Anaerobic biotransformation of fluorene and phenanthrene by sulfate-reducing bacteria and identification of biotransformation pathway

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ABSTRACT

In the present study, anaerobic biotransformation of fluorene and phenanthrene by sulfate-reducing bacteria (SRB) was investigated and biotransformation pathways were proposed. SRB was enriched from anaerobic swine wastewater sludge and its abundance was determined by the fluorescence in situ hybridization (FISH) technique. Batch anaerobic biotransformation studies were conducted with fluorene ($5 \, mg \, L^{-1}$), phenanthrene ($5 \, mg \, L^{-1}$) and a mixture of the two ($10 \, mg \, L^{-1}$). After 21 d of incubation, 88% of fluorene and 65% of phenanthrene were biotransformed by SRB. In contrast to previous studies, a decrease in biotransformation efficiency was observed in the presence of both fluorene and phenanthrene. Throughout the study, sulfate reduction was coupled with biotransformation of fluorene and phenanthrene. However, no increase in bacterial cell density was observed in the presence of an inhibitor, i.e. molybdate. Identification of metabolites by gas chromatography–mass spectrometry (GC–MS) revealed that fluorene and phenanthrene were biotransformed through a sequence of hydration and hydrolysis reactions followed by decarboxylation with the formation of p-cresol (only in the phenanthrene system) and phenol. The metabolites identified suggest novel biotransformation pathways of fluorene and phenenthrene.

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a recalcitrant group of contaminants and are composed of two or more fused benzene rings. They are mainly derived from coal processing, and incomplete combustion of petroleum fuels [1–3]. Because of their toxicity, persistence in the environment, low volatility, resistance to microbial degradation, and potential carcinogenic effects, PAHs pose an environmental threat to humans and animals [4].

Generally, high molecular weight PAHs (four or more rings), such as pyrene, benzo[b]fluoranthene and benzo[g,h,i]perylene, are strongly sorbed to particles, and thus greatly reduce their adverse effects to the environment. However, low molecular weight PAHs (two or three rings), such as naphthalene, fluorene and phenanthrene are water soluble and may be transported with groundwater or surface water resulting in a significant threat to the environment and humans [5]. Thus, the treatment of low molecular weight PAHs is more important. Among the low molecular weight USEPA pri-ority PAHs, fluorene ($C_{13}H_{10}$) and phenanthrene ($C_{14}H_{10}$) are most commonly found at contaminated sites [6–8].

Evidence has been presented for anaerobic degradation of fluorene and phenanthrene under nitrate-reducing [9], iron-reducing [10], sulfate-reducing [11–13] and methanogenic conditions [13,14]. Among these, the highest reported degradation rates are under sulfate-reducing conditions [13,15,16]. In past studies, researchers have controlled the sulfate-reducing conditions for PAHs biotransformation by adding sodium sulfate as the electron acceptor to activate the sulfate-reducing bacteria (SRB) but not to inoculate the SRB enrichment culture. There have been only a few reports investigating PAH biotransformation with sulfate-reducing enrichment culture [17–19]. In theory, by increasing the density of microbial populations and acclimation of microorganisms, the extent and rate of PAH biotransformation can be promoted. It is also generally accepted that the biotransformation is linked with the communities of microorganisms present during the process. Fluorescence in situ hybridization (FISH) detects acid sequences by a fluorescently labeled probe that hybridizes specifically to its complementary target sequence within the intact cell. It provides information about the presence, number and morphology of microorganisms, and has been widely applied to analyze microbial communities of SRB [20–22]. In addition, it has been reported that biotransformation of fluorene can be enhanced with

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the simultaneous presence of phenanthrene via a co-metabolic process [23]. Pathways for the microbial degradation of PAH compounds have been well studied under aerobic conditions. However, the anaerobic degradation pathway has only been investigated in detail for naphthalene under sulfate-reducing conditions. The proposed reaction mechanisms for naphthalene are hydroxylation [17], hydrogenation [24] and carboxylation [11,18]. Nevertheless, no detailed description has been proposed for the associated degradation pathways of fluorene and phenanthrene under sulfate-reducing conditions. Hence, more work is required to elucidate the degradation pathways of fluorene and phenanthrene under sulfate-reducing conditions. This study focused on the enrichment of SRB for the biotransformation of fluorene and phenanthrene. Subsequently, FISH technique was applied to confirm the abundance of SRB in the enriched microbial community. In addition, this study focused to examine the biodegradability of fluorene and phenanthrene (single and a mixture of the two) and to identify the biotransformation pathways of fluorene and phenanthrene.

2. Materials and methods

2.1. Chemicals

Fluorene (purity >99%) and phenanthrene (purity >96%) were purchased from Fluka Chemical (Neu-Ulm, Germany). Dichloromethane (DCM) and *N,N*-dimethylformamide (DMF) were purchased from Sigma–Aldrich Corporation (St. Louis, MO) with 99.9% and 100% purity, respectively. The stock solutions of both fluorene and phenanthrene were prepared in DMF with a concentration of $5000\,mg\,L^{-1}$. The other reagents used for the chemical analysis were of HPLC grade. All glassware were cleaned with distilled water and dried at $110\,^{\circ}C$ before each experiment.

2.2. Enrichment of sulfate-reducing bacteria

2.2.1. Bacterial source and composition of medium

A mesophilic sulfate-reducing bacterial culture growing in the presence of lactate (carbon source) for nearly 4 years (initially enriched from anaerobic swine wastewater sludge) was used as the source of inoculum for the enrichment process. A modified Postgate's C medium [25] was used for the growth and maintenance of SRB cultures. The medium contained the following constituents in deionized water: $2.52\,g\,L^{-1}$ $NaHCO_3$, $0.5\,g\,L^{-1}$ KH_2PO_4, $1\,g\,L^{-1}$ NH_4Cl, $0.06\,g\,L^{-1}$ $MgCl_2\cdot6H_2O$, $0.06\,g\,L^{-1}$ $CaCl_2\cdot6H_2O$, $2.84\,g\,L^{-1}$ Na_2SO_4, $0.02\,g\,L^{-1}$ $FeSO_4\cdot7H_2O$, and $0.5\,g\,L^{-1}$ yeast extract.

2.2.2. Bacterial enrichment

A specially designed 1200 mL serum bottle (Schott, Germany) fitted with a pH probe (Suntex, Taiwan) and an oxidation–reduction potential (ORP) probe (Orion, Japan) was used as an incubation reactor. Exactly 100 mL of mesophilic sulfate-reducing bacterial culture was added into the incubation reactor containing 900 mL of medium (final medium pH 7.2 ± 0.2; sterilized by autoclave (Eastern Medical, EA-635, Germany) at $121\,^{\circ}C$ for 30 min and cooled to room temperature). The mixture was then purged with filter-sterilized high purity nitrogen for 10 min. Fluorene and phenanthrene were added into the incubation reactor to achieve a final concentration of $10\,mg\,L^{-1}$ (each compound). Immediately after the addition of PAH compounds, the reactor was sealed with a Teflon-lined cap and the headspace of the incubator was replaced with pure nitrogen. The reactor was incubated in a temperature-controlled chamber (Advantec C1-612, Taiwan) at $30\,^{\circ}C$ (in dark) for 21 d. The enriched SRB cultures obtained at the end of 21 d was transferred into fresh Postgate's C medium for subsequent

sub-cultivation (10%, v/v). The pH and ORP were monitored continuously over the incubation period. Samples were collected periodically to measure PAH concentrations and to analyze the presence and relative abundance of SRB in the enrichment culture.

2.3. Biotransformation study

Six sets of biotransformation experiments were carried out simultaneously including one biotic set, two inhibition-control sets (molybdate, Na_2MoO_4, was added on days 0 and 6), one abiotic control set and two bacterial control sets. Fourteen experimental runs were performed in duplicate in 1200 mL bioreactors with a working volume of 1000 mL (Table 1).

The SRB suspension from the incubation reactor was harvested, concentrated by centrifugation at 6000 rpm for 10 min and washed thoroughly with sterile medium to remove residual PAHs. In the biotic experiments, 10 mL of the concentrated SRB suspension (approximately $1000\,mg\,L^{-1}$) was inoculated into the bioreactors containing 990 mL of mineral medium resulting in an initial biomass concentration of approximately $10\,mg\,L^{-1}$. Prior to the addition of PAH, bioreactors were purged with filter-sterilized nitrogen for 10 min at a flow rate of $10\,L\,min^{-1}$. PAHs from the stock solution prepared in DMF ($5000\,mg\,L^{-1}$) were added to the medium to achieve a final concentration of $5\,mg\,L^{-1}$. For studies in which fluorene and phenanthrene were added as a mixture, the concentrations of each were kept at $5\,mg\,L^{-1}$. To ensure anaerobic conditions, the headspace was replaced with nitrogen and the reactors were sealed with Teflon-lined stoppers. Reactors were incubated for 21 d in the dark without shaking in a temperature-controlled chamber at $30\,^{\circ}C$. Inhibition of sulfate reduction in selected reactors (inhibition-controls) was achieved by addition of Na_2MoO_4 ($4000\,mg\,L^{-1}$). Abiotic controls were prepared in the same manner as the biotic assays, but without the addition of SRB. Additionally, the abiotic control medium was autoclaved at $121\,^{\circ}C$ for 30 min and adjusted to a final concentration of $10,000\,mg\,L^{-1}$ sodium azide (NaN_3) to inhibit growth. A biotic experiment containing SRB and no PAH was prepared as bacterial control. The other bacterial control was performed with addition of DMF, but without PAH. From both biotic and abiotic reactors, samples were collected at time zero and at the end of 3, 6, 9, 12, 18 and 21 d to measure the pH, sulfate concentration, PAH concentration and bacterial density. At the end of the biotransformation process, ORP of the systems was measured. Furthermore, the selected samples from the bioreactors were analyzed by the gas chromatography-mass spectrometry (GC–MS) to identify the biotransformation metabolites.

2.4. Analytical techniques

2.4.1. PAH extraction and analysis

For PAHs extraction, 8 mL of sample from the bioreactor was transferred to a glass centrifugation tube (IWAKI, Japan) with 2 mL of DCM, and immediately closed with Teflon-lined caps. The tube was shaken vigorously in a rotary shaker (Shin Kwang, Taiwan) at 200 rpm for 16 h, and then centrifuged at 4000 rpm for 10 min. The supernatant was carefully decanted and 1.5 mL of the DCM extract was transferred to a 2-mL gas chromatograph (GC) vial and stored at $4\,^{\circ}C$ until analysis. Fluorene and phenanthrene concentrations were determined using a GC (Hewlett-Packard (HP) 6890 Plus) equipped with a flame ionization detector (FID) and DB-5 fused capillary column (30 m length, 0.53 mm inner diameter and $1.5\,\mu m$ film thickness). Hydrogen gas and airflow rates were maintained at 40 and $400\,mL\,min^{-1}$, respectively. Nitrogen was used as carrier and make-up gas supplied at a rate of 3.5 and $30\,mL\,min^{-1}$, respectively. The oven temperature was maintained at $120\,^{\circ}C$ for 1 min, and then increased at $20\,^{\circ}C\,min^{-1}$ to $280\,^{\circ}C$,

Table 1
Experimental conditions of biotransformation study

Run	PAH		Biomass (mg L^{-1})	Sulfate (mg L^{-1})	Molybdate (mg L^{-1})	Sodium azide (mg L^{-1})
	Compound	Concentration (mg L^{-1})				
1	FLU[a]	5	10	1920 (2335)[b]	–	–
2	PHE[c]	5	10	1920 (2301)[b]	–	–
3	FLU & PHE	5	10	1920 (2347)[b]	–	–
4[d]	FLU	5	10	1920 (2318)[b]	4000	–
5[d]	PHE	5	10	1920 (2323)[b]	4000	–
6[d]	FLU & PHE	5	10	1920 (2327)[b]	4000	–
7[e]	FLU	5	10	1920 (2289)[b]	4000	–
8[e]	PHE	5	10	1920 (2331)[b]	4000	–
9[e]	FLU& PHE	5	10	1920 (2296)[b]	4000	–
10	FLU	5	–	1920 (2310)[b]	–	10,000
11	PHE	5	–	1920 (2307)[b]	–	10,000
12	FLU & PHE	5	–	1920 (2312)[b]	–	10,000
13	–	–	10	1920 (2195)[b]	–	–
14[f]	–	–	10	1920 (2127)[b]	–	–

[a] Fluorene.
[b] The measured sulfate concentrations in the systems.
[c] Phenanthrene.
[d] Molybdate was introduced to bioreactor at the start of experiment.
[e] Molybdate was introduced to bioreactor on day 6.
[f] Bacterial control experiment with addition of DMF.

where it was held at that temperature for 5 min. Both injector and detector temperatures were maintained at 280 °C. At these conditions, fluorene and phenanthrene peaks were observed at 6.92 and 8.17 min, respectively. The recovery percentages of fluorene and phenanthrene were 91.3 ± 5.6% and 95.3 ± 7.5%, respectively and the corresponding method detection limits (MDL) were 1.11 ± 0.16 and 1.47 ± 0.18 µg L^{-1}.

2.4.2. Gas chromatography–mass spectrometry

For identification of the biotransformation intermediates, the GC–MS analyses were performed on a HP 6890 GC equipped with a 5973 mass selective detector. The column used was a HP-5 MS fused silica capillary column (30 m length, 0.25 mm inner diameter and 0.25 µm film thickness). Helium was used as the carrier gas at a flow rate of 1.6 mL min^{-1}. The injector temperature was 280 °C. The oven was maintained at 50 °C for 1 min, increased at 20 °C min^{-1} to 180 °C, then increased at 5 °C min^{-1} to 280 °C, where it was held for 1 min. The transfer line and ion trap manifold were set at 280 and 230 °C, respectively.

2.4.3. Spectrophotometry

Bacterial cell density was measured either gravimetrically or spectrophotometrically. The mixed liquor volatile suspended solid (MLVSS) measurement was made gravimetrically, according to the Standard Methods [26]. The spectrophotometric measurement was correlated with respective MLVSS concentration in a culture suspension at 660 nm (OD$_{660}$) using a UV digital spectrometer (Hitachi U-3010, Japan) [27]. Finally, a calibration graph was developed between MLVSS and absorbance (result not shown) and used thereafter for bacterial cell measurements.

2.4.4. Fluorescence in situ hybridization

At the end of sub-cultivation process, the presence and relative abundance of SRB in the system was determined by the FISH technique using the previously reported oligonucleotide probes, i.e. SRB385 (for δ-Proteobacteria including SRB) and SRB385Db (for Desulfobacteriaceae) [20]. The sum of SRB detected by probes SRB385 and SRB385Db was regarded as the total SRB population [20,28]. A positive control was conducted with a universal probe EUB338, which detected all eubacteria, and used to test the hybridization conditions [29]. In addition, two negative controls

were prepared; one of these controls was used to assess possible nonspecific binding (with probe NON338), and the other (lacking of probe) was used to monitor autofluorescence of microorganisms themselves [29,30]. All probes used in the present study were 5′-end labeled with fluorescein isothiocyanate (FITC) (Boehringer Mannheim, Germany).

The experimental procedures for fixation, hybridization, and microscopic counting of hybridized and 4′,6′-diamidino-2-phenylindole (DAPI)-stained cells in the present study were modified from that described by Amann et al. [31]. For fixation, 0.25 mL of concentrated cell suspension was mixed with 0.75 mL of 4% (w/v) paraformaldehyde (PFA) in phosphate-buffered saline (PBS) (10 mM sodium phosphate, 130 mM NaCl and pH 7.2), and the mixture was incubated at 4 °C for 2 h. After fixation, the cells were centrifuged at 10,000 rpm for 3 min and washed twice with 1 mL of PBS to remove the residual fixative (i.e. PFA). The fixed cells were then preserved in a mixture of 50% PBS–ethanol (v/v) and stored at −20 °C for less than 4 weeks until hybridization.

Hybridization was performed on Teflon-covered slides with 10 glass windows. Aliquots of the fixed cell suspensions (5 µL) were spread on each of the windows and air dried. The cells were fixed to the slides using 50%, 80%, and 100% ethanol, 3 min each, and were air dried. 10 µL of hybridization buffer (0.9 M NaCl, 20 mM Tris–HCl buffer [pH 7.2], 0.1% sodium dodecyl sulfate (SDS), and 30% [v/v] formamide) with fluorescent probe (5 ng µL^{-1}) was pipetted into the fixed wells. The slides were incubated in a temperature-controlled chamber for 2 h at 46 °C and then washed twice with washing buffer at 48 °C (0.07 M NaCl, 20 mM Tris–HCl buffer [pH 7.2], and 0.1% SDS) to remove the excess probe and salts. All hybridization and washing steps were performed in the dark.

Hybridized cells were visualized on an epifluorescence microscope (Olympus, Japan). For enumeration of the cells, 5–10 microscopic fields were randomly selected. The counting was performed based on fluorescence intensity by using the digital image analysis software Image-Pro Plus 5.1® (Washington, DC, USA) to measure the specific cell area after in situ probing. The cells with fluorescence intensity higher than 100 were regarded as hybridized cells. Nonspecific or background fluorescence was manually eliminated. The relative abundance of SRB was determined by dividing the area of hybridized cells by that of DAPI-stained cells.

2.4.5. Other analytical techniques

ORP and pH of the homogenized samples were measured using ORP (Suntex PC-300, Taiwan) and pH (Suntex SP-701, Taiwan) meters, respectively. For sulfate measurement, the sample was filtered through a 0.2 μm (Advantec No. A020A047A, Japan) nylon membrane filter and the concentration was determined by Standard Method [26]. The methane concentration was detected by a GC (China Chromatography 8900, Taiwan) equipped with a thermal conductivity detector (TCD). A stainless steel column (4.57 m length, 0.32 cm inner diameter) packed with Carboxen 1000 (60/80 mesh, Supelco) was used to separate the methane. Helium was supplied as a carrier gas at a rate of $25 \, mL \, min^{-1}$. The injector and detector temperatures were maintained at 70 °C and 80 °C, respectively. The oven was set at 120 °C. Certified methane gas standard was used for reference.

## 3. Results and discussion

3.1. Bacterial enrichment

The enrichment of the sulfate-reducing PAH-degrading bacteria was carried out with fluorene and phenanthrene concentrations of $10 \, mg \, L^{-1}$ (each). During the fifth sub-cultivation, the degradation of fluorene and phenanthrene occurred simultaneously without any lag phase. After 21 d of incubation, fluorene and phenanthrene concentrations decreased from 10.2 to $2.6 \, mg \, L^{-1}$ (74.5% removal)

and $10.7-4.2 \, mg \, L^{-1}$ (60.7% removal), respectively. However, 14.7% of fluorene and 17% of phenanthrene were lost in the abiotic controls. The loss of PAHs in the abiotic controls can be attributed to the adsorption of PAHs to the inner surfaces of the incubator, volatilization, photodegradation and/or leakage during sampling.

Throughout the enrichment process, no significant pH variation was observed in neither the biotic (pH around 7.3–7.4) nor the abiotic (pH around 7.2–7.3) control systems. The ORP of the biotic system decreased quickly from −210 to −350 mV within 3 d of incubation. This ORP was reported elsewhere as a favorable growth condition of SRB (−200 to −350 mV) [25].

3.2. Observations of FISH analysis

The presence and relative abundance of SRB in the enrichment culture was determined by the FISH. The total cell counts were performed by a DAPI staining approach. In the present study, two different SRB enrichment cultures were tested in triplicate. The epifluorescence micrographs of the DAPI-stained and hybridized bacteria with probes EUB338, SRB385, and SRB385Db are shown in Fig. 1. As evident from this figure, the major portion of the detected cells was rod-shaped. Fig. 1(a) and (b) illustrate that the universal probe EUB338 hybridized with approximately $88 \pm 8\%$ of the total DAPI-stained cells (Fig. 1(a) and (b)). This observation demonstrates that there is no false negative result due to methodological problem, and the fixation, probe penetration, and rRNA content of bacterial
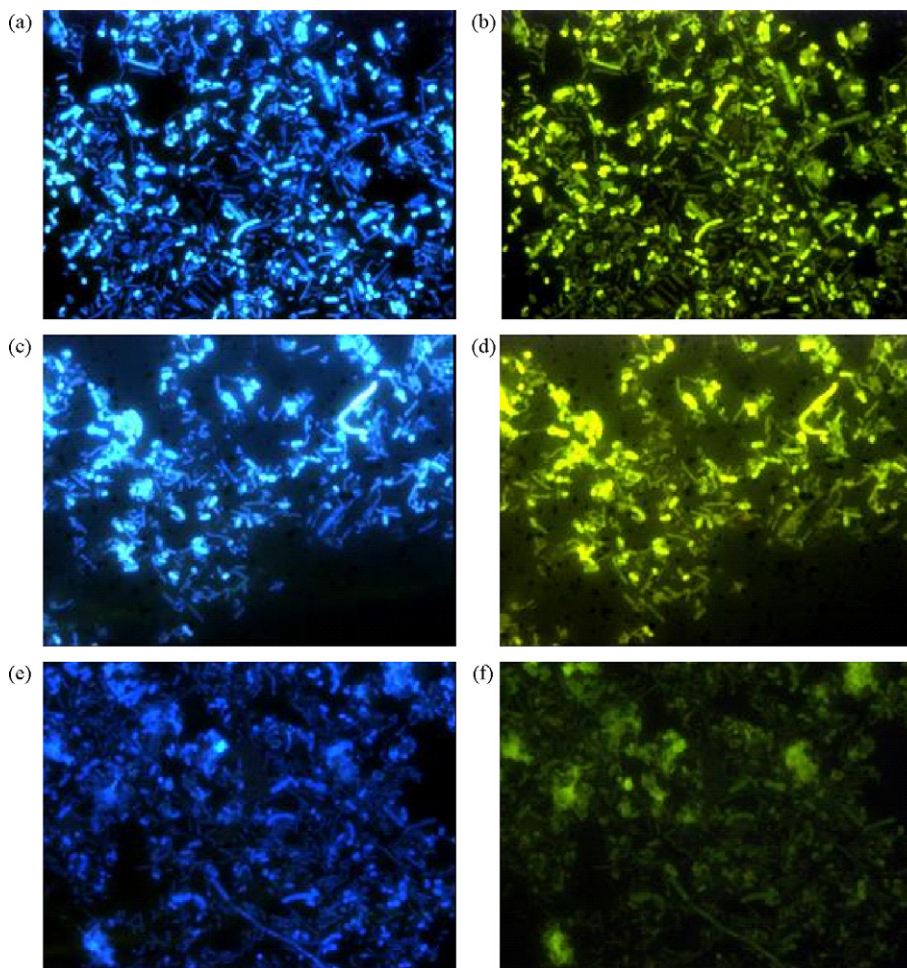
Fig. 1. Epifluorescence micrographs of bacteria. (a) DAPI-stained cells, (b) hybridization with probe EUB338 (same microscopic field as (a)), (c) DAPI-stained cells, (d) hybridization with probe SRB385 (same microscopic field as (c)), (e) DAPI-stained cells, and (f) hybridization with probe SRB385Db (same microscopic field as (e)).
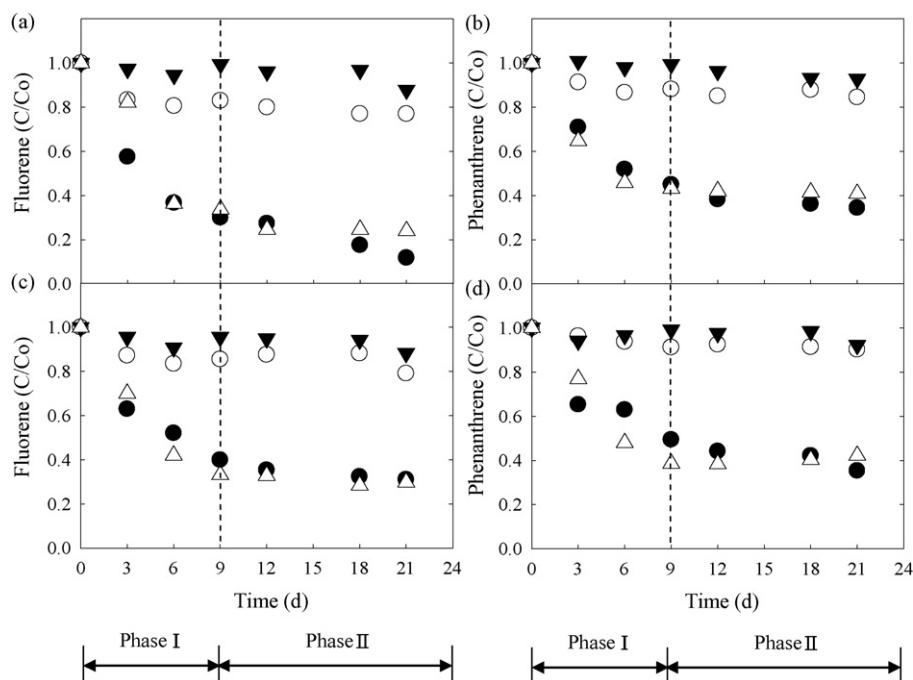
Fig. 2. Biotransformation of PAHs during 21 d of incubation. (a) Fluorene (single), (b) phenanthrene (single), (c) fluorene (mixture), and (d) phenanthrene (mixture). *Symbols*: biotic (●); inhibition-control (d 0) (○); abiotic control (▼); inhibition-control (d 6) (△). The presented data are mean values of duplicate incubations.

cells are not the limiting factors. The result of assay conducted with NON338 showed no nonspecific fluorescence (data not shown). In addition, results of control without probe revealed a low autofluorescence of microorganisms themselves (less than 1%) (data not shown). In this study, the sum of SRB detected by probes SRB385 and SRB385Db was regarded as the total SRB population. As shown in Fig. 1(c) and (d), up to $65 \pm 2\%$ of the total DAPI-stained cells hybridized to the SRB385 probe. The SRB385Db probe hybridized with around $22 \pm 4\%$ of the total DAPI-stained cells (Fig. 1(e) and (f)). These observations suggest that SRB is the dominant bacteria (ranged from 81% to 93%) in the enrichment culture.

3.3. Biotransformation experiments

3.3.1. Fluorene and phenanthrene biotransformation

The degradation of fluorene and phenanthrene in single and mixture compound systems are shown in Fig. 2. The reported degradation percentage of PAHs in the present study was determined based on their total concentrations (PAHs remaining in the aqueous solution and that adsorbed onto biomass) before and after the experiment. As seen in Fig. 2, more than 60% of initial fluorene and phenanthrene was biotransformed at the end of 6 d and 12 d, respectively, in the biotic system. However, no significant degradation of either PAHs was observed in the inhibition-control (0 d) and abiotic reactors. The lower degradation of PAHs in molybdate-added systems suggests that SRB was the major group of microorganisms involved in the biotransformation of fluorene and phenanthrene. This observation was in good agreement with the FISH results. After 21 d of incubation, almost 90% of fluorene was biotransformed by SRB whereas only 66% of phenanthrene was biotransformed in the biotic systems (without any inhibition).

From Fig. 2, it is clear that a two-phase degradation was observed in the biotic runs. The degradation rates of both fluorene and phenanthrene showed a rapid initial phase from 0 to 9 d (represented as k_1) followed by a slower and longer phase from 10 to 21 d (represented as k_2). The degradation rate constants of fluorene and phenanthrene in the biotic runs were determined by using a first-order kinetic equation [13].

$$C = C_0 \exp(-kt) \qquad (1)$$

where C_0 is the initial PAH concentration (mg L^{-1}), C is the substrate concentration (mg L^{-1}), k is the degradation rate constant (d^{-1}) and t is the time (d). The kinetic coefficients of fluorene and phenanthrene biotransformation are shown in Table 2. As seen in this table, the degradation rates of fluorene and phenanthrene in the single compound systems in the first stage (k_1) were 0.136 and 0.09 d^{-1}, respectively, and 0.091 and 0.011 d^{-1} for the second stage (k_2). When both fluorene and phenanthrene were spiked into the system, the k_1 of fluorene and phenanthrene were reduced to 0.098 and 0.072 d^{-1}, respectively. The rate of fluorene and phenanthrene degradation was higher in the single compound system compared to the mixed one. This particular observation contradicts results obtained by previous studies, where it is reported that fluorene biotransformation was enhanced in the presence of phenanthrene [23]. The reason for this observation is inhibition of microbial activity by higher total PAH concentrations.

The biodegradation of fluorene and phenanthrene, with molybdate initially added at 0 d (runs 4–6), was restricted in the inhibition-control system. In other separate experiments, molybdate was added at the end of 6 d (runs 7–9) in order to examine the response of highly active SRB towards inhibition. Both fluorene and phenanthrene biotransformations were reduced drastically

Table 2
First-order degradation rate constants and half-lives of fluorene and phenanthrene in single and mixture compounds in the anaerobic biotransformation studies

Run	$k_1{}^a$ (d^{-1})	$t_{1/2}$ (d)	r^2	$k_2{}^b$ (d^{-1})	$t_{1/2}$ (d)	r^2
FLU only	0.136	5.09	0.96	0.077	9.00	0.96
FLU in mixture	0.098	7.07	0.96	0.019	36.48	0.94
PHE only	0.090	7.70	0.97	0.019	36.48	0.86
PHE in mixture	0.072	9.63	0.90	0.024	28.88	0.88

^{a} Data from days 0 to 9.
^{b} Data from days 10 to 21.
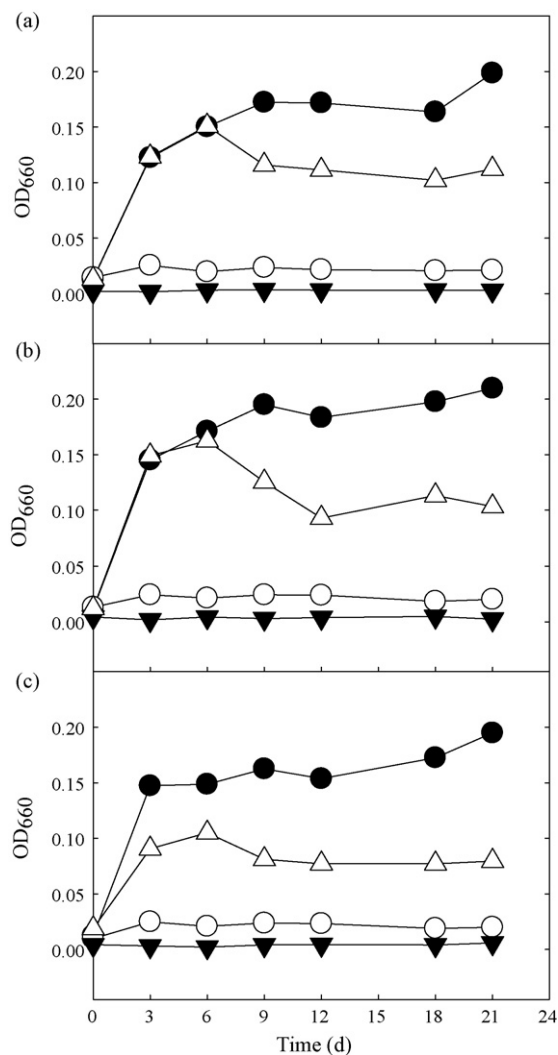
Fig. 3. Variation of bacterial cell density during 21 d of incubation. (a) Fluorene (single), (b) phenanthrene (single), and (c) fluorene and phenanthrene (mixture). *Symbols*: biotic (●); inhibition-control (d 0) (○); abiotic control (▼); inhibition-control (d 6) (△). The presented data are mean values of duplicate incubations.
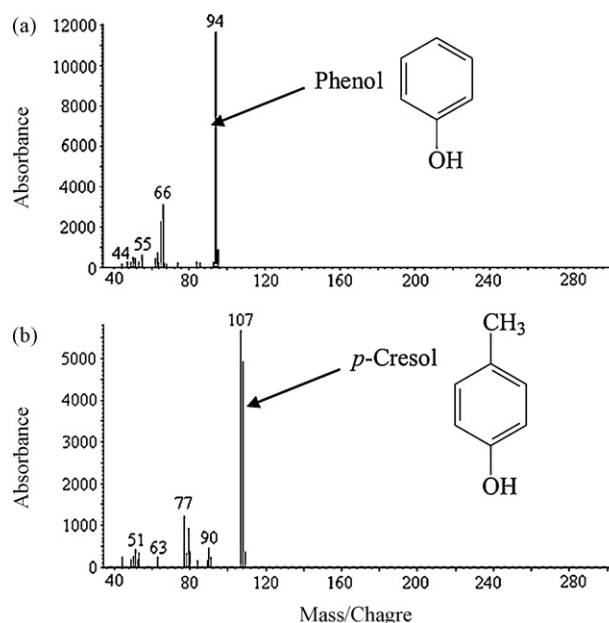
Fig. 4. Mass spectrum of metabolites detected in biotransformation studies conducted with SRB. (a) Phenol and (b) *p*-cresol.

following the application of molybdate into the systems. In addition, no methane production was observed in any of the biotic runs. These results infer that sulfate was the predominant terminal electron acceptor and the PAHs were metabolized by SRB.

3.3.2. Bacterial cell density

During the study, the bacterial cell densities in both single compound systems followed similar profiles (Fig. 3(a) and (b)). It is observed that the growth of biomass occurred immediately without a lag time. At the end of 6 d, the initial bacterial cell densities in both single compound systems increased from 0.01 to 0.16 (corresponds to 10–80 mg L^{-1}). The rapid bacterial growth in the above systems were in good correlation with the PAH biotransformation (Fig. 2). Comparing these results with the abiotic and inhibition-control systems reflects that PAH was biotransformed mainly by the microbial action. Conversely, no bacterial cell growth was observed in the inhibition-control systems. In the other control experiment, the increasing bacterial cell density ceased

Table 3
PAH biotransformation and sulfate reduction in the anaerobic biotransformation studies

Run	PAH degradation (%)			Sulfate reduction (%)		
	Phase I (0–9 d)	Phase II (10–21 d)	Total (0–21 d)	Phase I (0–9 d)	Phase II (10–21 d)	Total (0–21 d)
1	70.1	18.1	88.2	9.3	4.0	13.3
2	54.9	10.7	65.6	10.2	7.6	17.8
3	60.1/50.6[a]	8.8/14.1[a]	68.9/64.7[a]	9.7	6.0	15.7
4	17.1	6.0	23.1	0	0	0
5	11.9	3.6	15.5	0	0	0
6	14.4/8.8[a]	6.5/1.0[a]	20.9/9.8[a]	0	0	0
7	66.3	9.6	75.9	9.9	0.6	10.5
8	56.8	3.1	59.9	10.0	0.2	10.2
9	66.8/52.2[a]	3.4/5.6[a]	70.2/57.8[a]	10.3	0.1	10.4
10	3.9	8.3	12.2	0	0	0
11	3.7	3.5	7.2	0	0	0
12	4.4/3.4[a]	7.3/4.4[a]	11.7/7.8[a]	0	0	0
13	–	–	–	8.2	0.6	8.8
14	–	–	–	8.8	3.7	12.5

[a] Fluorene/phenanthrene degradation.

immediately after molybdate was added at the end of day 6. This observation reveals that SRB was the predominant bacteria in the biotic systems.

The bacterial cell density measured in the mixture compound system was significantly less compared to the single compound systems at the end of 21 d (Fig. 3(c)). Results indicate that the initial concentration of PAH had direct influence on the SRB growth rate. As a whole, it can be observed that PAH biotransformation of SRB was predominant when the target PAH compound was inoculated individually rather than in a mixture.
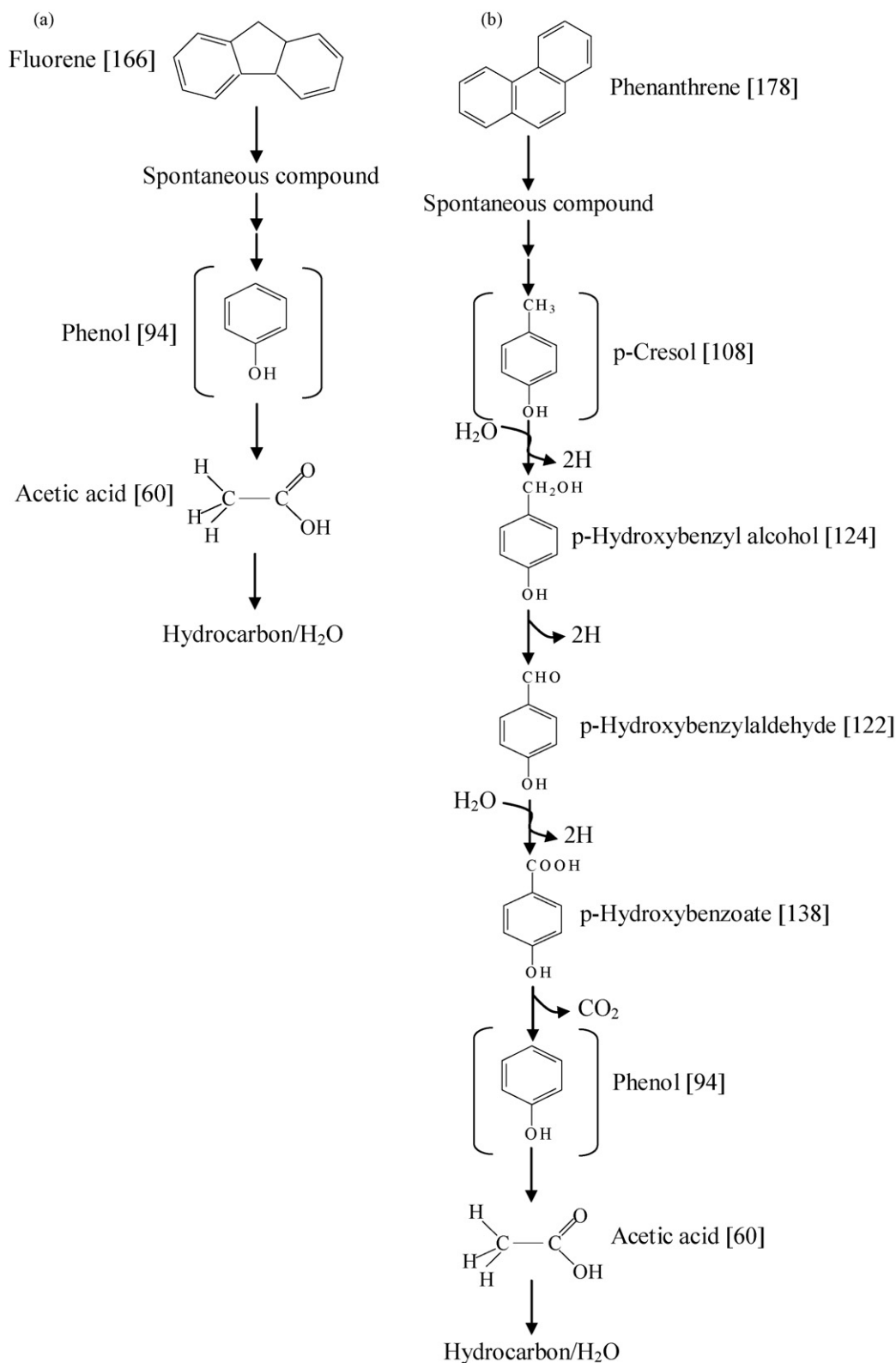

Fig. 5. Proposed anaerobic biotransformation pathways of (a) fluorene and (b) phenanthrene by SRB.

3.3.3. Sulfate reduction

In PAH biotransformation by SRB, sulfate is utilized as a final electron acceptor and the consumption of sulfate throughout the experiment reflects the performance of the system. The experimental results showed that sulfate reduction was observed in biotic, inhibition-control (6 d) and bacterial control reactors. A reduction of 8.8–17.8% of the initial sulfate concentration was recorded (Table 3). However, no change in initial sulfate concentration was observed in the abiotic and inhibition-control (0 d) systems. This result implies that PAH was biotransformed concurrently while sulfate was reduced in the biotic experiments. The sulfate reduction and PAH degradation percentages in all systems are shown in Table 3. The most sulfate was reduced when phenanthrene was added in the biotic system whereas lowest sulfate reduction was observed when no PAH was added into the system. The result of bacterial control experiment with addition of DMF and without PAH showed a 12.5% sulfate reduction indicating partial degradation of yeast extract and DMF. This would mean a 0.8% (13.3–12.5%) of the sulfate reduction is related to biotransformation of fluorene.

3.4. Biotransformation pathways of fluorene and phenanthrene

In the present study, samples collected at various time intervals from both biotic and abiotic fluorene and phenanthrene biotransformation studies were analyzed by a GC–MS operated under positive and negative ionization conditions. One unknown peak with a mass/charge ratio (m/z) of 94 (denoted as M, where M is molecular ion) was identified in the samples collected from biotic system using fluorene as their growth substrate (Fig. 4(a)). This metabolite showed the spectrum of phenol, which is identified by the mass spectrum of the reference library and the authentic standard compound. Interestingly, Boopathy [32] has reported the degradation of phenol into acetic acid by SRB. Hence, it can be proposed that fluorene is converted into a spontaneous compound, and further biotransformed via phenol and acetic acid.

On the other hand, the GC–MS analysis of the samples collected from the biotic systems with phenanthrene has shown two unknown peaks at m/z 107 (M − 1) and 94 (M) (Fig. 4(a) and (b)). The first peak was identified as 4-methylphenol (*p*-cresol). The only published metabolite from phenanthrene under sulfate-reducing conditions was phenanthrenecarboxylic acid [16]. Nevertheless, it was not found in our study. The second unknown peak was identified as phenol. Considering the fact that *p*-cresol has been reported to be biotransformed by SRB [33], the formation of phenol in the system might be due to the hydroxylation of the methyl group of *p*-cresol resulting in the formation of *p*-hydroxybenzyl alcohol or *p*-hydroxybenzylaldehyde. Further, *p*-hydroxybenzylaldehyde can be sequentially converted to *p*-hydroxybenzoate and phenol. However, no derivatives of *p*-cresol (excluding phenol) were observed in the system. The metabolic breakdown of phenanthrene into *p*-cresol by the enriched SRB was unclear at this particular time. None of the identified metabolites were observed in the abiotic transformation studies conducted with both fluorene and phenanthrene. Based on these results, the proposed anaerobic biotransformation pathways of fluorene and phenanthrene by SRB are shown in Fig. 5. Fluorene and phenanthrene were biotransformed by a sequence of hydration and hydrolysis reactions followed by decarboxylation resulting in mineralization of both these compounds by SRB under anaerobic conditions. However, studies with putative intermediates of fluorene and phenanthrene, i.e. phenol and *p*-creosol, as growth substrates are in progress and the analysis of samples from these systems by GC–MS can give more evidence to support the proposed pathway. In addition, studies are in progress to identify the central metabolite (represented as "spontaneous compound" in Fig. 5) in both the fluorene and phenanthrene biotransformation systems.

4. Conclusions

The enriched SRB culture was successful in the biotransformation of fluorene and phenanthrene. Biotransformation of both compounds occurred in sulfate-reducing conditions, which was evident from the ORP of the system. The sulfate reduction in the system was coupled with PAH biotransformation. As a whole, the enriched culture will be a useful tool in the biotransformation of PAHs. However, further studies are required before promoting the sulfate-reducing PAH-degrading bacterial culture for field scale application.

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