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Degradation of metabolites of benzo[a]pyrene by coupling *Penicillium chrysogenum* with $KMnO_4$

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Abstract

Several main metabolites of benzo[a]pyrene (BaP) formed by *Penicillium chrysogenum*, Benzo[a]pyrene-1,6-quinone (BP 1,6-quinone), *trans*-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene (BP 7,8-diol), 3-hydroxybenzo[a]pyrene (3-OHBP), were identified by high-performance liquid chromatography (HPLC). The three metabolites were liable to be accumulated and were hardly further metabolized because of their toxicity to microorganisms. However, their further degradation was essential for the complete degradation of BaP. To enhance their degradation, two methods, degradation by coupling *Penicillium chrysogenum* with $KMnO_4$ and degradation only by *Penicillium chrysogenum*, were compared; Meanwhile, the parameters of degradation in the superior method were optimized. The results showed that (1) the method of coupling *Penicillium chrysogenum* with $KMnO_4$ was better and was the first method to be used in the degradation of BaP and its metabolites; (2) the metabolite, BP 1,6-quinone was the most liable to be accumulated in pure cultures; (3) the effect of degradation was the best when the concentration of $KMnO_4$ in the cultures was 0.01% (w/v), concentration of the three compounds was 5 mg/L and pH was 6.2. Based on the experimental results, a novel concept with regard to the bioremediation of BaP-contaminated environment was discussed, considering the influence on environmental toxicity of the accumulated metabolites.

Key words: BaP; metabolites; accumulated; coupling; potassium permanganate

Introduction

Benzo[a]pyrene (BaP), a 5-ring polycyclic aromatic hydrocarbon (PAH), is well known as an ubiquitous xenobiotic that causes mutagenesis and carcinogenesis (Scheepers, 1992; Sims and Overcash, 1983). It can be co-metabolically oxidized by several microorganisms that grow on other substrates (Cerniglia *et al*., 1980; Cerniglia, 1993; Datta and Samanta, 1988; Gibson *et al*., 1975), and therefore several metabolites are formed simultaneously. Benzo[a]pyrene-1,6-quinone (BP 1,6-quinone), *trans*-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene (BP 7,8-diol), and 3-hydroxybenzo[a]pyrene (3-OHBP) are metabolites produced from degradation of BaP by certain fungi (Albert and Ravendra, 2000), and they have similar chemical structure with BaP-"K" region and "bay" region, which is related to toxicity, mutagenesis, and carcinogenesis (Cerniglia and Gibson, 1979). Their chemical structure showed that these oxidation products are generally stable and hence are hardly further metabolized by ring cleavage (Schneider *et al*., 1996). Therefore, even when high degradation rate of BaP is achieved, perhaps much BP 1,6-quinone, BP 7,8-diol, and 3-OHBP are often accumulated and lead to ecological threat.

Hitherto about ten BaP metabolites (with uncleaved ring) that were degraded by fungi have been identified (Cerniglia and Gibson, 1980b). Liu (1983) identified six ring-uncleaved products of BaP formed by *Penicillium chrysogenum* including *trans*-9,10-dihydroxy-9,10-dihydro-benzo[a]pyrene, *trans*-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene, benzo[a]pyrene-1,6-uinone, benzo[a] pyrene-3,6-quinone, 9-hydroxybenzo[a]pyrene, and 3-hydroxybenzo[a] pyrene.

Although degradation of BaP has been widely studied, there has been no report on the degradation of its accumulated metabolites. The main methods for eliminating BaP from the environment include: microbiological transformation and degradation, volatilization, photo-oxidation, chemical oxidation, bioaccumulation, and adsorption on sediment particles (Wilson and Jones, 1993). BP 1,6-quinone, BP 7,8-diol, and 3-OHBP as representative metabolites produced from degradation of BaP by fungus, conventional methods for removing them are often ineffective or time-consuming due to their inherent toxicity. Recently, considerable attention has been paid to couple

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chemical and biological methods for the remediation of contaminated soils by PCBs (Mackinnon and Thomson, 2002; Li, 2004). In 2002, the research on Nadamjal also showed that the degradation rate of the mineral was further enhanced if the PAHs were preoxidized by a chemical reagent before biodegradation. $KMnO_4$, as chemical reagent, exhibits oxidation effect especially under acidic conditions. On the one hand, it can efficiently oxidize quinone, diol, and phenol. On the other hand, $KMnO_4$ exhibits a certain degree of toxicity toward microoganisms. However, several microoganisms can still sustain in lower concentration ranges of $KMnO_4$ after domestication.

With the objective of removal or lowering of accumulated metabolites of BaP, it is essential to find an effective method as well as the optimal conditions for degradation of metabolites of BaP.

1 Materials and methods

1.1 Chemicals

The PAHs used in this study were BaP, BP 1,6-quinone, BP 7,8-diol, and 3-OHBP. BaP with 98% purity was purchased from Fluka Chemical Company. BP 1,6-quinone, BP 7,8-diol, and 3-OHBP standards were purchased from Aldrich Chemical Co Ltd. All solvents were of HPLC grade, except dichloromethane that was of analytical grade; NH_4NO_3, $MgSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$, $FeSO_4{\cdot}7H_2O$, KH_2PO_4, $Na_2HPO_4{\cdot}12H_2O$, $MgSO_4{\cdot}H_2O$, $KMnO_4$, sodium succinate and glucose were of reagent grade.

1.2 Growth conditions for fungus

Penicillium chrysogenum was screened, identified, and separated from crude oil-contaminated soil in the Liaohe Oil Field in China. Before the experiments were begun, the organism was cultured using a mixture of BaP, BP 1,6-quinone, BP 7,8-diol, and 3-OHBP for two weeks. The method used for continual growth and maintenance of the fungus has been reported previously (Liu *et al*., 1983). Basidiospores were stored at room temperature on agar slants and were transferred to fresh slants every 8 weeks. The experimental medium consisted of (in g/L distilled water): NH_4NO_3, 1; $MgSO_4{\cdot}7H_2O$, 0.2; $CaCl_2{\cdot}2H_2O$, 0.01; $FeSO_4{\cdot}7H_2O$, 0.01; KH_2PO_4, 0.4; $Na_2HPO_4{\cdot}12H_2O$, 0.6; $MgSO_4{\cdot}H_2O$, 0.02; and glucose, 0.5.

1.3 Degradation experiments of the three metabolites

1.3.1 Comparison of the two methods for degradation of the three metabolites

All operations were conducted in dim yellow light to avoid photodegradation. Two methods for degradation of the three metabolites are described below:

First method: the three metabolites were degraded by coupling the fungus *Penicillium chrysogenum* with $KMnO_4$; $KMnO_4$ was added into Erlenmeyer flasks (125-ml), the final concentration of $KMnO_4$ in the culture was 0.5% (w/v), and then mixture of the three metabolites (5 mg/L each separately dissolved in acetone) and sodium succinate (50 mg/L) as co-substrates were added together into the cultures. The pH of the culture solution was maintained at 6.5 of the culture solution by adding 1.0 mol/L HCl and measured by pHs-29A acidity meter. The final volume of culture was 30 ml. After sterilization, Erlenmeyer flasks were cultured for 48 h on a rotary shaker at 28°C at a rate of 160 r/min, and then inoculant (5% v/v) was added. Control experiments were operated under the same conditions except without $KMnO_4$ and *Penicillium chrysogenum*. The degradation rates of metabolites were measured at different time intervals (0, 3, 6, 9, 12, and 15 d) by HPLC.

Second method: the three metabolites were degraded only by the fungus *Penicillium chrysogenum*. Control experiments were operated under the same conditions except without *Penicillium chrysogenum*. Other procedures were the same as described above.

1.3.2 Effect of $KMnO_4$ concentration on degradation of the three metabolites

The Erlenmeyer flasks (125-ml) were divided into 3 groups and each group consisted of 6 Erlenmeyer flasks. A series of concentrations $KMnO_4$ were prepared as 0.01%, 0.5%, 1%, respectively. The control was operated in the absence of $KMnO_4$ and the fungus. All other conditions were as same as those described above.

1.3.3 Effect of the concentration of three metabolites on degradation

The Erlenmeyer flasks (125-ml) were divided into 3 groups and each group consisted of 6 Erlenmeyer flasks. A series of concentrations of the three metabolites in each group was prepared as 1, 5 and 10 mg/L, respectively. The $KMnO_4$ concentration was the optimum value described in the previous paragraph. All other conditions were as described previously.

1.3.4 Effect of pH value on degradation of the three metabolites

The Erlenmeyer flasks (125-ml) were divided into 3 groups and each group consisted of 6 Erlenmeyer flasks. The pH value of the culture solution was regulated by adding 1.0 mol/L HCl, and the final pH value of different groups was 6.2, 6.5, and 6.8, respectively. All other conditions were as described previously.

1.4 Degradation experiment of BaP

BaP was degraded by two methods as described in section 1.3.1. The Erlenmeyer flasks (125-ml) were divided into 3 groups and each group consisted of 6 Erlenmeyer flasks. BaP (50 mg/L dissolved in acetone) and sodium succinate (50 mg/L) as co-substrates were added into each Erlenmeyer flask. The BaP experiments were carried out under the optimum conditions on the results of the three metabolites. Controls were carried out under the same conditions except in the absence of $KMnO_4$ and *Penicillium chrysogenum*.

1.5 Methods used for analysis

Samples of the medium were extracted (Robert *et al*., 1997; Eschenbach *et al*., 1994; Song *et al*., 2002) as

follows. The samples were extracted with 3×8 ml CH_2Cl_2. The organic extracts were combined after the separation of organic and water phases, and the organic phase was eluted from a chromatography column filled with 2 g of deactivated aluminum oxide (deactivated by addition of 15% water), 4 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 sulfate. The chromatography column was eluted with 10 ml of hexane : dichloromethane (4:1, v:v). The eluate was concentrated at 50°C by a rotary evaporator and dried by nitrogen gas and finally dissolved in methanol for HPLC analysis. The analysis was performed with an HPLC equipped with a gradient pump (KNAUER K1001), an autosampler (TSP AS100), and a reverse-phase C-18 column. The concentrations of these compounds were calculated using HPLC software CHEM EURO 2000.

In this article, degradation rate of metabolites and BaP at certain time point, d_n (%), was calculated according to

$$d_n = (C_0 - C_n)/C_0 \quad (1)$$

where C_0 and C_n are the initial concentration of BaP and the concentration at certain time point (mg/L), respectively.

Accumulation rate of metabolites at certain time point, a_n(%), was calculated according to

$$a_n = C_e/C_b \quad (2)$$

where C_b is the initial concentration (mg/L) of BaP, and C_e is the equilibrium concentration (mg/L) of metabolites at certain time point .

2 Results and discussion

2.1 Identification of BaP and its metabolites

The BaP and its metabolites formed by *Penicillium chrysogenum* were identified and quantified with standards by reversed-phase HPLC. The extraction recovery of BaP under given conditions was higher than 88%, and the three metabolites ranged from 74% to 90%. Elution conditions were as follows: 1:9 (v:v) mixture of water and methanol 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. BaP, BP 1,6-quinone, BP 7,8-diol, and 3-OHBP were identified based on their retention times. At a wavelengh of 254 nm, retention time of BaP, BP 1,6-quinone, BP 7,8-diol, and 3-OHBP were 10.361 min, 3.58 min, 4.02 min, and 4.92 min, respectively. Under the experimental conditions described in this study, the main metabolites produced from degradation of BaP by Penicillium chrysogenum were BP 1,6-quinone, BP 7,8-diol, and 3-OHBP. Fig.1 shows chemical structures of the three metabolites and their relationship with parent compound (BaP).

2.2 Comparison of the two methods for degradation of the three metabolites

To enhance the degradation rate of the three toxic metabolites, selection of a suitable method was crucial.

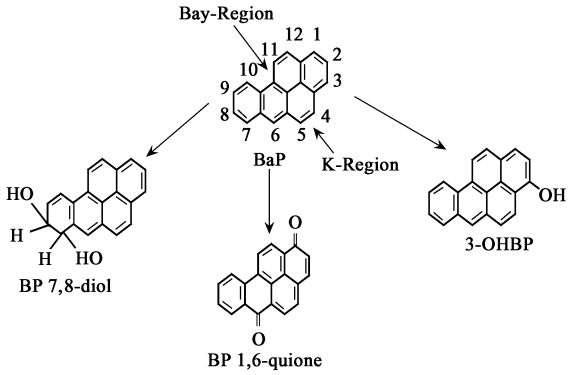

Fig. 1 Chemical structure of the three metabolites.

Fig.2 shows that degradation rates of BP 1,6-quinone, BP 7,8-diol, and 3-OHBP were much higher by coupling *Penicillium chrysogenum* with $KMnO_4$ compared with *Penicillium chrysogenum* alone. The reason is that the potent oxygenation of $KMnO_4$ has partly oxidized the three contaminants and hence reduces the inherent toxicity of metabolites. The fungus can more easily degrade the metabolites after detoxification by $KMnO_4$. Consequently, the method of coupling *Penicillium chrysogenum* and $KMnO_4$ was adopted in subsequent experiments described below.

2.3 Selection of concentration of $KMnO_4$

$KMnO_4$, as chemical oxidant, first oxidizes BP 1,6-quinone, BP 7,8-diol, and 3-OHBP during pretreatment, and continuously reacted after *Penicillium chrysogenum* was inoculated. Fig.3 shows that during the first 3 d, degradation rates of the three metabolites were much lower in 0.01% $KMnO_4$ culture than in 1% $KMnO_4$. However, after 3 d, their degradation rates showed a rapid increase in 0.01% $KMnO_4$ culture, whereas the increase was slower in 1% $KMnO_4$ culture. It could be explained that the higher concentration of $KMnO_4$ results in stronger oxidation of the metabolites, but it also has a certain toxicity, which *Penicillium chrysogenum* needs more time to adapt. During the first 3 d, the degradation rates of the three metabolites mainly depended on $KMnO_4$, and later, mainly depend on

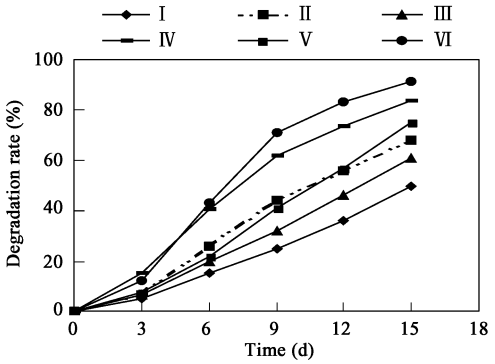

Fig. 2 Comparison of different methods for degradation of the three accumulated metabolites; II, IV, and VI are degradation curves of BP 1,6-quinone, BP 7,8-diol, and 3-OHBP for coupling *Penicillium chrysogenum* and $KMnO_4$ method, respectively; I, III, and V are curves of BP 1,6-quinone, BP 7,8-diol, and 3-OHBP for the method using only *Penicillium chrysogenum*, respectively.

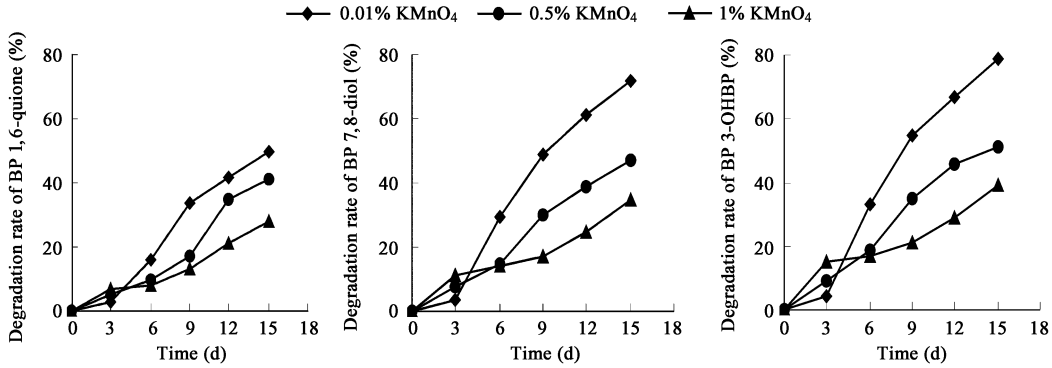

Fig. 3 Degradation of BP 1,6-quinone, BP 7,8-diol, and 3-OHBP at different concentrations of $KMnO_4$

the fungus because *Penicillium chrysogenum* can more easily degrade the metabolites after detoxification by $KMnO_4$. Therefore, 0.01% $KMnO_4$ in culture seems to be the best concentration for these experiments; it can provide maximum oxidation potential and mitigate the effect of toxicity on the fungus. On the day 15, the degradation rates of BP 1,6-quinone, BP 7,8-diol, and 3-OHBP were 50%, 72%, and 79%, respectively.

2.4 Effect of degradation of the three metabolites at different concentrations

It can been seen from Fig.4 that during the first 3 d, there was little obvious difference in the degradation rates of the three metabolites when they were at different concentrations. After 3 d, their degradation rates showed a rapid increase when their concentrations were all 5 mg/L; in contrast, there was a slow increase when their concentrations in the cultures were 10 mg/L because higher concentrations of the three metabolites have stronger toxicity to the fungus initially, and more time is required for the fungus to adapt to the toxicity of the metabolites. However, extremely low concentration would reduce usability to microorganisms. So, 5 mg/L was the optimum concentration of the three metabolites. On the day 15, the degradation rate of BP 1,6-quinone, BP 7,8-diol, and 3-OHBP was 51%, 70%, and 82%, respectively.

2.5 Effect of pH

Suitable pH value may play an important role in coupling chemical and biological methods. Higher acidity can strengthen oxidation of $KMnO_4$. But fungus *Penicillium chrysogenum* can only survive under condition of weak acidity. Fig.5 shows that during the first 3 d, there was little obvious difference in the degradation rates of the three metabolites when the cultures were at different pH values. After 3 d, their degradation rates showed a rapid increase at a pH value of 6.2, which was considered the most suitable in this experiment. On the day 15, the degradation rates of BP 1,6-quinone, BP 7,8-diol, and 3-OHBP was 59.5%, 75.9%, and 88.6%, respectively. Furthermore, the degradation rates of BP 1,6-quinone was the lowest, whereas the 3-OHBP was the highest at all time points.

In summary, as the metabolites of BaP, BP 1,6-quinone is more liable to be accumulated. Optimum conditions of degradation of BP 1,6-quinone, BP 7,8-diol, and 3-OHBP are: concentration of $KMnO_4$ was 0.01% (w/v), concentration of each of the three metabolites was 5 mg/L, and pH was 6.2.

2.6 Degradation of BaP and accumulation of metabolites

Fig.6 shows that BP 1,6-quinone, BP 7,8-diol, and 3-OHBP were gradually generated and degraded with the degradation of BaP. Fig.6a shows the method involving coupling *Penicillium chrysogenum* and $KMnO_4$, and Fig.6b shows the method involving only *Penicillium chrysogenum*. The rate of accumulation of the three metabolites depends on their rates of production and degradation. Generally, a maximum accumulation rate was reached after 6–12 d.

Fig.6a shows that accumulation of BP 7,8-diol and 3-OHBP were more lower than that in Fig.6b. Accumulation

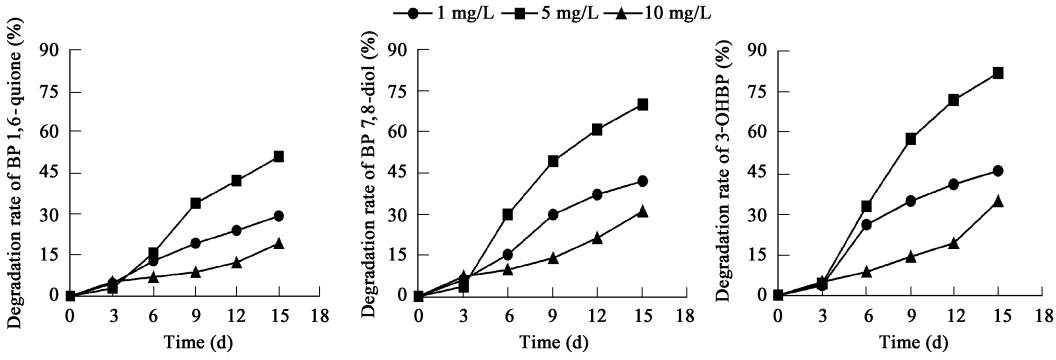

Fig. 4 Degradation of BP 1,6-quinone, BP 7,8-diol, and 3-OHBP when they are of different concentrations.

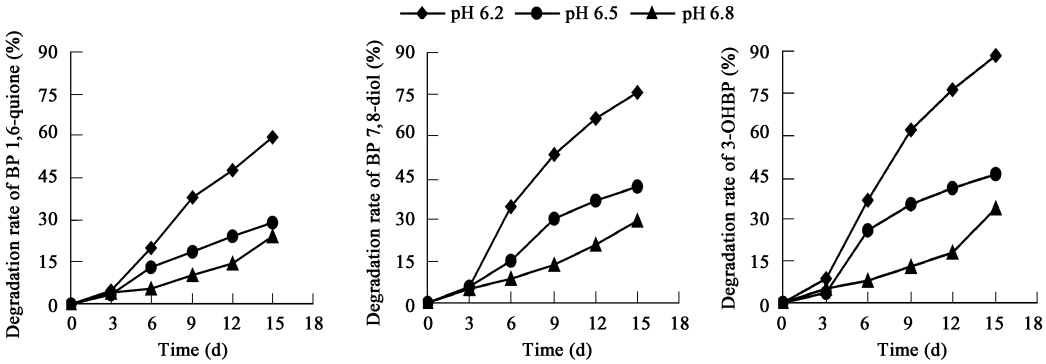

Fig. 5 Degradation of the BP 1,6-quinone, BP 7,8-diol, and 3-OHBP at different pH values.

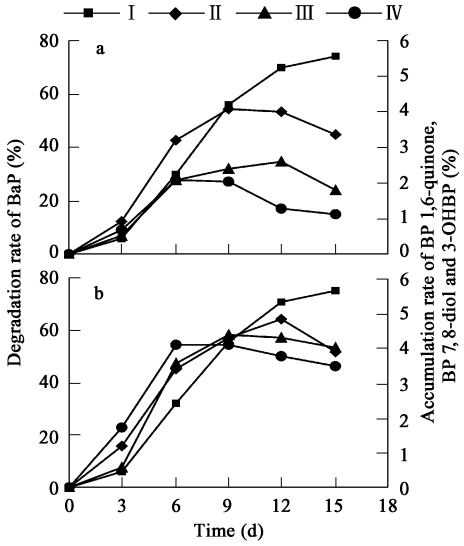

Fig. 6 Degradation of BaP and the accumulation of the three compounds with degradation of BaP under different conditions. (a) BaP was degraded by coupling the *Penicillium chrysogenum* and $KMnO_4$; (b) BaP was degraded only by *Penicillium chrysogenum*. (I) degradation of BaP; (II) accumulation of BP 1,6-quinone; (III) accumulation of BP 7,8-diol; (IV) accumulation of 3-OHBP.

of BP 1,6-quinone with time was higher, which further confirmed that BP 1,6-quinone was more liable to be accumulated. In the experiment, however, there was little difference in the degradation rates of the parent compound (BaP).

From the experimental results, it is proposed that the degradation of these metabolites would be of great significance in view of remediation of BaP contamination in environment, if the optimum conditions of degradation of BaP and its metabolites are coupled with the above method.

3 Conclusions

Three main metabolites of BaP formed by degradation by *Penicillium chrysogenum*, BP 1,6-quinone, BP 7,8-diol, and 3-OHBP, were identified by HPLC with standards; coupling the *Penicillium chrysogenum* and $KMnO_4$ was an effective method for the degradation of BP 1,6-quinone, BP 7,8-diol, and 3-OHBP, which was first used in the degradation of PAHs; as one of the metabolites, BP 1,6-quinone was the most liable to be accumulated, whereas 3-OHBP was more liable to be degraded in pure cultures; in coupling method, optimum parameters were as follows: the concentration of $KMnO_4$ was 0.01% in the cultures, concentration of the three metabolites were all 5 mg/L, and pH value of the cultures was 6.2; the coupling method has been successfully applied for the degradation of BaP, and the accumulation rates of the three metabolites was much lower than the degradation only by *Penicillium chrysogenum*. It is thus evident that degradation and aging of BaP and accumulation of its metabolites are interesting areas of research in future.

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