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Biodegradation of anthracene by *Aspergillus fumigatus*

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ABSTRACT

An anthracene-degrading strain, identified as *Aspergillus fumigatus*, showed a favorable ability in degradation of anthracene. The degradation efficiency could be maintained at about 60% after 5 d with initial pH of the medium kept between 5 and 7.5, and the optimal temperature of 30 °C. The activity of this strain was not affected significantly by high salinity. Exploration on co-metabolism showed that the highest degradation efficiency was reached at equal concentration of lactose and anthracene. Excessive carbon source would actually hamper the degradation efficiency. Meanwhile, the strain could utilize some aromatic hydrocarbons such as benzene, toluene, phenol etc. as sole source of carbon and energy, indicating its degradation diversity. Experiments on enzymatic degradation indicated that extracellular enzymes secreted by *A. fumigatus* could metabolize anthracene effectively, in which the lignin peroxidase may be the most important constituent. Analysis of ion chromatography showed that the release of anions of *A. fumigatus* was not affected by addition of anthracene. GC-MS analysis revealed that the molecular structure of anthracene changed with the action of the microbe, generating a series of intermediate compounds such as phthalic anhydride, anthrone and anthraquinone by ring-cleavage reactions.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of chemical compounds consisting of carbon and hydrogen, arranged in the form of two or more aromatic rings. As a result of incomplete combustion of organic matter in automobile exhaust, petrochemical industry, or accidental spills during the transportation of petroleum, PAHs become ubiquitous contaminants in the environment [1,2]. Since PAHs exhibit carcinogenic, teratogenic, mutagenic and other toxic properties [3], as well as their characteristics such as bioaccumulation, biomagnification and persistent toxicity, PAHs have posed serious risks to the environment and human health. Consequently, PAHs have raised great environmental concern all over the world, and the USEPA has listed 16 PAHs as priority pollutants [4].

Although PAHs released into the environment may undergo chemical oxidation, photolysis, volatilization and adsorption on sediment and soil particles, the main pathway for their removal is probably through microbial transformation and degradation [4]. Compared with other physical and chemical methods such as combustion, photolysis, landfill and ultrasonic decomposition, biodegradation is expected to be an economic and environmentally friendly alternative for removal of PAHs [5], thus, more and more research interests are turning to the biodegradation of PAHs.

Some microorganisms can utilize PAHs as a source of carbon and energy so that PAHs can be degraded to carbon dioxide and water, or transformed to other nontoxic or low-toxic substances [6,7]. Also, the indigenous microorganisms that are able to tolerate and degrade PAHs could exist in long PAHs contaminated waters, soils or sediments [8,9]. Accordingly, isolating high-efficient degrading microorganisms from natural environment is a feasible and important technique [10]. Experiments on evaluating the degradative ability of microorganisms have been conducted, meanwhile, several microorganisms that exhibit outstanding ability to degrade PAHs have been screened out, including bacteria, fungi, and even algae [11,12]. Among these microorganisms, some could utilize PAHs (mainly low molecular weight PAHs) as the sole carbon and energy source, however, high molecular weight PAHs rarely served as the sole carbon and energy source. In this case, co-metabolic degradation may occur. Herwijnen et al. examined the strain *Sphingomonas* sp. LB126, which could use fluorene as the sole carbon and energy source and further degrade phenanthrene, fluoranthene, anthracene and dibenzothiophene co-metabolically [13]. Luan et al. suggested that a consortium of bacteria could degrade fluorene, phenanthrene and pyrene [14]. It was also reported that strains *Pseudomonas aeruginosa* and *Pseudomonas citronellolis* could grow in media containing phenanthrene and pyrene, illustrating that

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these two bacteria could metabolize PAHs [15]. Besides these bacteria, some fungi are also good degraders of PAHs, among which the white rot fungi have received extensive attention. Leonardi et al. found that two white rot fungi—*Irpex lacteus* and *Pleurotus ostreatus* performed well in degrading PAHs [16]. From these studies, it is obvious that the biodegradation has become a promising and attractive approach to deal with contamination of PAHs.

Anthracene is a three-ring PAH with relatively serious toxicity. Once anthracene enters the body, it appears to target the skin, stomach, intestines and the lymphatic system, and it is a probable inducer of tumors [17]. In this study we choose anthracene as a model compound of the PAH family to study its biodegradation.

The main objective of the present work was to study anthracene biodegradation using *Aspergillus fumigatus* isolated from the aged crude petroleum-contaminated soil. The investigation was primarily focused on certain environmental factors which may affect the degradation efficiency, including pH, temperature and concentration of NaCl. Meanwhile, the degradation of different substrates by *A. fumigatus* was also discussed. Additionally, we also explored co-metabolism as well as extracellular enzymatic degradation of anthracene. Ion chromatography (IC) was utilized to investigate the relationship between biodegradation and the release of anions of *A. fumigatus* in the process of anthracene biodegradation. Moreover, the degradation mechanism of anthracene was revealed utilizing gas chromatography-mass spectrometry (GC-MS) and high performance liquid chromatography (HPLC).

2. Materials and methods

2.1. Chemicals

Anthracene (purity ≥ 97%) and methanol (HPLC-grade) were obtained from Sigma–Aldrich. Other reagents were of analytical grade and purchased from Guangzhou Chemical Reagent Factory.

2.2. Preparation of culture medium

Mineral salts medium (MSM) was composed of (g L^{-1} water) 3 NH_4NO_3, 0.5 KH_2PO_4, 0.5 $K_2HPO_4{\cdot}3H_2O$, 0.008 $MgSO_4{\cdot}7H_2O$, 0.002 $CuSO_4{\cdot}5H_2O$, 0.002 $MnSO_4{\cdot}H_2O$, 0.002 $FeSO_4{\cdot}7H_2O$ and 0.002 $CaCl_2{\cdot}2H_2O$ [18]. The pH was adjusted to 7.5 with either HCl or NaOH solutions. Potato-sucrose medium was composed of 20 g L^{-1} sucrose and 500 mL L^{-1} potato extract prepared from 200 g potatoes. If the strain needed to be maintained on agar slants, agar was added to medium at a concentration of 16 g L^{-1}. Culture media were sterilized in an autoclave at 110 °C for 30 min.

2.3. Microorganism and culture condition

Anthracene-degrading strain was isolated from contaminated soils near a gas station and identified as *Aspergillus fumigatus* based on morphology and 18s rRNA gene sequencing.

For enrichment of *A. fumigatus*, one loop of culture maintained on slants was inoculated in 100 mL of potato-sucrose medium in a 250 mL Erlenmeyer flask and incubated at 30 °C with shaking at 130 r min^{-1} for 36 h. There were some pellets formed in the medium. The cell suspension was centrifuged at 10,000 × *g* for 10 min at 4 °C, then the pellets were collected and washed with distilled water for removal of some impurities and nutrient materials. After these procedures, the pellets could be used in anthracene biodegradation.

2.4. Biodegradation activity of A. fumigatus

In this section, all biodegradation experiments were carried out in MSM to which anthracene was added at certain concentration. The flask with 20 mL of the above solution was inoculated with 1 g *A. fumigates* (wet weight) which was prepared according to Section 2.3. This dosage was based on our previous experiment which showed that the addition of 1 g *A. fumigatus* could degrade anthracene efficiently.

2.4.1. Effect of certain factors on anthracene biodegradation

The influence of pH, temperature and concentration of NaCl on anthracene biodegradation was assessed. The concentration of anthracene was 10 mg L^{-1} in these experiments. The initial pH of MSM was adjusted to 4.0, 5.0, 6.0, 7.0, 7.5, 8.0, 9.0 and 10.0 and incubated at 30 °C. The incubation temperatures were set at 20, 25, 30, 35 and 40 °C, while the different NaCl concentrations were 0, 2, 4, 6, 8 and 10 g L^{-1}, individually. Controls were run in parallel where flasks were un-inoculated. All solutions were agitated in the dark at 130 r min^{-1} for 5 d. After incubation, samples of the whole flask were collected to measure the residual anthracene.

2.4.2. Effect of lactose on anthracene biodegradation

The effect of lactose concentration on degradation was investigated by using lactose concentrations at 0, 2, 5, 10, 15, 20, 30 and 50 mg L^{-1}. After inoculation of *A. fumigatus*, all samples were incubated in a rotary shaker for 5 d.

2.4.3. Degradation of different substrates by A. fumigatus

The different substrates including benzene, toluene, phenol, salicylic acid, dimethylbenzene, benzoic acid, hydroquinone, catechol, 1-naphthol and 2-naphthol were added to the sterilized MSM, with the concentrations of each substrate being 5, 10, 25, 50 and 100 mg L^{-1} individually. The pH values were adjusted to 7.5 with either HCl or NaOH solutions. These solutions were inoculated with *A. fumigatus* and incubated at 30 °C in a rotary shaker in the dark at 130 r min^{-1} for 5 d. Then, the culture medium was centrifuged at 10,000 × *g* for 10 min at 4 °C and the pH value of supernatant was measured by pH meter. The sample without any substrates was set as blank control.

2.5. Role of intra- and extra-cellular enzymes in biodegradation of anthracene

Based on the preliminary work which had verified *A. fumigatus* could effectively metabolize anthracene [19], the enzymatic degradation was further explored in order to find out the distribution of enzyme produced by *A. fumigatus* as well as its degradation function.

The culture medium which had inoculated with *A. fumigatus* and incubated for 36 h was centrifuged at 10,000 × *g* for 10 min at 4 °C. Then the pellet was resuspended in distilled water and broken up ultrasonically, followed by centrifugation for 20 min. The supernatant was filtered with 0.22 μm pore-size filters and then the filtrate was considered the crude intracellular enzyme extract. The free cell suspension of the culture medium was collected simultaneously, and also filtered with 0.22 μm filters, then the filtrate was considered the crude secreted extracellular enzyme.

A. fumigatus, intracellular and extracellular enzymes were added separately to anthracene (10 mg L^{-1}) containing aqueous system, and flasks with intracellular and extracellular enzymes did not inoculate with *A. fumigatus*. The concentrations of enzymes were determined by the weight of *A. fumigatus*. The heavier the weight of *A. fumigatus* is, the higher the concentrations of enzymes. The anthracene biodegradation was carried out at 30 °C with shaking. Samples were withdrawn periodically to determine residual concentrations of anthracene. Un-inoculated flasks were served as control at each time point.

2.6. Ion chromatography

The samples degraded for 5 d were centrifuged at 10,000 × g for 10 min at 4 °C, then the resultant supernatant was eluted with C18 column to remove anthracene. One mL clear supernatant was diluted and filtered with 0.22 μm pore-size filters. Afterwards, F^-, Cl^-, Br^-, NO_2^-, NO_3^-, PO_4^{3-} and SO_4^{2-} were detected by an ICS-900 Ion Chromatography System consisting of a pump, an injection valve, and a conductivity cell (Dionex, Sunnyvale, USA). Eluent flow rate was set at 1.2 mL min^{-1} with an injection volume of 10 μL. An Dionex IonPac® AS14 (4 mm × 250 mm) analytical column was used to separate the anions.

Eluent: 3.5 mM Na_2CO_3, 1.0 mM $NaHCO_3$; flow rate: 1.2 mL min^{-1}; detection: suppressed conductivity using a CD25; suppressor: Anion Self-Regenerating Suppressor (ASRS®-ULTRA II, 4 mm), AutoSuppression® Recycle Mode; applied current: 100 mA; injection volume: 10 μL; storage solution: eluent.

Concentrations of anions released by *A. fumigatus* in solution without anthracene within 30 min and 5 d were studied as control, and all these tests were performed using three replicates.

2.7. Research on degradation mechanism of anthracene

In our former experiments, FTIR observation of the extracted samples which had been degraded by *A. fumigatus* for 5 d exhibited that the structure of anthracene changed with the action of *A. fumigatus*, generating a series of metabolites, such as aromatic acid, aromatic ketone, aromatic aldehyde with one or two benzene rings, as well as saturated hydrocarbons [19]. Here, GC-MS was utilized to further explore the metabolites of anthracene biodegradation.

After biodegradation for certain time, the potential metabolites in anthracene degradation were extracted with CH_2Cl_2. The extracts were dried over Na_2SO_4, concentrated and filtered prior to analysis by GC-MS [20]. GC-MS analyses were carried out on QP2010 (Shimadzu, Japan) equipped with a type Rxi-5MS GC column (30 m × 0.25 mm × 0.25 μm). Helium was used as the carrier gas with a constant flow of 1.50 mL min^{-1}. The column temperature program started at 80 °C, held for 5 min, then the oven was heated to 250 °C at a rate of 10 °C min^{-1}, followed by an increase to 280 °C at 15 °C min^{-1} and held for 5 min. The solvent cut time was set to 4 min. The GC-MS interface temperature was maintained at 250 °C. Mass spectra were recorded at 1 scan s^{-1} under electronic impact with electron energy of 70 eV, mass range 50–550 amu (atom to mass unit). The temperature of ion source was set at 220 °C. One μL sample was injected directly.

2.8. Analytical method of anthracene

Anthracene was extracted with CH_2Cl_2 for estimation of anthracene left in medium. The pH of medium was adjusted to 2.0, and an equal volume of CH_2Cl_2 was added in a separation funnel. The mixture was vigorously shaken for 10 min and allowed to set until phase separation. The organic phase was removed and then the operation was repeated again. The organic part was collected and water was removed with Na_2SO_4, followed by concentrating using a rotary evaporator at 35 °C. Subsequently, the residues were dissolved in CH_2Cl_2. Finally, this solution was diluted to a proper concentration with HPLC-grade methanol and filtered with 0.22 μm pore-size filters.

Anthracene was analyzed by HPLC (Shimadzu, Japan) using an ultraviolet detector. Chromatographic separation was achieved with a C18 reverse-phase column (dimensions 0.25 mm × 150 mm). Samples of 20 μL were injected via an autosampler and were eluted under isocratic conditions with 85% methanol and 15% water, at a constant flow rate of 1.0 mL min^{-1}. Peaks eluting from the column were detected by their absorbance at 254 nm by an ultraviolet detector. The average recovery percentage of anthracene by this method ranged from 75.7% to 107.49%, with a detection limit of 5.79 μg/L.

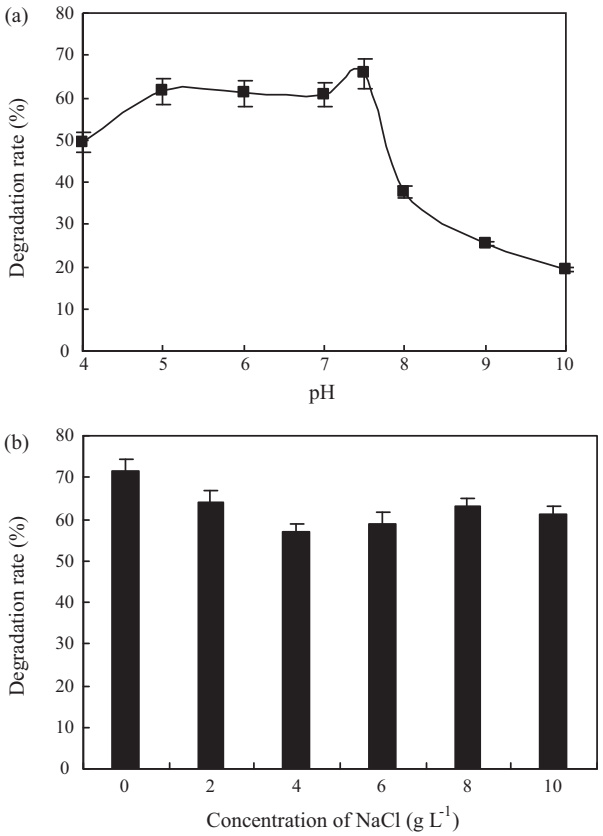

Fig. 1. Effect of pH and NaCl concentration on anthracene biodegradation. Cells were grown for 5 d with shaking at 130 r min^{-1} (30 °C) at different pH (a) or different NaCl concentration (b) in liquid MSM medium consisting of 10 mg L^{-1} anthracene.

2.9. Statistical analysis

All of these experiments were performed in triplicates and the results presented were average values of the three replicates. The standard deviations for all measurements ranged from 1% to 8%.

3. Results and discussion

3.1. Biodegradation activity of A. fumigatus

3.1.1. Relationship between anthracene biodegradation and incubation conditions

The effect of pH on anthracene biodegradation by *A. fumigatus* was depicted in Fig. 1(a). It suggested that the pH value of MSM could affect anthracene degradation to a certain extent. When pH ranged from 5.0 to 7.5, the biodegradation efficiency maintained above 60%, showing no significant difference, and reached the highest point at pH 7.5, while as pH was reduced to 4.0, the degradation efficiency dropped to 49%. However, the most significant effect on anthracene degradation was observed over the pH range of 7.5–10.0. Thus, it could be inferred that the *A. fumigatus* could exhibit better efficacy on biodegradation of anthracene in pH range of 5.0-7.5 than in the relative more acidic or alkaline conditions and that this strain had favorable adaptability to the pH change to a certain extent. Jacques et al. studied biodegradation by *Pseudomonas* sp. isolated from a petrochemical sludge landfarming site and also indicated that this strain could degrade anthracene

faster over the pH range 5.0–9.0, while the degradation efficiency obviously reduced at pH 10.0 [15]. This conclusion was similar to the current study.

Temperature is an important factor affecting the growth and activity of microorganisms. The optimum temperature for biodegradation of anthracene by *A. fumigatus* in our experiment was 30 °C with a degradation efficiency of 65%. The degradation ability increased with temperature from 20 to 30 °C. Nevertheless, higher degradation efficiencies could not be further achieved by continuing increase of temperature. The degradation efficiency was only 27% at 35 °C. In our experiment at 40 °C, the medium was nearly dried after shaking for 5 d. This was attributed to the evaporation of water and anthracene at higher temperatures, then, the anthracene in water was also reduced through this physical process.

As illustrated in Fig. 1(b), biodegradation efficiency all decreased to some extent after the addition of NaCl. Although NaCl resulted in the decrease of degradation rate compared with control, the degradation efficiencies were generally kept around 60%. This result indicated that the high salinity tested did not significantly reduce the activity of *A. fumigatus*.

3.1.2. Co-metabolism of anthracene

Because PAHs are toxic to microorganisms, and they are not the essential nutrients for microorganism growth, the microorganisms require a period of time to adapt to the PAHs contaminated environment. In order to shorten this adaption period and accelerate degradation of contaminants, some other carbon sources which microorganisms can easily utilize may be added. But there exists another problem that addition of a carbon source may inhibit the biodegradation of contaminants and result in diauxic growth [21]. Hence, it is necessary to explore the effects of different additional carbon sources as co-metabolic substances. According to our previous study, lactose was proved to be the optimal additional carbon source [19].

The effect of lactose concentration on degradation was investigated by using lactose concentrations at 0, 2, 5, 10, 15, 20, 30 and 50 mg L^{-1}. After inoculation of *A. fumigatus*, all samples were incubated in a rotary shaker for 5 d.

Fig. 2(a) showed that degradation efficiency increased with increasing concentration of lactose ranged from 0 to 10 mg L^{-1}, then the further increase of lactose exerted suppressive effect on anthracene biodegradation. Therefore, the lactose concentration was not the higher, the better. This experiment suggested that the highest degradation rate could be achieved with the addition of 10 mg L^{-1} lactose when dealing with 10 mg L^{-1} anthracene, i.e., the ratio of anthracene and lactose was 1:1.

It was worth further exploring why the microorganism did not perform well under high concentration of carbon source. There may be two explanations. One was that when there existed high concentration of additional carbon source, the strain would mainly metabolize this carbon source, consequently reducing the degradation of the target contaminants. The other was excessive carbon source would pose detrimental effect on microorganism growth due to the changes in pH. So a series of degradation experiments were carried out in MSM with lactose concentrations at 0, 2, 5, 10, 15, 20, 30 and 50 mg L^{-1}. After 5 d incubation, the pH value of MSM was measured (Fig. 2(b)).

The change of pH in MSM with different lactose concentrations in Fig. 2(b) was in accordance with the variation of degradation efficiency reflected by Fig. 2(a) that the higher the degradation efficiency, the lower the pH of MSM. The pH value reached the lowest point with the lactose concentration at 10 mg L^{-1}, while the degradation efficiency of anthracene was at its summit as shown in Fig. 2(a), indicating that there was an accumulation of certain quantity of acid substances during the degradation process. It can be seen

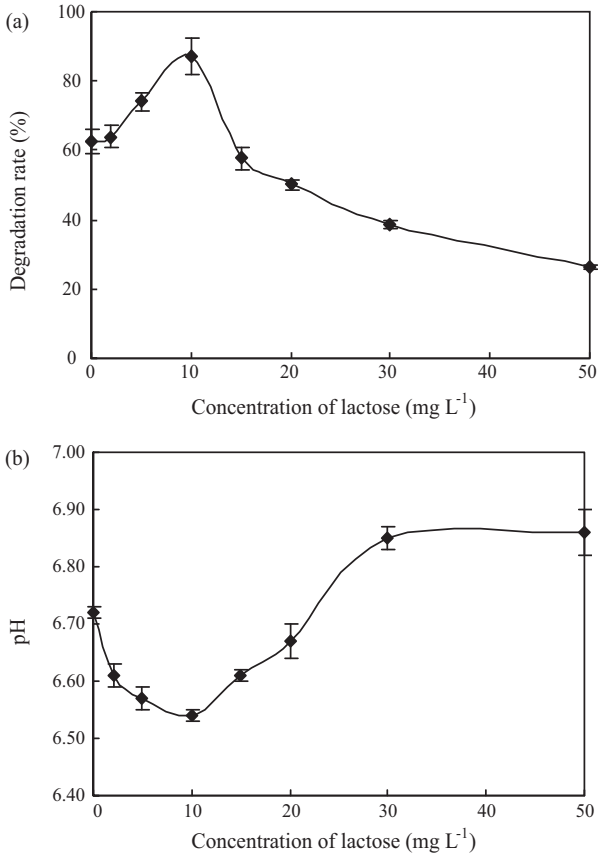

Fig. 2. (a) Degradation efficiency under different lactose concentration. Cells were grown at 30 °C for 5 d with shaking at 130 r min^{-1} in liquid MSM medium (pH 7.5) consisting of 10 mg L^{-1} anthracene. (b) Change of pH under different lactose concentration. Cells were grown at 30 °C for 5 d with shaking at 130 r min^{-1} in liquid MSM medium (pH 7.5) consisting of 10 mg L^{-1} anthracene.

from Fig. 2(b) that pH value was not low even when lactose concentration was quite high. Therefore, the low degradation efficiency of anthracene did not result from the death of microorganisms caused by the lowering of pH, but probably from the excessive usage of lactose at higher concentrations.

Then, the effect of lactose on degradation speed in several successive days was taken into account. *A. fumigatus* was inoculated into MSM with 10 mg L^{-1} lactose and anthracene, while the un-inoculated flasks and flasks without lactose were served as control. The biodegradation was carried out for 7 d and the residual anthracene as well as pH value of MSM was monitored each day. The degradation efficiency of anthracene and pH values were plotted versus time as depicted in Fig. 3.

It was evident from Fig. 3(a) that in the samples with lactose, the degradation was delayed during the first 3 d. This was because *A. fumigatus* mainly utilized lactose at this time interval. From the 3rd day on, the degradation efficiency sharply increased. From day 5 to day 6, in the samples with lactose, degradation efficiency was higher than the control. However, the final degradation efficiencies were not significantly different. These phenomena indicated that the appropriate additional carbon source could accelerate degradation, but the final degradation efficiency was directly dependent on the ability of microorganisms. Nevertheless, it was still significant that the biodegradation of this organic contaminant with low bioavailability could be accelerated by adding some carbon source.

The two curves in Fig. 3(b) showed similar trend that pH declined during biodegradation. It indicated that there were differences between the degradation speed of contaminants and the accumulation of metabolites in these two situations, especially in

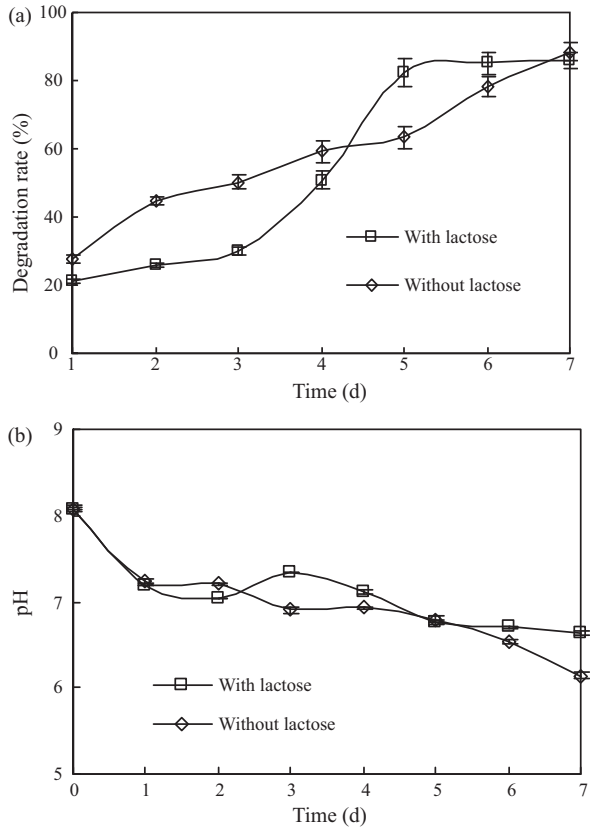

Fig. 3. (a) Change of degradation rate with degradation time in process of anthracene degradation with/without lactose. Cells were grown at 30 °C for 5 d with shaking at 130 r min^{-1} in liquid MSM medium (pH 7.5) consisting of 10 mg L^{-1} anthracene. (b) Change of pH with degradation time in process of anthracene degradation with/without lactose. Cells were grown at 30 °C for 5 d with shaking at 130 r min^{-1} in liquid MSM medium (pH 7.5) consisting of 10 mg L^{-1} anthracene.

day 7, the variation in pH was obvious since the different pH values of 6.64 and 6.14 were observed in the samples with or without lactose. It can be explained that there were some acidic metabolites such as organic acid produced in the degradation of anthracene.

3.1.3. Biodegradation range of substrate by A. fumigatus

The pH values of media with different substrates were exhibited in Table 1. Compared with blank control, the pH values all changed to some extent in medium with each substrate. It could be explained that *A. fumigatus* had utilized these substrates and yielded some products, thereby the pH was changed. In addition, it was found that in these ten substrates, the pH of each medium declined except benzoic acid, and the pH of medium containing

Table 1
pH values of medium with different substrates.

Substrate	Concentration (mg L^{-1})				
	5	10	25	50	100
Benzene	6.82	6.90	6.79	6.95	6.82
Toluene	6.51	6.58	6.76	6.22	6.71
Phenol	7.06	7.05	6.87	6.99	7.02
Salicylic acid	6.76	6.78	6.46	6.72	6.74
Dimethylbenzene	6.47	6.32	6.57	6.38	4.26
Benzoic acid	7.32	7.15	7.33	7.44	8.00
Hydroquinone	6.69	6.57	6.70	6.60	6.51
Catechol	6.80	6.70	6.76	6.53	6.49
1-Naphthol	6.46	6.45	6.48	6.41	6.57
2-Naphthol	6.43	6.56	6.36	6.49	6.46

The pH of blank control was 7.52.

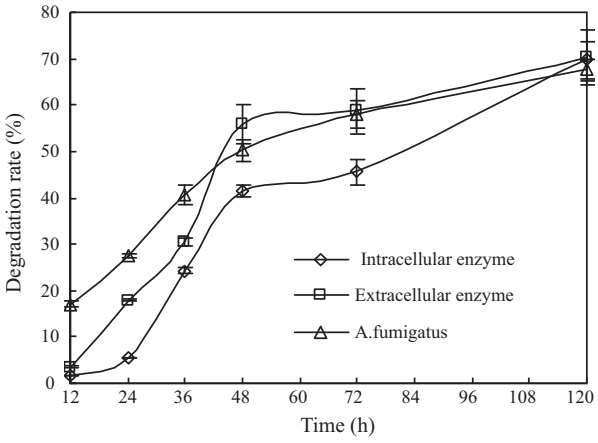

Fig. 4. Degradation process of anthracene by *A. fumigatus*, intracellular and extracellular enzymes. The temperature was 30 °C, and the initial concentration of anthracene was 10 mg L^{-1}, shaking at 130 r min^{-1}.

phenol changed little, which was very close to 7.00. Change of pH could reflect the degradation of some substrates to a certain extent. Aromatic hydrocarbons are usually weak-ionizing or non-ionizing, if they are degraded, acid substances will be produced, resulting in declining of medium pH. The weaker the degradation of substrates is, the less obvious the change of pH. The experimental results showed that when phenol was used as substrate, pH of medium changed little, which illustrated the strain might barely degrade this substance. However, for benzoic acid, pH seemed nearly no change or even went up after biodegradation. Because this substance was acidic itself, the rising of pH showed it was degraded by *A. fumigatus*. From these groups of experiments it was seen that *A. fumigatus* could utilize some aromatic hydrocarbons as sole source of carbon and energy, indicating its degrading diversity.

3.2. Role of intra- and extra-cellular enzymes in biodegradation of anthracene

The experiment was conducted according to Section 2.5. The degradation curves by *A. fumigatus* intact cells, intracellular and extracellular enzymes in 7 d were presented in Fig. 4. From 0 to 36 h, the degradation rate by *A. fumigatus* cells was higher than those of enzymes, which was ascribed to the adsorption of anthracene by the cells. After 36 h, the effect of enzymes, especially extracellular enzymes, became obvious. Between 36 and 48 h interval, the degradation by extracellular enzymes was faster than the other two samples. After 48 h, the increasing of degradation rate of extracellular enzymes slowed down. This result also indicated that although there was an action of adsorption by the cells, the final degradation rates by cells or enzymes exhibit no significant differences.

It was initially inferred that the degradation of anthracene was mainly due to the activities of extracellular enzymes. Eibes et al. indicated that two extracellular enzymes, manganese peroxidase (MnP) and lignin peroxidase (LiP), were associated with the degradation of PAHs [22]. The presence and activity of these extracellular enzymes are highly dependent on manganese and nitrogen in the medium, and these biochemical materials can be triggered by nitrogen limited medium. In order to explore whether the degradation was affected by MnP and/or LiP, nitrogen limited medium was used in *A. fumigatus* culture.

In the MSM used in early experiments, all nitrogen came from NH_4NO_3 at 3.0 g L^{-1}. Here, NH_4NO_3 concentrations were set at 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 and 5.0 g L^{-1} in the following tests.

The degradation rate was the highest in medium without any addition of NH_4NO_3, and 87% of anthracene was removed in

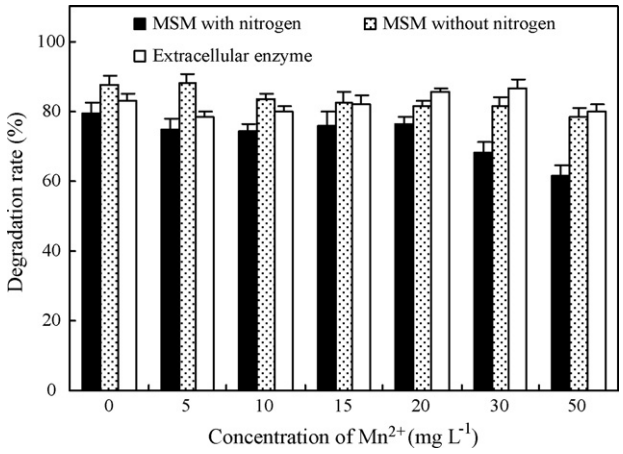

Fig. 5. Degradation efficiency under different Mn^{2+} concentrations. The temperature was 30 °C, and the initial concentration of anthracene was 10 mg L^{-1}, shaking at 130 r min^{-1} for 5 d.

5 d. Then, the degradation ability decreased with the increase of NH_4NO_3, but when NH_4NO_3 concentration ranged from 0.5 to 2.0 g L^{-1}, the degradation efficiencies were maintained around 78%. Thus, it can be confirmed that a nitrogen supply in the medium could affect the production of enzymes, thereby reducing the degradation of anthracene. Nitrogen limited medium could induce the production of enzymes. It was inferred that the biodegradation of anthracene by *A. fumigatus* was controlled by MnP and/or LiP. Since the formation of MnP is based on Mn^{2+}, it is necessary to consider how Mn^{2+} affects the biodegradation of anthracene in order to infer whether it is controlled by MnP.

The biodegradation process was tested in the media with different concentrations of Mn^{2+} (0, 5, 10, 15, 20, 25, 30, 50 mg L^{-1}) with or without the addition of NH_4NO_3. In addition, whether Mn^{2+} could affect the activity of extracellular enzymes was also tested simultaneously. All tests were performed for 5 d. The result was sketched in Fig. 5.

It can be seen from Fig. 5 that degradation efficiencies of anthracene were higher in MSM without nitrogen, supporting the conclusion that nitrogen limited media could favor anthracene biodegradation again. These experiments also indicated that the addition of Mn^{2+} could restrain anthracene degradation to a certain extent, especially in MSM with nitrogen, there exhibited obvious degressive trend when Mn^{2+} concentration was high (above 20 mg L^{-1}). This may be due to the toxicity of excessive Mn^{2+} to *A. fumigatus*. Besides, it has been reported that the activities of MnP increased with the increasing concentrations of Mn^{2+}, while in contrast, Mn^{2+} could restrain secretion of LiP. Only when concentration of Mn^{2+} is in an appropriate range, higher activities of LiP and MnP can be exerted simultaneously. The higher initial concentrations of Mn^{2+} would repress the expression of MnP [23], and decrease the enzymatic activities of both LiP and MnP [23,24], resulting in lower degradation rate of anthracene.

Table 2b
Analysis of variance of anion concentrations.

	F	*F*-crit	*P*-value
F^-	0.6925	5.1433	0.5363
Cl^-	0.1903	5.1433	0.8315
NO_2^-	12.9474	5.1433	0.0067
Br^-	–	–	–
NO_3^-	1.0741	5.1433	0.3993
PO_4^{3-}	0.3233	5.1433	0.7356
SO_4^{2-}	0.6609	5.1433	0.5503
All of anions	0.0095	3.5546	0.9905

In MSM without nitrogen, despite that the degradation efficiencies decreased with increasing Mn^{2+} concentrations, this inhibiting effect was slight. Even when Mn^{2+} concentration was up to 50 mg L^{-1}, degradation rate could still reach 78%, while this ability was the highest (88%) when Mn^{2+} concentration was 5 mg L^{-1}. Therefore, it can be inferred that lower concentrations of Mn^{2+} could promote anthracene degradation in the nitrogen limited condition.

Mohammadi and Nasernejad also reported that nitrogen-limited medium could trigger higher activity of these two extracellular enzymes secreted by *Phanerochaete chrysosporium*, but their results showed that 10 mg L^{-1} of Mn^{2+} achieved highest degradation efficiency [25]. The difference between the literature work and the present study were due to the discrepancy of the biodegradation time, anthracene concentrations and temperature.

3.3. Result of IC analysis

There were seven anions to be tested, including F^-, Cl^-, NO_2^-, Br^-, NO_3^-, PO_4^{3-} and SO_4^{2-}. The average concentrations of these anions were presented in Table 2a. From Table 2a, it was observed that the concentrations of samples with *A. fumigatus* and/or anthracene changed to a little extent compared with blank control, but the trend was not obvious. Therefore, analysis of variance was employed to evaluate these data in order to estimate whether the release of anions of *A. fumigatus* was related with anthracene biodegradation. $\alpha = 0.05$ was selected, and the analysis results were summarized in Table 2b.

The difference of each group is significant if $F > F$-crit. Additionally, the *P*-value approach could be used—if *P* is considerably smaller than $\alpha = 0.05$, there is evidence to conclude that the effect is significant. According to Table 2b, it was clear that the difference of concentrations for each anion was insignificant except for NO_2^-, meanwhile, the difference of the whole concentrations of anions was also insignificant. Hence, it could be inferred that the release of anions of *A. fumigatus* was not affected by addition of 10 mg L^{-1} contaminant anthracene, indicating that anthracene of this concentration had little toxicity to degrading strain. It was reflected from this aspect that *A. fumigatus* had favorable ability in anthracene degradation.

Table 2a
Concentrations of anions in process of anthracene degradation.

Anions (mg L^{-1})	Concentrations of anions released by *A. fumigatus* in solution without anthracene within 30 min	Concentrations of anions released by *A. fumigatus* in solution without anthracene within 5 d	Concentrations of anions released by *A. fumigatus* after degrading anthracene for 5 d
F^-	0.0000	0.0108	0.0045
Cl^-	0.8429	0.6225	0.9373
NO_2^-	0.0791	0.0354	0.0000
Br^-	0.0000	0.0000	0.0000
NO_3^-	29.2425	25.6821	25.3066
PO_4^{3-}	7.0206	6.6194	6.5432
SO_4^{2-}	0.5911	0.3593	0.5597

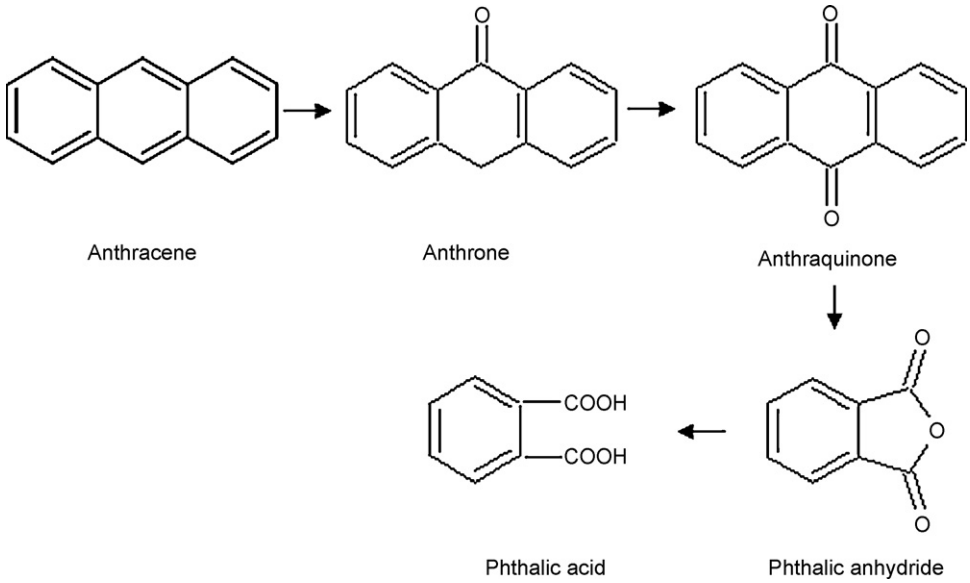

Fig. 6. Proposed pathway of anthracene degradation by *A. fumigatus*.

3.4. Degradation mechanism of anthracene

Initially we conducted biodegradation experiment with *A. fumigatus* and performed GC-MS analysis of extract samples according to Section 2.7. However, multiple peaks were indistinguishable. It was inferred that these peaks came from the secretion of *A. fumigatus*. Since the extracellular enzyme could degrade anthracene, enzymatic degradation was then studied. GC-MS chromatograms of samples from anthracene biodegradation showed peaks with the *m*/*z* of 148, 194 and 208. According to GC-MS library search, they were confirmed as phthalic anhydride, anthrone and anthraquinone, respectively. These compounds were suggested as the products of ring-cleavage reactions. From the structures of anthracene and these three substances, we inferred that phthalic acid and anthraquinone were metabolites of phthalic anhydride and anthrone, individually. Later, we conducted biodegradation experiment on phthalic acid and anthrone using extracellular enzyme. These two experiments lasted for 2 d and the degradation effect was detected using HPLC. The experimental data were shown in Table 3. We can find that phthalic acid and anthrone could be quickly degraded in 2 d, indicating that these metabolites were intermediates, not dead-end metabolites.

The GC-MS analysis of degradation intermediates were in agreement with results of FTIR that the three rings of anthracene opened, producing compounds with one or two rings[19]. Possible degradation sequence is given in Fig. 6.

There were a few reports on the degradation pathway of anthracene. Eibes et al. investigated the enzymatic degradation of anthracene and found several degradation products of anthracene [26]. Their results were similar to ours. Herwijnen et al. elucidated metabolic pathway of anthracene by *Sphingomonas* sp. LB126 [13]. They pointed out a different pathway in which mono- and dihydroxyanthracene in the products were observed and transformed to naphthalene-2, 3-dicarboxylic acid via meta-cleavage. This pathway was quite different from ours and this may be ascribed to the different microorganisms using different enzymes [13]. Moreover, *Sphingomonas* sp. was a prokaryotic bacterium, while *A. fumigatus* was a eukaryotic fungus. This result also indicated that the process and metabolites of anthracene biodegradation were different with individual microorganisms.

Table 3
Degradation of anthrone and phthalic acid.

Time (d)	1	2
Degradation efficiency of anthrone (%)	23.31	82.40
Degradation efficiency of phthalic acid (%)	20.95	71.85

4. Conclusions

This work demonstrated that the *A. fumigatus* isolated from contaminated environment exhibited exceptional ability in degrading anthracene. Maximum biodegradation rate of about 65% was achieved in our experiments when the initial pH was controlled between 5.0 and 7.5, and temperature was set at 30 °C. The NaCl concentration in the media did not affect biodegradation efficiency greater than 20%. These results indicated that this degrading strain could perform well under a wide range of environmental conditions, which warranted further study on bioremediation of anthracene. Our research suggested that when lactose was used as an additional carbon source and its concentration was made equal to that of anthracene, *A. fumigatus* performed best. Except anthracene, *A. fumigatus* could utilize some aromatic hydrocarbons such as benzene, toluene, phenol etc. as sole source of carbon and energy, indicating its degrading diversity.

Research on enzymatic degradation showed that the biodegradation of anthracene was mainly contributed by extracellular enzyme. Between the two possible extracellular enzymes (MnP and LiP), it could be inferred that LiP played the primary role in biodegradation of anthracene. Analysis of IC indicated that addition of anthracene did not affect the release of anions of strain itself. A possible degradation pathway was proposed in which anthracene was firstly degraded to anthrone, which was easily transformed to anthraquinone, and then phthalic acid was produced by ring cleavage.

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