no free electrons are present:

$$\frac{1}{66}C_{14}H_{10} + \frac{1}{8}SO_4^{2-} + \frac{3}{16}H^+ = \frac{14}{66}CO_2 + \frac{1}{66}H_2S + \frac{1}{16}HS^- + \frac{5}{66}H_2O \quad (4)$$

Table 3
Comparison of anaerobic removal rates of PHE.

Reference	Electron acceptor	R_s (mg g^{-1} VSS d^{-1})	Initial PHE concentration (mg L^{-1})	Description
Rockne and Strand [10]	Sulfate	0.09–0.15[a]	0.8	Complete degradation within 140 d; creosote-contaminated sediments
Rockne and Strand [10]	Nitrate	0.90–1.30[a]	0.8	Complete degradation within 13 d; creosote-contaminated sediments
Rockne and Strand [34]	Nitrate	0.15[a]	0.18	Almost complete mineralization within 20 d; creosote-contaminated sediments
Chang et al. [35]	Nitrate	0.02–0.04[b]	2[c]	66–100% degradation within 56 d; petroleum-contaminated sediments
This study	Sulfate	2.36–7.57[a]	2–50	10–77% degradation within 21 d; sulfate-reducing enrichment culture

[a] Biodegradation of PHE by enrichment cultures in aqueous systems.
[b] Unit = mg kg^{-1} sediment d^{-1}.
[c] Unit = mg kg^{-1} sediment.

The stoichiometry of PHE degradation and sulfate reduction was determined from the ratio of the mass of the sulfate consumed to the mass of PHE degraded at the end of the experimental period. Using Eq. (4), the theoretical ratio of sulfate reduction to PHE removal was found to be 8.25. In biotic run 2, the theoretical sulfate reduction based on the mass of PHE degraded (13.8 mg L^{-1}) was calculated to be approximately 61.5 mg L^{-1}, whereas the actual sulfate reduction was 147.8 mg L^{-1}. This implies that sulfate might have utilized as a terminal electron acceptor in the removal of additional organic compounds present in the medium (i.e. yeast extract and DMF). On the other hand, 94.0 mg L^{-1} of sulfate reduction was observed in the bacterial control with addition of yeast extract and DMF indicating that a portion of bacterial growth was from the utilization of these organic compounds. The mechanism of co-metabolism is envisaged in this degradation process. This indicates that the difference in sulfate concentration between biotic (147.8 mg L^{-1}) and control (94.0 mg L^{-1}) systems is directly related to the PHE oxidation and the amount of sulfate reduction (53.8 mg L^{-1}) was nearly equivalent to the stoichiometric amount of PHE degraded with a ratio of 7.18. In addition, the bacterial cell densities increased significantly in both biotic experiments (Fig. 2c). As a whole, sulfate is utilized as the principal electron acceptor for the biodegradation of PHE and the PHE removal mainly results from the activity of SRB.

Moreover, the results of present study suggest that biodegradation is the dominant removal mechanism of PHE. In addition to this mechanism, the adsorption of PHE onto bacterial biomass (biosorption) should be considered as another significant mechanism for the removal of PHE from aqueous media since the PHE added in the experiments was higher than its water solubility (1.3 mg L^{-1}). In such case, the PHE would possibly precipitate out of solution or may be adsorbed onto the biomass due to its recalcitrant and hydrophobic characteristics. To quantify the adsorbed amount of PHE by SRB biomass, samples from the confirmation experiments were taken at the end of the experimental period to determine the PHE concentrations in both resident biomass and aqueous solution. Results showed that 26.6 ± 2.6% and 35.0 ± 0.1% of the total PHE were removed by biosorption in biotic runs 1 and 2, respectively, while low levels were detected in the aqueous solutions (10.4 ± 0.3% and 15.1 ± 3.3%, respectively). A higher biosorption percentage was observed in the biotic run with the higher initial PHE concentration. The increase in biosorption percentage with increases in initial PHE and resident biomass concentrations (Fig. 2c) may be due to the higher probability of contact between the substrate and biomass.

3.3. Metabolic pathway of PHE

In order to identify the metabolites directly related to PHE oxidation, controls containing SRB and no PHE (bacterial control) and controls of sterilized medium with PHE (abiotic control) were carried out [37]. The GC–MSD chromatograms of the biotic run, abiotic bacterial and controls were overlapped and compared. Peaks found only in the biotic run were targeted as the possible metabolites. The GC–MSD chromatograms of degradation products of PHE exhibited four major peaks at mass/charge ratio (m/z) 136, 117, 107 and 94, respectively. The full scan mass spectra are shown in Fig. 3. Peaks at m/z 107 (M−1) and 94 (M) (Fig. 3c and d) have been identified as *p*-cresol and phenol, respectively, in our previous study [22]. The presence of these two compounds in cell suspensions provides further evidence for their role as metabolites of PHE degradation by SRB. In the present study, two novel metabolites of PHE were identified. The first metabolite was identified as 2-methyl-5-hydroxybenzaldehyde or a close analogue (Fig. 3a) with a molecular ion at m/z 136 and fragment ions at m/z 121 (M−15), m/z 107 (M−29) and m/z 93 (M−43). The other metabolite was identified as 1-propenyl-benzene with an intensive

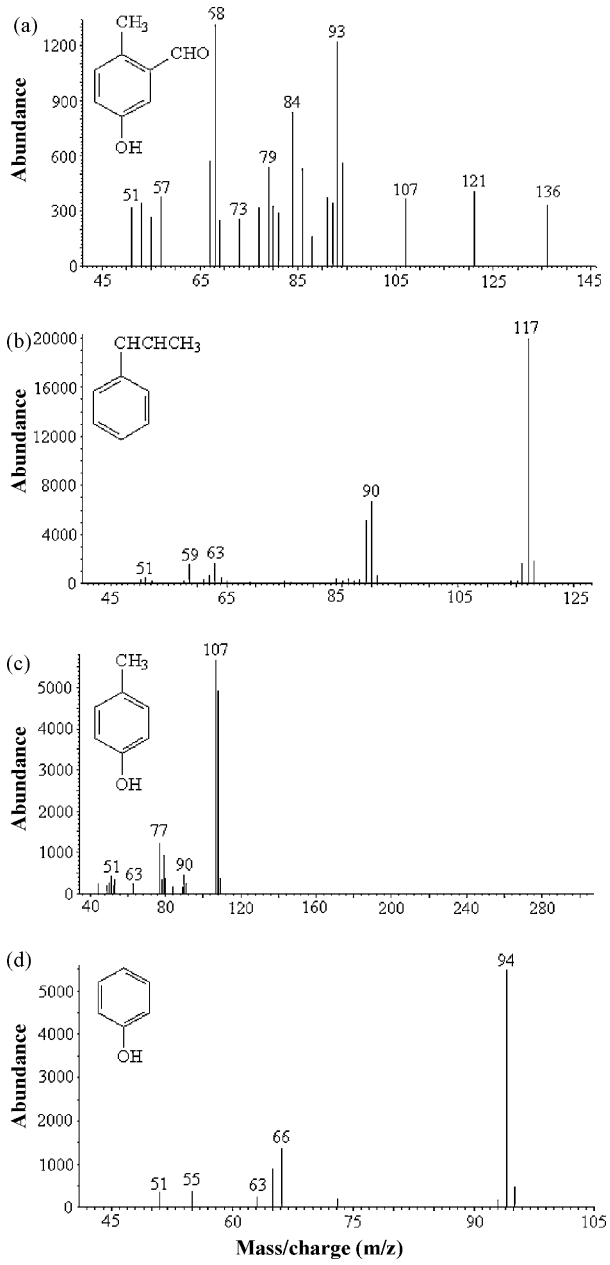

Fig. 3. Mass spectrum of metabolites detected in PHE biodegradation experiments conducted with SRB. (a) 2-methyl-5-hydroxybenzaldehyde, (b) 1-propenyl-benzene, (c) *p*-cresol and (d) phenol.

base peak at m/z 117 (M−1) and fragment ion at m/z 90 (M−28) (Fig. 3b). To our knowledge, this is the first report of accumulation of 2-methyl-5-hydroxybenzaldehyde and 1-propenyl-benzene as metabolites of PHE. Phenanthrenecarboxylic acid, the only published metabolite of PHE under the sulfate-reducing conditions, however, was not detected in this study. The formation of 2-methyl-5-hydroxybenzaldehyde and 1-propenyl-benzene can be explained by the opening and partial removal of the aromatic rings of PHE through a sequence of hydration and hydrolysis reactions. In addition, Fig. 4 shows the changes in GC–MSD peak areas of the detected intermediates during the degradation. The amounts of phenol and *p*-cresol increased gradually, reached the maximum levels at day 3, and then decreased slowly until day 21. After 3 d of incubation, 1-propenyl-benzene was identified as the major metabolite (peak area of 1×10^6), and then decreased to undetectable level at the end of day 6. On the other hand, 2-methyl-5-hydroxybenzaldehyde

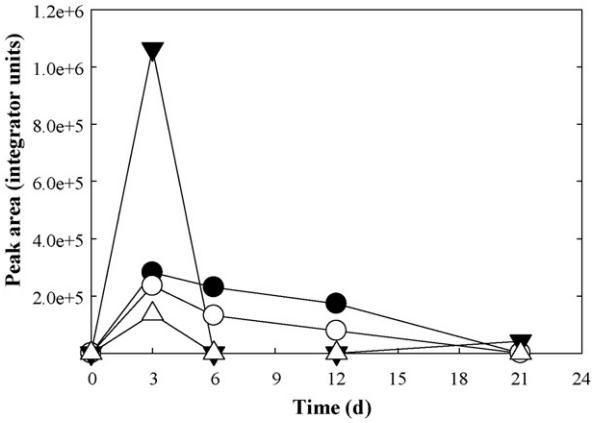

Fig. 4. Changes of GC–MS peak areas of PHE metabolites during the biodegradation process. Symbols: phenol, ●; *p*-cresol, ○; 1-propenyl-benzene, ▼; 2-methyl-5-hydroxybenzaldehyde, △.

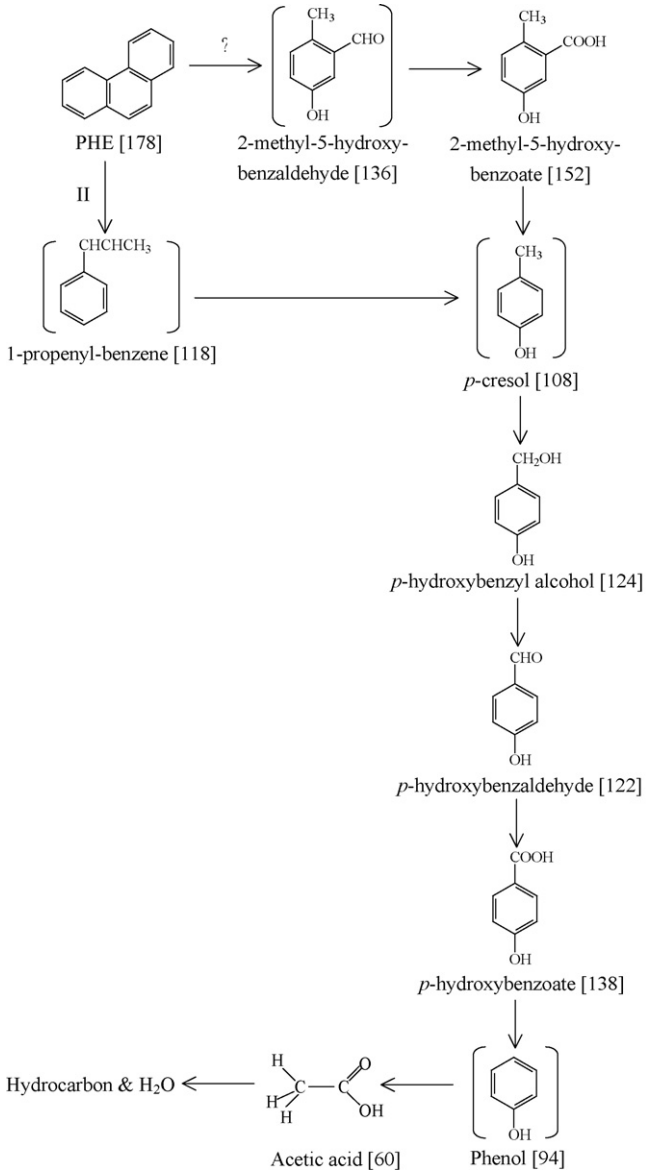

Fig. 5. Proposed anaerobic metabolic pathways of PHE by SRB. Compounds mentioned in brackets were identified in the present study.

was detected within 3 d of incubation, accumulated transiently and then decreased to undetectable level at day 6. These results suggest that 1-propenyl-benzene and 2-methyl-5-hydroxybenzaldehyde are unstable intermediates of PHE, which can be transformed rapidly to other compounds.

The metabolic pathways of PHE by SRB were proposed based on the identification of metabolites detected during biotransformation of PHE (Fig. 5). One possible route for PHE biotransformation by SRB was confirmed by the presence of 2-methyl-5-hydroxybenzaldehyde ("I" in Fig. 5). PHE can be converted to 2-methyl-5-hydroxy-benzaldehyde and 2-methyl-5-hydroxybenzoate, and further to form *p*-cresol by hydrolysis followed by decarboxylation. An alternative pathway ("II" in Fig. 5) suggests that PHE can be biotransformed to 1-propenyl-benzene, and further to *p*-cresol via hydrolysis. *p*-Cresol can be sequentially metabolized through *p*-hydroxybenzoate to phenol by consecutive methyl group oxidation steps followed by decarboxylation. The enzymes for these metabolic pathways involve *p*-cresol methylhydroxylase, benzyl alcohol dehydrogenases, aldehyde dehydrogenases and hydroxybenzoate decarboxylase [38,39]. The proposed metabolic pathways from *p*-cresol to phenol and metabolic mechanisms were also supported by previous reports in anaerobic degradation of cresol with sulfate-reducing enrichment cultures [39,40]. In summary, the present study indicated that there were two pathways for the biotransformation of PHE to phenol using SRB. Hydration, hydrolysis and decarboxylation were proposed as metabolic mechanisms that occur during the biotransformation process. However, the identified metabolites were not quantified in the present study.

In another separate study, biodegradation of putative intermediates of PHE, i.e. phenol and *p*-cresol, by SRB was examined individually at an initial concentration of 5 mg L^{-1} (data not shown). Results showed that approximately 67% (excluding abiotic loss) of *p*-cresol was biodegraded after 21 d of incubation. On the other hand, only 3% of phenol was degraded as compared to the abiotic control. Phenol was detected during the time course of *p*-cresol biodegradation experiments with SRB. This observation strongly evidences the biotransformation of *p*-cresol into phenol by the SRB. Further studies are required to investigate the fate and extent of the putative intermediates formed in the biodegradation of PHE by SRB.

4. Conclusions

This study specifically examined the effects of PHE, sulfate and biomass concentrations on anaerobic degradation of PHE by SRB and identified the metabolic intermediates of PHE. Experimental results demonstrate that the PHE degradation rate was directly related to these factors. The optimal conditions for biodegrading PHE are 18.5 mg L^{-1} for the PHE concentration, 841 mg L^{-1} for the sulfate concentration and 50 mg L^{-1} for the biomass concentration. Results obtained provide a basis for further study and may be useful in selecting appropriate design and operating parameters for in situ anaerobic bioremediation of PAH-contaminated systems. In this study, four PHE metabolites were detected and the changes of their peak areas with time were recorded. The identified metabolites provide new information to better elucidate the metabolic mechanisms and pathways of PHE. This finding also clarifies the fate of PHE under sulfate-reducing conditions.

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