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Versatility of fluorene metabolite (phenol) in fluorene biodegradation by a sulfate reducing culture

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A B S T R A C T

Biodegradability of fluorene and the versatility of fluorene metabolite (i.e. phenol) in fluorene biodegradation by a sulfate-reducing enrichment culture were investigated. Batch experiments (with 5 mg l^{-1} fluorene) were designed via the central composite design to examine the effects of sulfate (5–35 mM) and biomass (5–50 mg l^{-1}) concentrations (variables) on fluorene degradation (response). The experimental results revealed that fluorene removal was more influenced by the biomass concentration than the sulfate concentration. The optimal sulfate and biomass concentrations for fluorene biodegradation (90% removal) were found to be 14.4 mM and 37.8 mg l^{-1}, respectively. Under the optimal conditions, a set of biodegradation experiments were repeated to evaluate both the biodegradability of fluorene metabolite and the potential effect of phenol accumulation on fluorene degradation. The outcomes indicated a slow phenol degradation rate, i.e. 0.02 mg l^{-1} d^{-1}. Moreover, a small reduction in the fluorene biodegradation efficiency was observed in the presence and accumulation of phenol. However, this sulfate reducing culture is a valuable resource for the simultaneous degradation of fluorene and phenol.

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1. Introduction

In the past two decades, biodegradation of polycyclic aromatic hydrocarbons (PAHs) under sulfate-reducing conditions or by using sulfate-reducing bacteria (SRB) has been investigated in detail (Coates et al. 1996; Bedessem et al. 1997; Rockne and Strand 1998; Hayes et al. 1999; Meckenstock et al. 2000; Chang et al. 2002; Lei et al. 2005; Kumar et al. 2009). Numerous environmental factors including pH, temperature, availability and nature of nutrients, the concentrations of electron donors used for microbial degradation and the microbial concentration have been found to influence the biodegradation of PAHs (Bamforth and Singleton 2005; Mohan et al. 2006). Among these, the sulfate concentration is a significant factor in the biodegradation of PAHs under sulfate-reducing conditions. Borden et al. (1995) found that the rate and extent of intrinsic bioremediation were limited by the concentration of electron acceptor. Yuan et al. (2001) reported that the biodegradation of phenanthrene was significantly influenced by the addition of sulfate. Biodegradation of phenanthrene under high sulfate concentration can inhibit the microbial enzyme synthesis and subsequently reduce the biodegradation rate of phenanthrene. On the other hand, the presence of active microorganisms capable of degrading PAHs plays a key factor for efficient and rapid biodegradation of PAHs. To achieve the best biodegradation efficiency, the microorganisms should be present in sufficient numbers and should have the capability to produce the necessary enzymes for the degradation process. It is widely accepted that the rate and extent of PAH biodegradation are related to the biomass concentration. The inoculation of sufficient contaminant-degrading bacteria may speed up the degradation process (Rothermich et al. 2002). Therefore, it is essential to study the effect of biomass concentration on PAH biodegradation.

The central composite design (CCD) and response surface methodology (RSM) have been widely used for the design and investigation of complex experiments (Hagenimana et al. 2006; Wang et al. 2008). The main advantage of this experimental strategy is the reduction of experimental trials required to obtain a statistically acceptable result. In addition to RSM, the numerical optimization method, i.e. desirability functions methodology (DFM), can be applied to optimize the experimental factors for a desired response (Corzo et al. 2008). This approach transforms an estimated response into an individual desirability that varies from 0 to 1. The desirability increases as the corresponding response is more desirable, and the overall desirability is equal to the geometric average of all individual desirability.

In addition, most of the PAH biodegradation studies in the past have focused on the removal of parent compound; while relatively

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little attention has been paid to the potential formation of metabolites from the partial transformation of the parent compound. In our previous study, we pointed out that fluorene is metabolized to phenol by a SRB enrichment culture (Tsai et al. 2009). The metabolite can also serve as an electron donor or a competitor in the biodegradation process, which may result an increase or decrease in the PAH degradation rate. Therefore, it is necessary to evaluate the fate and extent of the metabolite during the biodegradation of parent compound by the SRB enrichment culture.

The objective of the present study was to apply the RSM and DMF for the determination of optimal concentrations of sulfate and biomass for fluorene biodegradation. Also, the study was extended to investigate the potential biodegradation of the formed metabolite in the presence of fluorene by the SRB.

2. Material and methods

2.1. Chemicals and reagents

Analytical grade fluorene (99.0% purity) was purchased from Fluka Chemical (Neu-Ulm, Germany). Both dichloromethane (DCM) and *N*,*N*-dimethylformamide (DMF) were supplied by Sigma–Aldrich Chemical (St. Louis, MO, USA) with 99.9% and 100% purities, respectively. Fluorene stock solution (0.5%) was prepared in DMF and stored in dark at 4 °C. Other chemicals and reagents used for medium preparation and chemical analysis were of HPLC grade.

2.2. Biomass

A previously reported sulfate-reducing bacterial culture for the degradation of fluorene and phenanthrene (Tsai et al. 2009) was utilized for the present study. The presence of SRB in the enriched culture was about 85% of the total cells. The exact makeup of the bacterial consortium is not identified; however, *Desulfosarcina*, *Desulfococcus* and *Desulforhopalus* spp, *Desulfobacteriaceae* and *Desulfovibrionaceae* were identified using the fluorescence *in-situ* hybridization (FISH) with group and genus specific 16S rRNA-targeted oligonucleotide probes as the dominant species of the enriched SRB. The bacterial culture was utilized as such without any isolation/purification.

2.3. Biodegradation of fluorene

A series of batch experiments were designed using the CCD to examine the effects of initial sulfate and biomass concentrations on fluorene biodegradation. The statistical software, i.e. MINITAB 14 (Minitab Inc., USA), was used to design the experimental conditions. The experimental design consisted of eleven biotic runs, a molybdate-inhibition control and an abiotic control (i.e. cell-free reactor) (Table 1). The experiments were carried out in 1.2 liter bioreactors with a working volume of 1 liter at a fluorene concentration of 5 mg l^{-1}. The concentrated SRB suspension was inoculated into the bioreactors containing sterile medium (Tsai et al. 2009) with various sulfate concentrations (Table 1). The incubation mixture was then flushed with filter-sterilized nitrogen gas for 10 min at a flow rate of 10 l min^{-1} to remove the residual dissolved oxygen in the medium. Subsequently, the pH of the medium was adjusted to 7.2 ± 0.2 and placed in a temperature-control chamber at 30 °C for 21 d without shaking. At regular time intervals, samples were collected from the bioreactors for measuring the pH, ORP, bacterial cell density and concentrations of sulfate and fluorene.

The experimental outcome of the fluorene biodegradation study was modeled using the RSM. Subsequently, the DFM was used to find out the optimal conditions for fluorene degradation. In order to verify the modeling efficiency and the accuracy of DFM in the identification of optimal fluorene biodegradation conditions, laboratory experiment was conducted under the optimal biodegradation conditions established using DFM.

2.4. Biodegradation of fluorene in the presence of phenol

Batch experiments were designed to examine the ability of the sulfate-reducing bacterial culture in the degradation of putative fluorene metabolite (i.e. phenol) and the simultaneous degradation of fluorene and phenol. The sulfate and biomass concentrations for both the experiments were selected based on the maximum fluorene removal percentage observed in the optimization study.

A stock solution of phenol was prepared in deionized water at 5 g l^{-1} and stored in an air-tight brown glass bottle to avoid photo-oxidation. Subsequently, phenol biodegradation experiment was conducted following the procedures as stated above at 1 mg l^{-1} of phenol (Tsai et al. 2009). The fluorene biodegradation study in the presence of phenol was conducted at 5 mg l^{-1} of fluorene and 1 mg l^{-1} of phenol. Both the experiments were conducted for 21 d in duplicate. At different time intervals, samples were withdrawn and analyzed for phenol and fluorene concentrations. The difference between the first-order degradation rate constants of the two experiments (i.e. the experiment containing fluorene and phenol simultaneously, and the one containing fluorene only) was used to analyze the effect of phenol on fluorene biodegradation.

Table 1
The experimental conditions and results of CCD.

Run	Fluorene concentration (mg/L)	Sulfate concentration (mM)	Biomass concentration (mg/L)	Fluorene removal (%)	
				Experimental	Predicted
1	5	5	27.5	85.9	83.3
2	5	9.4	11.6	69.6	69.8
3	5	9.4	43.4	84.5	90.1
4	5	20	5	57.2	60.9
5[a]	5	20	27.5	85.6	87.5
6[a]	5	20	27.5	85.2	87.5
7[a]	5	20	27.5	84.3	87.5
8	5	20	50	89.2	89.7
9	5	30.6	11.6	70.0	65.5
10	5	30.6	43.4	79.6	85.8
11	5	35	27.5	69.8	77.2
Inhibition	5	20	27.5	27.4	87.5
Abiotic	5	20	0	2.6	–

[a] Center point.

2.5. Analytical methodology

The pH, ORP, bacterial cell density and concentration of sulfate were performed as per the procedures reported by Tsai et al. (2009). Fluorene concentration was measured using the GC-FID equipped with a HP-5 fused capillary column (30 m length, 0.53 mm inner diameter and 1.5 μm film thickness). The flow rates of hydrogen gas and air were maintained at 40 and 400 mL/min, respectively. A high purity of nitrogen gas was used as the carrier and make-up gases supplied at the rates of 3 and 22 mL/min, respectively. The oven was maintained initially at 120 °C for 1 min, and then increased at 20 °C/min to 280 °C; finally held for 5 min, which corresponds to a total runtime of 14 min. The temperatures of injector and detector were maintained at 280 °C. Sample injection was carried out under splitless mode. Exactly, 1 μL of the DCM extract was injected into the GC-FID system using a 5 μL syringe. Under these conditions, fluorene peak was observed at 4.8 min.

On the other hand, the phenol concentration was quantified by the high performance liquid chromatography (HPLC). Prior to the analysis, the particulates in the aqueous samples were removed by centrifugation at 10,000 rpm for 3 min. The cell-free supernatants were analyzed in the HPLC fitted 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}. The phenol concentration was quantified from the measurement of UV absorbance at 254 nm using a single-wavelength UV-VIS detector (Hitachi L-2420, Japan) (Spence et al. 2001; Aleksieva et al. 2002). Under these conditions, phenol was detected after a retention time of 5.8 min. The recovery efficiency and method detection limit of phenol were 103.9 ± 7.0% and 36.8 μg l^{-1}, respectively.

3. Results and discussion

3.1. Optimization of fluorene biodegradation

3.1.1. Results of CCD experiments

Table 1 shows the outcomes of CCD experiments. A maximum fluorene removal of 89% was observed under the experimental conditions of run 8 (sulfate ~ 20 mM; biomass ~ 50 mg l^{-1}). A minimum fluorene removal of 57% was observed in run 4 with the lowest biomass concentration, i.e. 5 mg l^{-1}. On the other hand, no appreciable loss of fluorene was observed in the inhibition and abiotic controls. Comparison of runs 4 and 8 demonstrates that fluorene removal can be increased with the increase in biomass concentration. The statistical software, MINITAB 14 (Minitab Inc., USA) was employed to perform the experimental design, regression analysis and analysis of variance (ANOVA), as well as to generate the response surfaces and contour plots. To interpret the relationship between the independent variables (i.e. sulfate and biomass concentrations) and their response (i.e. fluorene biodegradation), regression analysis was performed based on the experimental data and was fitted into an empirical second-order polynomial model as shown in Eq. (1).

$$R = 43.074 + 1.078A + 1.957B - 0.032A^2 - 0.024B^2 - 0.005AB \quad (1)$$

where R is fluorene removal (%), A is sulfate concentration (mM) and B is biomass concentration (mg l^{-1}). The Eq. (1) can be useful to predict the fluorene removal for a biodegradation study operated under the designed ranges. To evaluate the suitability and the statistical significance of the model, an ANOVA test was performed with an F-test (95% probability). The result of ANOVA test is shown in Table 2. The sulfate concentration, biomass concentration and the interaction between these two factors are significant items in the model (Table 2). The high correlation coefficient ($R^2 = 0.93$), low PRESS (<1000) and high adequacy precision (>4) values of the developed model demonstrate its adequacy in the prediction of fluorene removal.

Table 2
ANOVA for the predicted model.

Item	Coefficient	SS[d]	F value	p-Value[e]
Constant	43.074			
A[a]	1.078	108.03	6.87	0.047
B[b]	1.957	644.16	40.97	0.001
A^2	−0.032	74.39	4.73	0.697
B^2	−0.024	200.87	12.78	0.082
AB	−0.005	2.68	0.17	0.016
Fitted R^2	0.93			
PRESS[c]	554.51			
Adequacy precision	9.73			

[a] Sulfate concentration.
[b] Biomass concentration.
[c] PRESS: predication error sum of squares.
[d] SS: sum of squares.
[e] p-Value less than 0.05 indicates that the model terms are significant.

A three-dimensional contour plot was prepared to observe the relationship between the factors (sulfate and biomass concentrations) and the response (fluorene removal). Fig. 1 shows that fluorene removal is more influenced by the biomass concentration compared with the sulfate concentration. The increase in biomass concentration shows an increase in the fluorene removal. The reason for higher fluorene removal could be due to the enhanced microbial concentration, growth rate and activity at the early stage of the biodegradation process (Rothermich et al. 2002). Furthermore, lower fluorene removal is observed under high sulfate concentrations than at low sulfate concentrations. This could be attributed to the inhibition of microbial enzyme synthesis under high sulfate concentrations (Yuan et al. 2001). The fluorene removal is maximum (>88%) when the sulfate and biomass concentrations are in the ranges of 10–15 mM and 35–45 mg l^{-1}, respectively.

Table 3 lists the predicted fluorene removals using the DFM under various sulfate and biomass concentrations. Among the solutions, the maximum fluorene removal is observed in solution 8 corresponding to the biomass and sulfate concentrations of 14.4 mM and 37.8 mg l^{-1}, respectively. Therefore, the confirmation

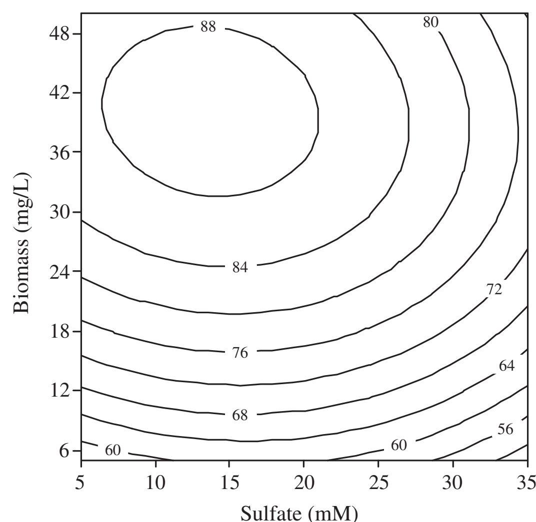

Fig. 1. Contour plots showing the removal percentage of fluorene vs. sulfate and biomass concentrations.

Table 3
The optimal fluorene biodegradation conditions estimated by the desirability functions methodology.

Solution	Sulfate (mM)	Biomass (mg/L)	Removal (%)	Desirability
1	13.2	34.8	89	1
2	16.8	39.8	89	1
3	10.6	40.4	89	1
4	13.2	34.8	89	1
5	16.8	39.8	89	1
6	16.1	41.9	89	1
7	9.9	40.8	89	1
8[a]	14.4	37.8	90	1
9	19.1	36.7	89	1
10	12.9	29.9	87	1
11	10.6	40.4	89	1
12	20.2	32.6	87	1

[a] Optimal run.

experiment was conducted under these sulfate and biomass concentrations.

3.1.2. Results of confirmation experiment

The confirmation experiment exhibited an identical fluorene removal as observed in solution 8. The profiles of fluorene degradation, sulfate concentration and bacterial cell density during the degradation process are shown in Fig. 2a–c, respectively. It can be seen in Fig. 2a that the fluorene concentration decreases from 5.6 to 0.6 mg l^{-1} in the biotic run, whereas no decline in the fluorene concentration is observed in the abiotic control. On the other hand, the sulfate concentration reduces from 14.8 to 11.7 mM in the biotic run without a lag time (Fig. 2b). A significant increase in the bacterial cell density is also observed in the biotic run experiment (Fig. 2c), which is proportional to the decreases of fluorene and sulfate concentrations. These observations reveal that fluorene biodegradation is coupled to sulfate reduction, and the fluorene depletion in the biotic runs is due to the activity of SRB. In addition, no significant pH variation was observed. The ORP of the biotic reactor decreased quickly from −60 to −300 mV within 3 d of incubation, which indicates that the system was maintained under the favorable growth conditions of SRB (Postgate 1984).

3.2. Biodegradation of phenol by the SRB enrichment culture

In our earlier study, we identified that fluorene was mineralized via a sequence of hydration and hydrolysis reactions followed by decarboxylation with the formation of trace amount of phenol (Tsai et al. 2009). In this study, phenol degrading ability of the SRB enrichment culture and its effect on fluorene biodegradation were evaluated. Fig. 3 shows the profile of phenol degradation over the incubation period. The degradation rate of phenol in the biotic run is found to be 0.02 mg l^{-1} d^{-1}. On the other hand, no significant loss of phenol is observed in the corresponding abiotic control. The degradation of phenol by the enrichment culture could be envisaged due to the presence and activity of phenol-degrading anaerobic bacteria in the enrichment culture. However, the degradation rate of phenol in the present study is very less compared with the other phenol degradation studies conducted under similar conditions. Wang et al. (1988) reported rapid phenol biodegradation rates, i.e. 12–168 mg l^{-1} d^{-1}, while using a phenol-enriched methanogenic culture. Moreover, a rapid and complete degradation of phenol (0.18–1.5 mg l^{-1} d^{-1}) was observed while using nitrate-enriched cultures (Broholm and Arvin 2000). Interestingly, phenol was completely biodegraded by a sulfate-reducing enrichment culture (26.7 mg l^{-1} d^{-1}) within 48 h in the study conducted by Boopathy (1995). However, only less phenol degradation (C/C_0 of phenol around 0.75 at the end of 21 days in biotic experiments) was observed in the present study (Fig. 3), which is likely due to the low population of phenol-degrading bacteria in the enrichment culture used for the experiments.

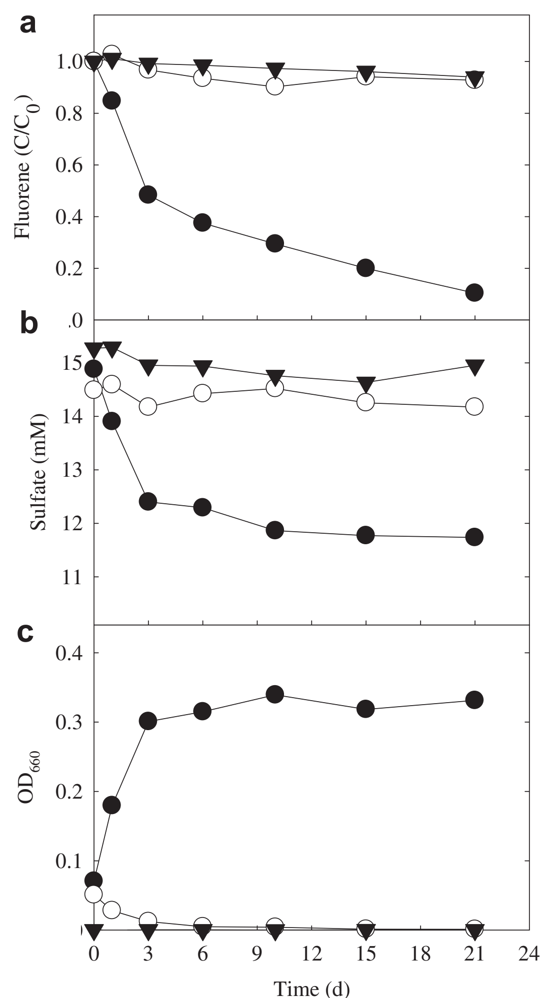

Fig. 2. Variations of fluorene (C/C_0) (a), sulfate concentration (b) and bacterial cell density (c) during the confirmation experiments. Symbols: biotic, ●; inhibition-control, ○; abiotic control, ▼. 0.1 OD_{660} = 50.1 mg/L biomass (data based on the mean values of duplicate incubations).

In a separate experiment, the versatility of phenol on fluorene biodegradation was investigated. A gradual decrease in fluorene concentration with proportionate increase in phenol concentration

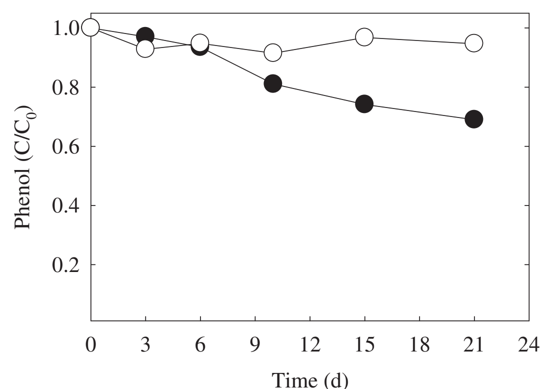

Fig. 3. Biodegradation of phenol by the SRB enrichment culture. Symbols: biotic, ●; abiotic control, ○ (data based on the mean values of duplicate incubations).

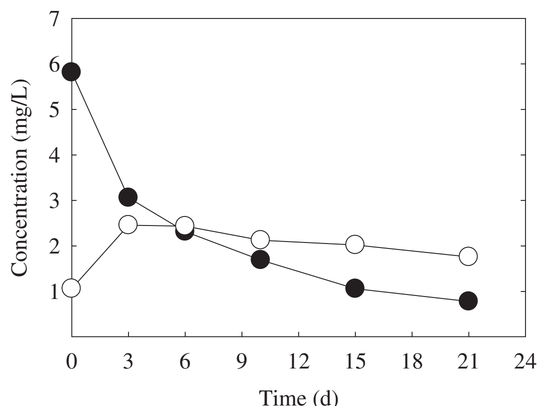

Fig. 4. Biodegradation of fluorene in the presence of phenol. Symbols: fluorene, ●; phenol, ○ (data based on the mean values of duplicate incubations).

was observed in the experiment (Fig. 4). The phenol degradation rate is found to be 0.04 mg l^{-1} d^{-1} (data from d 3 to d 21). This result strongly evidences the biotransformation of fluorene into phenol by the SRB. On the other hand, the first-order degradation rate constant without the addition of phenol was 0.1 d^{-1}. A lower first-order degradation rate constant for fluorene degradation (0.09 d^{-1}) was observed in the presence of phenol at 1 mg l^{-1}. The decrease in fluorene degradation rate could be due to the phenol-inhibition. A similar inhibition of PHE degradation in the presence of *cis*-4,5-dihydro-4,5dihydroxypyrene (a metabolite of pyrene) was also reported by Kazunga and Aitken (2000). However, the influence of phenol-inhibition is not apparent in this study.

4. Conclusions

The optimal sulfate and biomass concentrations for fluorene removal within the designed experimental ranges were found to be 14.4 mM and 37.8 mg l^{-1}, respectively. The first-order degradation rate constant for fluorene biodegradation was estimated as 0.1 d^{-1}. A decrease in fluorene biodegradation rate (0.09 d^{-1}) was observed in the presence of its metabolite, i.e. phenol. The biotic run conducted with phenol alone has resulted in a phenol degradation rate of 0.02 mg l^{-1} d^{-1}. The data acquired from the metabolite biodegradation experiments can complement and augment the present knowledge on the effect of metabolite accumulation on PAH biodegradation.

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