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Influence of Chemical Oxidant on Degradation of Benzo[a]pyrene Metabolites by the *Bacterium-Zoogloea* sp.

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ABSTRACT

It is neither comprehensive nor appropriate that the bioremediation of a benzo[a]pyrene (BaP)-contaminated environment be assessed only by its high degradation extent because its metabolites’ chemical structures are similar to the parent compound and maybe equally toxic. Therefore, further degradation of BaP metabolites is significant. Three methods, combining the *Zoogloea* sp. with potassium permanganate, combining the *Zoogloea* sp. with H2O2, *Zoogloea* sp. alone, were investigated to degrade cis-BP4,5-dihydrodiol and cis-BP7,8-dihydrodiol, which are the metabolites of BaP formed by *bacterium-Zoogloea* sp. Optimum parameters of degradation in the best method are that: of the three methods, coupling the *Zoogloea* sp. and KMnO4 is the best; compared with cis-BP7,8-dihydrodiol, cis-BP4,5-dihydrodiol is the more liable to be accumulated in pure cultures; the degradation effect of the two metabolites is optimal when the initial concentration of KMnO4 in the cultures is 0.05%; initial concentration of cis-BP4,5-dihydrodiol and cis-BP7,8-dihydrodiol is 4 mg L⁻¹, 8 mg

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INTRODUCTION

The environment fate of polycyclic aromatic hydrocarbons (PAHs) is motivated by their wide distribution, high persistence, and potentially deleterious effect on human health (Li et al., 2005). Mineral fuels, coal-derived oils, tobacco smoke, and vehicle exhaust contain PAHs, which released to the environment, has resulted in the presence of detectable levels of PAHs in air, water, and soil (Yao et al., 1998). BaP, a five-ring PAH, is regarded as a strong carcinogenic contaminant (Cerniglia, 1997). It can be cometabolically oxidized by a number of micro-organisms growing on other substrates (Zang et al., 2007).

Although degradation of BaP has been studied extensively (Moody et al., 2004), the metabolic pathways and the types of metabolites produced during the degradation process have received less attention, even though some of the metabolites may be more toxic and difficult to degrade than its parent PAHs compounds (Andersson and Henrysson, 1996; Mahro, 2000; Lafontaine et al., 2006), which might explain the elevated cytotoxicity and genotoxicity that has been observed during earlier bioremediation experiments (Belkin et al., 1994).

Usually, metabolites of BaP are cis- of trans-dihydrodiol compounds when degradation by bacterium or fungus, respectively. cis-BP4,5-dihydrodiol, cis-BP7,8-dihydrodiol, and cis-BP9,10-dihydrodiol are some metabolites of BaP by bacteria (Cerniglia et al., 1979). They have a similar chemical structure to BaP-“K” region and “bay” region (see Fig. 1), which are well known to relate to carcinogenesis (Wang, 1991). These oxidation products are generally stable, and hardly for their ring cleavage (Sutherland et al., 1990). Consequently, there is a need for developing robust remediation strategy of degradation BaP and its toxic metabolites.

Recently, combining of chemical with biological methods (Lee et al., 2001; Mackinnon and Thomson, 2002) have attracted considerable attention for remediation of poor bioavailability compounds like PCBs (Tunnicliffe and Thomson, 2004). Some chemical oxidants, like KMnO4 and H2O2, can well oxidize compounds of quinone, diol, and phenol. The advantages of using KMnO4 or H2O2 for pre-oxidized contamination are that they are nontoxic, inexpensive, and effective. It is reported that the mineralization extent is evidently higher if the PAHs are preoxidized by a chemical reagent before biodegradation (Nadamjal et al., 2002). This treatment technology (bacteria and oxidant in combination) can be used where contaminated by these compounds with high concentrations.

However, problems associated with their biodegradability in environmental media have received increasing attention (Doong and Lei, 2003, Luan et al., 2006). For low solubilization and bad bioavailability of BaP, many previous studies have reported that cometabolic substances may induce some micro-organisms to produce certain dioxygenase that can degrade BaP (Gong et al., 2002). These cometabolic substances may also be useful to degrade other similar (poly) aromatic structures (Heitcamp and Cerniglia, 1987). Suitable cometabolic substances for key contaminants are those that are low molecular weight and similar chemical structure to the parent compounds. In 2002, Gong et al. took phenanthrene as a cometabolic substance of BaP biodegradation, and his research showed that degradation extent was enhanced and initial lag period was reduced.

In this paper, the degradation of cis-BP4,5-dihydrodiol and cis-BP7,8-dihydrodiol by combining bacterium-Zoogloea sp. with chemical oxidant is reported.

MATERIALS AND METHODS

Chemicals

The PAHs used in this study were BaP, cis-BP4,5-dihydrodiol and cis-BP7,8-dihydrodiol. BaP with 98% purity

L−1, respectively; cometabolic substance is salicylic acid or sodium succinate. The degradation extent of cis-BP 4,5-dihydrodiol and cis-BP 7,8-dihydrodiol using combining the Zoogloea sp. and KMnO4 reach 76.1% and 85.9% after 12 days of cultivation, respectively, which were more than twice compared with conventional method.

Key words: degradation; metabolites; cis-BP4,5-dihydrodiol; cis-BP7,8-dihydrodiol; bacterium; Zoogloea sp.; chemical oxidant

Figure 1. Accumulated metabolites of BaP by Zoogloea sp.
was purchased from Fluka Chemical Company (Milwaukee, WI). cis-BP4,5-dihydrodiol and cis-BP7,8-dihydrodiol standards were purchased from Aldrich Chemical Co. Ltd. (Milwaukee, WI). All solvents were HPLC grade, except dichloromethane, which was analytical grade; MgSO\textsubscript{4} \cdot 7H\textsubscript{2}O, NH\textsubscript{4}NO\textsubscript{3}, KCl, glucose, potassium permanganate, sodium succinate, phthalic acid, biphenyl, salicylic acid, and glucose were reagent grade.

**Bacteria growth conditions**

In a previous study on the biodegradation of PAHs using indigenous bacteria conducted by Li et al. (2002), 10 dominant species of bacteria found in crude oil-contaminated soil in the Liaohe Oil Field in China were compared. For this reason the bacteria-Zoogloea sp. was used in this study. Before our experiments, the bacterium was domesticated with BaP for 6 weeks.

**Metabolites degradation experiments**

All operations were conducted in dim yellow light in order to avoid photodegradation.

Three methods were used. The first one (Method I) was to degrade by combining the Zoogloea sp. with KMnO\textsubscript{4}: 30 mL cultures were added into 125-mL Erlenmeyer flasks, the initial concentration of KMnO\textsubscript{4} in the culture was 0.1% (w/v), and then mixtures of the two metabolites (dissolved in acetone, with initial concentration in the culture was 8 mg L\textsuperscript{-1}) with sodium succinate (concentration 20 mg L\textsuperscript{-1}) as a cometabolic substrate were added into cultures together. The pH value of the cultures solution was regulated by adding 1.0 mol/L HCl and controlled by a pHs-29A acidity meter (with a final value of 6.9). After sterilization, Erlenmeyer flasks were cultured for 48 h on a rotary shaker at 28°C and 160 rpm, after which the inoculant (10% v/v) was added. Control experiments were operated in the same conditions except without the bacteria Zoogloea sp. and the oxidant. The degradation extent of metabolites were measured at different time intervals (0, 3, 6, 9, 12, and 15 days) by high-performance liquid chromatography (HPLC).

The second one (Method II) was to degrade by combining the Zoogloea sp. with H\textsubscript{2}O\textsubscript{2}. The final concentration of H\textsubscript{2}O\textsubscript{2} in the culture was 0.1% (w/v). The third one (Method III), conventional method, was to degrade BaP with only Zoogloea sp. All other experimental conditions were as above description.

**Selection of degradation parameters**

For selection of suitable initial KMnO\textsubscript{4} concentration, four groups of 125-mL Erlenmeyer flasks were selected and each group consisted of three flasks. The initial concentration of KMnO\textsubscript{4} (w/v) in each group was 0.01, 0.05, 0.1, and 0.5%, respectively. Control experiments were conducted in the same conditions except that the two contaminants were degraded in the absence of KMnO\textsubscript{4} and the bacteria. The degradation extent of metabolites were measured on day 12 by HPLC.

For selection of suitable initial metabolites concentration, four concentrations of the two metabolites in each group were carried out (1, 4, 8, and 12 mg L\textsuperscript{-1}). The KMnO\textsubscript{4} concentration was the optimum concentration obtained in the above experiment. For selection of cometabolic substrates,

![Figure 2. Comparison of the three methods for degradation. i, ii were degradation curve of cis-BP4,5-dihydrodiol and cis-BP7,8-dihydrodiol, respectively, by method I; iii, iv were degradation curve of cis-BP4,5-dihydrodiol and cis-BP7,8-dihydrodiol, respectively, by method II; v, vi were degradation curve of cis-BP4,5-dihydrodiol and cis-BP7,8-dihydrodiol, respectively, by method III.](image-url)
sodium succinate (SS), salicylic acid (SA), phthalic acid (PA), biphenyl (B) (each cometabolic substrates 20 mg L$^{-1}$, respectively) were studied. For selection of glucose concentration, three different glucose concentrations were set, which were 0.5, 2, and 5%, respectively. All other conditions were as described above.

**BaP degradation**

Degradation experiments of BaP were implemented by Method I and Method II. The initial concentration of BaP in the culture was 50 mg L$^{-1}$. Both the parent compound (BaP) and the two metabolites were monitored in the degradation experiments at different time intervals (0, 3, 6, 9, 12, and 15 days) by HPLC. All other conditions were as described previously.

**Analysis of extractable BaP and its metabolites**

Medium samples were extracted as follows (Robert *et al.*, 1997): the media was extracted with 3 × 8 mL CH$_2$Cl$_2$. Each sample in the Erlenmeyer flask was extracted for 5 min in an ultrasonic bath after adding CH$_2$Cl$_2$, which can avoid the adsorption to the flask. The organic extracts were combined after the separation of organic and water phases. The organic phase was cleaned in a chromatography column filled with 3 g of deactivated aluminum oxide (deactivated by 15% water addition), 5 g of activated silica gel (70–230 mesh, activated by placing in an oven at 130°C for 16 h, and cooled in a desiccator at least for 10 min before use), and 1 g of anhydrous sodium sulphate. The chromatography column was eluted with 10 mL 4:1 hexane:dichloromethane (v:v). The eluate was concentrated at 50°C by a rotary evaporator and dried by nitrogen gas and finally redissolved in acetonitrile storage in darkness at −20°C before further analysis.

The BaP and its metabolites were identified and quantified with standards by reversed-phase HPLC equipped with a gradient pump (KNAUER K1001), an autosampler (TSP AS100), and a reverse-phase C-18 column. Elution conditions were as follows: a 1:9 (v:v) mixture of water and acetonitrile was used as the solvent at a flow rate of 0.5 mL/min. In all cases, 20 μL of sample was injected to the HPLC by an autosampler.

**RESULTS AND DISCUSSION**

*Identify of BaP and its metabolites*

In pure cultures, some bacteria can hydroxylate BaP to cis-dihydriodols at 4, 5, 7, 8, or 9, 10 positions. Retention times of BaP, cis-BP4,5-dihydrdiol, and cis-BP7,8-dihydriodil were 10.361 min, 6.835 min, and 5.468 min in a wavelength of 254 nm, respectively. Figure 1 shows chemical structures and their relationship of the three compounds. Comparison with standards by retention times, cis-BP4,5-dihydriodil and cis-BP7,8-dihydriodil were identified, which were two metabolites of BaP by Zoogloea sp.

*Comparison of the methods*

From Fig. 2, the effect of Method I was the best among the three methods. This result is similar with continuing degradation of BaP and its metabolites (Zang *et al.*, 2006). On the contrary, Method III provided the poorest degradation of the two metabolites. It could been seen that the degradation extent of cis-BP4,5-dihydriodil and cis-BP7,8-dihydriodil were the highest at every time point by the method. It must be potent oxygenation of KMnO$_4$ that has partly oxidized the two contaminants and reduced inherent toxicity of metabolites. Method I provided better degradation over Method II because oxidation of KMnO$_4$ was stronger than...
H₂O₂. As Zoogloea sp. is a widely used bacteria in China (Li et al., 2002), and KMnO₄ is nontoxic, inexpensive, and readily available, this combined method provides a cost-efficient means to remediate BaP-contaminated environments. Consequently, the following experiments all adopted Method I.

Optimal initial KMnO₄ concentration

Suitable concentration of KMnO₄ was propitious to not only preoxidation but also continued degradation of the two metabolites by micro-organism. For method I, concentration of KMnO₄ plays an important role in further degradation of the two metabolites because reducing the toxicity of the two metabolites depends heavily on the initial concentration of KMnO₄. The higher the concentration of KMnO₄, the better effect the pretreatment. Figure 3 shows that 0.05\% KMnO₄ culture is the optimum for degradation of the two metabolites. This is because higher concentration KMnO₄, where the bacteria need more time to adapt, also has a certain influence on the bacteria. On day 12, the degradation extent of cis-BP4,5-dihydrodiol and cis-BP7,8-dihydrodiol were 39.0\% and 47.8\%, respectively.

Selection of initial concentration of metabolites

Initial concentrations of the metabolites are another important factor for degradation of the two metabolites. From their physical and chemical character, the two metabolites were hardly further metabolized for their toxicity to micro-organisms and H-bond in their molecule. Moreover, degradation of the two metabolites was the key step for full degradation of BaP. In order to enhance degradation extent, the contaminants must be in a certain range. Figure 4 shows that the optimum initial concentration of cis-BP4,5-dihydrodiol and cis-BP7,8-dihydrodiol is 4 and 8 mg L⁻¹ in the pure culture, respectively. The higher the concentration, the stronger the toxicity. However, an extremely low concentration would reduce usability for the micro-organisms. On day 12, the degradation extent of cis-BP4,5-dihydrodiol and cis-BP7,8-dihydrodiol was 52.6 and 56.1\%, respectively.

Effect of cometabolic substrates on degradation

Usually it is difficult for a micro-organism to directly take PAHs as only carbon source and energy source, especially high molecular weight PAHs. A cometabolic substance is an ideal bridge between them. Based on the principle of a selection cometabolic substance as described above, sodium succinate, phthalic acid, biphenyl, and salicylic acid were selected targets for biodegradation of cis-BP4,5-dihydrodiol and cis-BP7,8-dihydrodiol. The results are shown in Figure 5. As a cometabolic substance, the effect of sodium succinate was as good as salicylic acid after cultivation for 12
days, while the effect of phthalic acid and biphenyl was poor. In addition, it is interesting to observe that the cometabolic substance is different for different bacteria to degrade the same contamination comparison with our previous experiments.

**Influence of glucose**

Glucose is a very suitable energy source for the micro-organism growth. However, high glucose concentrations impede biodegradation because micro-organisms will choose glucose in prefernce to other food sources as it is easily ingested. From Fig. 6 a 0.5% glucose was optimum in our experiments. After 12 days, the degradation extent of \textit{cis}-BP4,5-dihydrodiol and \textit{cis}-BP7,8-dihydrodiol was 76.1 and 85.9%, respectively. With this amount of initial glucose, the degradation extent is obviously improved.

**Degradation of BaP**

Following BaP degradation, the metabolites \textit{cis}-BP4,5-dihydrodiol and \textit{cis}-BP7,8-dihydrodiol will gradually form and then be degraded. Figure 7 clearly shows that, Method I demonstrates the best overall degradation of both the parent BaP and its metabolites compared to Method III in the long term. However, in the first 6 days, there was little difference in the degradation extent of BaP between the two methods; but after that time, the degradation extent of BaP in Method I was quickly enhanced. In fact, there is no effect for pretreatment on BaP itself. Only when the degradation extent of the two metabolites were quickly enhanced, coherent toxicity to micro-organisms decline, and that ability of the micro-organism degradation BaP is increased.

In the past 40 years, the effects of various micro-organisms on degradation extent of BaP has been used to assess remediation of BaP-contaminated environments (Juhasz and Naidu, 2000). However, we now think that it is neither comprehensive enough nor appropriate to assess the bioremediation of a BaP-contaminated environment only by examination of the high degradation extent of BaP alone. The accumulation of its metabolites, degradation, and aging of BaP may also be the important factors to be considered in remediation of BaP-contaminated environments.

**CONCLUSIONS**

\textit{cis}-BP4,5-dihydrodiol and \textit{cis}-BP7,8-dihydrodiol were identified by HPLC. Degradation by coupling Zoogloea sp. with potassium permanganate was found to be the most effective method to degrade both BaP and its metabolites. Compared with \textit{cis}-BP7,8-dihydrodiol, \textit{cis}-BP4,5-dihydrodiol is the more liable to be accumulated in pure cultures. This result is the same with the continuing degradation of BaP and its metabolites (Zang et al., 2006). The optimum conditions were found to be as follows: the initial concentration of KMnO$_4$ in the culture at 0.05%; initial concentration of \textit{cis}-BP4,5-dihydrodiol and \textit{cis}-BP7,8-dihydrodiol is 4 and 8 mg L$^{-1}$, respectively; and the cometabolic substance was salicylic acid or sodium succinate. When this prescription was used the results indicated that the residue of BaP and the two metabolites was clearly

![Figure 7. Comparison of method I with method III for BaP degradation. i, iv were degradation curve of BaP by method I and method III, respectively; ii, v were accumulation curve of \textit{cis}-BP4,5-dihydrodiol by method I and method III, respectively; iii, vi were accumulation curve of \textit{cis}-BP7,8-dihydrodiol by method I and method III, respectively.](image-url)
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lower than that of the conventional method of biodegradation.

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