Annals of Microbiology, **57 (4)** 503-508 (2007)

Characterisation of a thermostable catechol-2,3-dioxygenase from phenanthrene-degrading *Pseudomonas* sp. strain ZJF08

Ying ZHOU[1], Jiashi WEI[2], Tao JIANG[1], Weifeng GAO[1], Yushu MA[1]*, Dongzhi WEI[1]*

[1]State Key Laboratory of Bioreactor Engineering, Institute of Biochemistry, East China University of Science and Technology, Shanghai 200237, P. R. China; [2]School of Life Science, FuDan University, Shanghai 200433, P.R. China

Received 22 May 2007 / Accepted 22 October 2007

Abstract - Four strains with high phenanthrene-degrading ability were isolated from petroleum badly polluted soil. The strain *Pseudomonas* sp. ZJF08 demonstrated the highest rate of degradation (138.1 $mg \cdot L^{-1} \cdot day^{-1}$) among them and degraded 97.1% of the phenanthrene in one week. The activities of two key enzymes of ZJF08, polycyclic aromatic hydrocarbon dioxygenase and catechol-2,3-oxygenase (C23O), were also assayed during the degradation of phenanthrene. Both of them reached their maximums on the 2nd day of degradation. The C23O gene (*C7*) of *Pseudomonas* sp. ZJF08 was cloned and expressed in *Escherichia coli*, and its gene product was purified by a Ni-NTA-agarose column. The optimum temperature for the purified C23O was 40 °C at pH 7.5 and the C23O activity could be still detected when the temperature reached 70 °C. The results showed that the C23O from *Pseudomonas* sp. strain ZJF08 exhibited better thermostability than its homologs reported.

Key words: biodegradation, catechol-2,3-dioxygenase, PAH dioxygenase, phenanthrene, thermostability.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants generated from incomplete combustion of organic matter by many anthropogenic activities and pose a serious concern on the health of aquatic organisms and human beings through bioaccumulation (Hughes *et al*., 1997). PAHs exposures occur through inhalation, ingestion and dermal contact. PAHs are highly lipid-soluble and can be quickly absorbed by the gastrointestinal tract of mammals. PAH metabolism in the human body produces epoxide compounds with mutagenic and carcinogenic properties, which were reported to cause cancers in lungs, livers and skins (Samanta *et al*., 2002). PAHs contains a skeleton of carbon with hydrogen atoms arranged in two or more aromatic rings of low solubility which makes them resistant to nucleophilic attack (Johnsen *et al*., 2005). Because of the low bioavailability of the complex mixtures, PAHs are highly limiting for conventional remediation techniques. Bioremediation is not a new strategy for PAH removal (Guieysse *et al*., 2004), and most of the results of *in situ* remediation showed a small rate of degradation. This could be associated with the inability of many microorganisms to degrade PAHs, low solubility of the contaminant and specific nutrient limitation of the biota (Boopathy, 2000). Phenanthrene is one among more than 100 PAHs and is chosen for the study of PAHs degradation because of its relatively strong carcinogenicity, mutagenicity and toxicity (Kanaly and Harayama, 2000; Langworthy *et al*., 2002).

In phenanthrene biodegradation, dihydroxylated aromatics and catechol are subjected to aromatic ring fission by intradiol- or extradiol-type dioxygenases. The intradiol-type dioxygenase opens the aromatic ring by cleavage between two hydroxylated carbons, while extradiol-type dioxygenases opens the aromatic ring by cleavage between the hydroxylated carbon and adjacent nonhydroxylated carbon. PAH dioxygenase (PDO) and catechol 2,3-dioxygenase (C23O) have been identified as two key enzymes during PAH biodegradation, (Resnik *et al.*, 1996; Meyer *et al*., 1999). Catechol and its derivatives are key metabolic intermediates in the aerobic degradation of PAH. C23O thus plays a major role in the metabolism of aromatic hydrocarbons. C23O is an extradiol-type dioxygenase opening the aromatic ring of catechol which serves as the common intermediate in catabolic pathways of monocyclic and polycyclic compounds (Nozaki *et al*., 1982; Kim *et al.*, 2005).

In this study, the C23O gene from chromosomal DNA of *Pseudomonas* sp. ZJF08, a soil bacterium isolated from soil samples heavily polluted with petroleum, was expressed in *Escherichia coli* BL21 (DE3), and its gene product was purified by a Ni-NTA-agarose column. Furthermore, the characteristics of this enzyme were also studied in detail.

* Corresponding authors. Phone: +86-21-64253287; Fax: +86-21-64250068; E-mail: myushu@ecust.edu.cn, dzhwei@ecust.edu.cn

MATERIALS AND METHODS

Sampling and phenanthrene-degrading bacteria isolation. Soil samples were collected aseptically from a layer 0-3 cm deep at the DaQing petroleum field in Northeastern China. Samples were allowed to dry before being crushed and sieved through a 2-mm mesh screen to remove plant and other debris. For the isolation of phenanthrene-degrading bacteria, soil samples (2 g) were added to 100 mL mineral medium (MM: 0.2 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.02 $g \cdot L^{-1}$ $CaCl_2$, 1.0 $g \cdot L^{-1}$ KH_2PO_4, 1.0 $g \cdot L^{-1}$ K_2HPO_4 and 0.05 $g \cdot L^{-1}$ $FeCl_3$, pH adjusted to 7.0 by 10 $mol \cdot L^{-1}$ NaOH) (Kahng *et al.*, 2002) containing 0.1% phenanthrene (97%, HPLC grade, purchased from Fluka). The cultures were incubated at 30 °C with orbital shaking (150 rpm). A 2 mL aliquot was transferred every six days to phenanthrene-containing MM and incubated under the same conditions. After five transfers, 0.5 mL culture was diluted in 5 mL sterilised deionised water, sprayed on MM agar plate (MM with 2% agar and 0.1% phenanthrene) and incubated at 30 °C for 36 h in dark. Colonies were transferred to LB plates for the further experiments.

Phenanthrene biodegradation by the bacterial isolates. The efficiency of the selected isolates in phenanthrene degradation was evaluated by GC analysis using diphenyl as the internal standard. The isolates were transferred to a 250 mL Erlenmeyer flask containing 150 mL LB and harvested at mid-exponential phase by centrifugation at 10000 *x g* for 10 min. The cell pellets were washed with MM and centrifuged again. The washing and centrifugation procedure was repeated for three times. Inocula were prepared by resuspending the cells with MM to give a spectrophotometric reading of 1.0 at 600 nm. The cells were inoculated in 30 mL MM containing 0.1% phenanthrene (six replicates) and incubated at 30 °C for 7 days with orbital shaking (180 rpm). After incubation, the pH was reduced to 2.5 in each flask containing MM, and the cultures were subjected three times to extraction with 15 mL benzene (> 99.5%). Excessive water was removed by adding sodium sulphate. Extracted material was quantified in a gas chromatography (GC model 6890N, Agilent, Palo Alto, CA) equipped with a DB5-5% phenyl methyl siloxane capillary column (30 m x 0.25 mm x 0.25 μm). The injector and transfer line temperature were set at 290 °C, and the temperature program was as follows: 2 min at 100 °C, and then increased to 280 °C with a rate of 20 °C min^{-1}. Aliquot (1 μL) was injected at a split of 1:13 (v/v).

Identification of phenanthrene-degrading isolates. The bacterial isolates were identified by 16S rDNA sequencing as described (Moffett *et al*., 2000). Universal bacterial primers corresponding to *Escherichia coli* positions 8F and 1541R were used for PCR amplification of the 16S rDNA. The DNA template was amplified as follows: initial denaturation at 94 °C for 4 min; followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min and extension at 72 °C for 1 min; and additional extension at 72 °C for 10 min after the cycles. The PCR product was analysed on 0.8% agarose gel and purified using a MK 005-2 gel extraction kit (BioDev, China) according to the manufacturer's instruction. The purified fragment was cloned into pMD18-T Vector (TaKaRa, Japan) and the partial DNA fragment was sequenced by AuGCT (Peking China). Homology analysis was performed by BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

Biodegradation conditions using bacterial isolates. The influence of pH and temperature on the phenanthrene biodegradation by the isolates was assessed using MM containing 0.1% phenanthrene, in triplicates. The biodegradation was carried out separately at 20, 30 and 37 °C. After the optimal temperature had been determined, the pH of MM was adjusted to 6.5, 7.0 and 7.5 by 10 $mol \cdot L^{-1}$ NaOH to determine the optimal pH. The degrading-rate was measured by GC as described above.

Key enzyme assays. The isolate ZJF08 was incubated with MM containing 0.1% phenanthrene. The culture broth was sampled at different time intervals and filtered through glass wools to remove the remaining phenanthrene. The filtrate was centrifuged at 10000 *x g* at 4 °C for 15 min and rapidly washed with 0.1 $mol \cdot L^{-1}$ NaCl. The cells harvested were separated into two parts: one for PDO activity assay, and the other for C23O activity detection.

PDO activity was measured as described by Chen and Aitken (1999). One unit of specific activity was defined as the amount of enzyme that produced 1 μmol product (indigo) $h^{-1} \cdot (mg\ protein)^{-1}$.

To determine C23O activity, the cell pellets were resuspended in 20 $mmol \cdot L^{-1}$ phosphate buffer (pH 7.3) containing 10% (v/v) glycerol, 10% (v/v) ethanol and 0.5 $mmol \cdot L^{-1}$ dithiothreitol. Cell suspension (15 mL) was disrupted with an ultrasonic oscillator at 200 W in an ice bath (3-s period followed by a 3-s interval, 90 times). Particulate matter was removed by centrifugation at 10000 *x g* for 25 min at 4 °C. The supernatant was used to determine the C23O activity by measuring the decrease of the substrate (catechol) by GC analysis using o-cresol as an internal standard. One unit of specific activity was defined as the amount of enzyme that converted 1 μmol substrate $min^{-1} \cdot (mg\ protein)^{-1}$.

The protein concentration was estimated on the basis of total cellular proteins, which were determined by the method of Bradford (1976) using 1 $mg \cdot mL^{-1}$ bovine serum albumin as a standard.

Cloning and nucleotide sequencing of C23O gene (*C7*). The *C7* gene was amplified by PCR (forward primer: 5′-GGAAAGCTTCAATGAAAAAAGGCGTA-3′ with a Hind III site; reverse primer: 5′-GTCTCGAGTTAGGTCAGAACGGTCAT-3′ with an Xho I site). The reaction was performed as described above. The DNA fragment amplified was digested by Hind III and Xho I and ligated with pET-28a plasmid digested by the same enzymes. The recombinant plasmid pET-*C7*, confirmed by restriction enzyme analysis and sequencing, was transformed into *E. coli* BL21 (DE3) for expression.

Expression and purification of the C23O in *Escherichia coli*. The preculture of *E. coli* BL21 (DE3) containing the pET-*C7* was prepared by inoculating one colony into 5 mL LB medium supplemented with kanamycin (100 $\mu g \cdot mL^{-1}$) and incubated overnight at 37 °C with orbital shaking (200 rpm). Two millilitres of the overnight culture was transferred to 200 mL LB medium supplemented with kanamycin and further incubated at

37 °C. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mmol·L^{-1} when the OD_{600} of the culture reached about 0.5. The incubation was carried out at 37 °C for another 3 h before the cells were harvested. The cell pellets were washed in a half volume of PBS (150 mmol·L^{-1} NaCl, 3 mmol·L^{-1} KCl, 10 mmol·L^{-1} Na_2HPO, and 2 mmol·L^{-1} KH_2PO, pH 7.4), and disrupted by sonication. The resultant supernatant was loaded onto a Ni-NTA-agarose column preequilibrated with PBS. The column was washed with the PBS containing 20 mmol·L^{-1} imidazole to remove unbound proteins and then eluted with a "stepwise" gradient of imidazole (60, 100, 300, 500 mmol·L^{-1}) in PBS. Eluates were collected and analysed by 12% SDS-PAGE. After dialysis against buffer A (20 mmol·L^{-1} Tris-HCl, 150 mmol·L^{-1} NaCl, pH 7.5), the purified enzyme was stored at -20 °C.

Catechol 2,3-dioxygenase assays. C23O activity was assayed in buffer A. To determine the influence of temperature on the purified enzyme activity, 5.4 x 10^{-3} mg purified enzyme was pre-incubated separately at 20, 30, 40, 50, 60, 70 and 80 °C for 10 min in buffer A, and then the reaction was performed at the corresponding temperatures by adding catechol to a final concentration of 50 mmol·L^{-1}. The activity was determined by GC analysis as described above. Five different buffers (with pH 5.5, 6.5, 7.5 8.5 and 9.5, respectively) were also used to test the effect of pH on the enzyme activity. One unit of specific activity was defined as the amount of enzyme that converted 1 μmol substrate min^{-1}·(mg protein)$^{-1}$.

RESULTS

Isolation and selection of phenanthrene-degrading bacteria

After 42 days of successive transfers under controlled conditions, four selected bacterial isolates grew well in MM containing 0.1% phenanthrene as the sole carbon source. The phenanthrene degrading rates of these four isolates were determined (Table 1). Isolate ZJF08 was the best one that possessed the highest degradation of 97.1 ± 2.8% in 7 days. The percentage of phenanthrene biotransformation by strains ZJF01, ZJF03 and ZJF05 were 93.3, 91.7 and 95.0% after 7 days respectively.

Identification of phenanthrene-degrading isolates

The four isolates were identified by the partial gene sequencing of 16S rDNA. Sequence analysis at NCBI indicated that the partial sequences from ZJF01, ZJF03 and ZJF08 shared 96, 98 and 99% homology with that from *Pseudomonas* sp. (Genbank accession number: DQ213044) respectively. Another one, ZJF05, was characterised as a *Rhizobium* sp. (Genbank accession number: AF345544), as the sequence amplified shared 96% homology with that from the species.

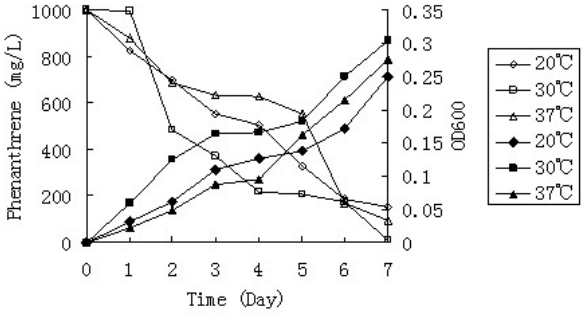

FIG. 1 - Effect of temperature on degradation of phenanthrene by ZJF08 and the growth of ZJF08 in MM containing 0.1% phenanthrene during 7 days. Three different temperatures, 20 °C (◇), 30 °C (□) and 37 °C (△) were used for the degradation and 20 °C (◆), 30 °C (■) and 37 °C (▲) were used for the growth of ZJF08. Data were means from three replicates.

Biodegrading conditions using bacterial isolates

In order to determine the optimal conditions for ZJF08, various temperatures and pH were tested for the degradation. Three different temperatures, 20, 30 and 37 °C, were tested in this study. The highest degrading-rate and best growth (OD_{600} reached 3.03 after 7 days) were observed at 30 °C (Fig. 1). ZJF08 had a better growing trend during incubation at 30 °C. Since pH was another important environmental factor affecting microbial diversity and activity, controlling enzyme activity, transport process and nutrient solubility in remediation (Wong *et al*., 2002), its effect on ZJF08 growing in the MM containing 0.1% phenanthrene was also assessed. The degrading rate was better at pH 6.5 during the first four days but the best degrading rate (98.4%) was observed at pH 7.5 at the end of the degrading process (Fig. 2).

TABLE 1 - Phenanthrene degradation of selected isolates estimated by gas chromatography during the incubation for 7 days in mineral medium containing 0.1% phenanthrene

Isolates	Initial concentration of phenanthrene (mg·L^{-1})	3rd day concentration phenanthrene (mg·L^{-1})	7th day concentration phenanthrene (mg·L^{-1})	Degradation rate (mg·L^{-1}·day^{-1})	Degradation (%)
ZJF01	986.2 ± 2.54	97.3 ± 2.07	66.1 ± 2.65	131.9 ± 0.22	93.3 ± 1.7
ZJF03	817.1 ± 0.37	448.5 ± 1.24	67.8 ± 1.65	107.7 ± 0.09	91.7 ± 2.5
ZJF05	990.6 ± 1.76	471.4 ± 0.67	49.5 ± 1.34	134.5 ± 0.13	95.0 ± 1.6
ZJF08	987.4 ± 1.68	279.8 ± 1.35	38.5 ± 2.13	138.1 ± 0.09	97.1 ± 2.8

Data are mean ± SD from three replicates.

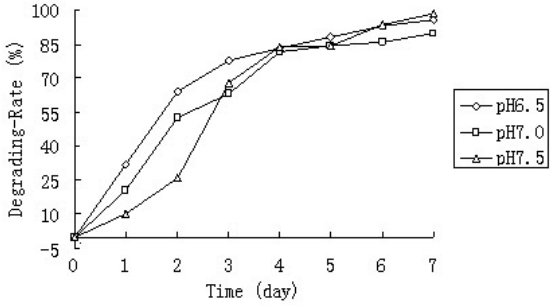

FIG. 2 - Effect of pH on biodegradation by ZJF08 in MM containing 0.1% phenanthrene during 7 days. Three different pH values, 6.5(◇), 7.0(□) and 7.5(△) were used in this study. Data were means from three replicates.

Key enzyme activity assay

When ZJF08 was grown in 0.1% phenanthrene, the PDO activity varied throughout the 7 days incubation. As shown in Fig. 3, on the 2nd day of degradation, the PDO activity reached its maximum of 2.15 U. There was a sharp drop of PDO activity during the 3rd day, reaching the lowest point of 0.63 U. Specific enzymatic activity slowly increased during the 4th day and reached its second peak (1.39 U) on the 5th day, then dropped gradually again.

As depicted in Fig. 4, during the initial two days of degradation, the C23O activity reached its maximum of 117 U, followed by a decrease to 2 U (3rd day) and 0 (4th day). From the 5th day, it began to increase slowly from 0 to 69 U then decreased to 23 U on the 6th day.

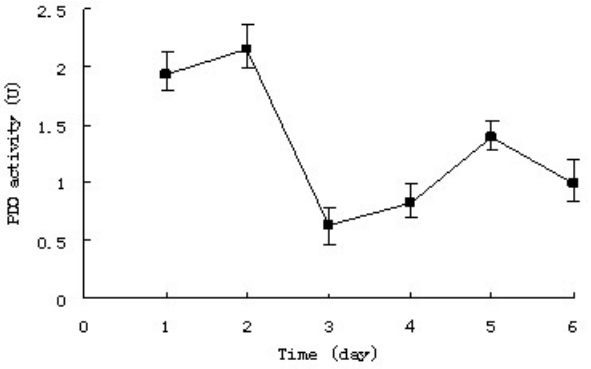

FIG. 3 - PAH dioxygenase (PDO) activity during degradation of 0.1% phenanthrene by ZJF08 at different time intervals. Data were means from three replicates.

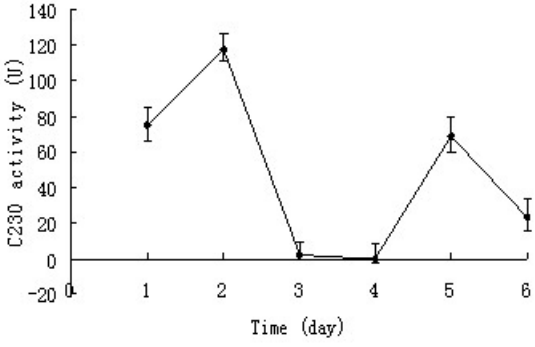

FIG. 4 - Catechol 2,3-dioxygenase (C23O) activity during degradation of 0.1% phenanthrene by ZJF08 at different time intervals. Data were means from three replicates.

Nucleotide sequence of the C23O gene (*C7*)

An open reading frame corresponding to the C23O gene was composed of 924 bp with an ATG initiation codon and a TAA termination codon (Genbank accession number: DQ517339). Sequence analysis by BLAST on GenBank showed that it shared 96% homology with *P. putida* plasmid pWW53 C23O gene (Genbank accession number: AF102891) and there was not any identity with that from stain ZJF05, which was also isolated in our group (data was not shown). The C23O gene *C7* exhibited 57.5% GC content. The open reading frame encoded a polypeptide of 35 kDa containing 308 amino acids, which was in agreement with the size of the C23O as determined by 12% SDS-PAGE.

The expression and purification of the C23O from *Pseudomonas* sp. strain ZJF08

The recombinant C23O could be expressed in *E. coli* BL21 (DE3) with high level after induction for 3 h at 37 °C. SDS-PAGE analysis of the eluates indicated that the fraction eluted by 100 mM imidazole contained a single band about 40 kDa (Fig. 5), which was further identified by activity assay.

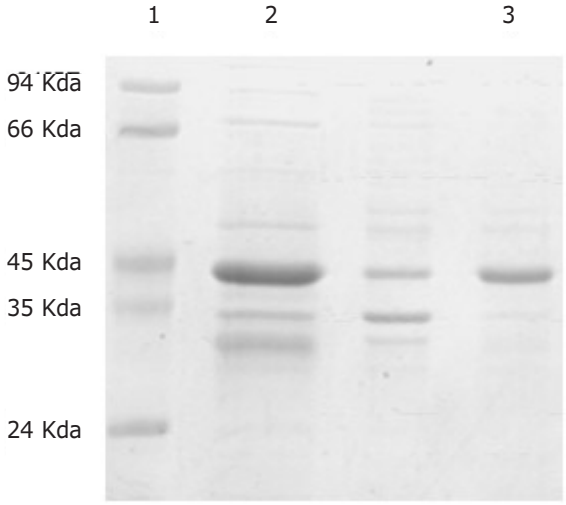

FIG. 5 - SDS-PAGE analysis of the fractions after purification. Lane 1: markers (94 KDa, 66 KDa, 45 KDa, 35 KDa, 24 KDa); lane 2: crude lysate prepared from *E. coli* BL21 (DE3) harboring pET-C7; lane 3: the purified C23O eluted by 100 mM imidazole.

Enzyme activities of the C23O

The aromatic ring-fission activity of the purified enzyme on catechol was tested at different temperatures and with various pH buffers. When the pH of the reaction mixture was 7.5, the maximal specific activity of the enzyme was 370.4 U at 40 °C and the C23O activity could be still detected even the temperature was increased to 70 °C (Fig. 6).

As depicted in Fig. 7, when the reaction was taken under the room temperature, the optimal pH for the purified enzyme was 7.5 (159.3 U), which was in agreement with the fact that the strain ZJF08 degraded phenanthrene more efficiently at pH 7.5.

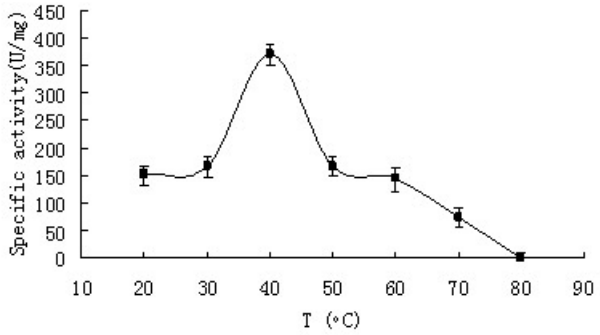

FIG. 6 - The specific activity of the purified C23O at the different temperatures. Data were means from three replicates.

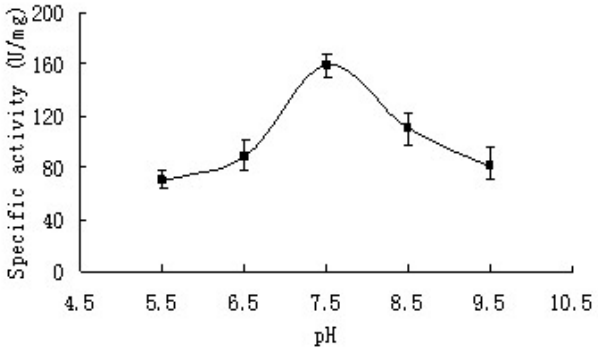

FIG. 7 - The specific activity of the purified C23O in different pH buffers. Data were means from three replicates.

DISCUSSION

In this study, four strains with high phenanthrene degrading ability were isolated and identified. Three of them were *Pseudomonas* sp. This genus was one of the most studied and reported as a PAH degrader which could also decompose many other organic recalcitrant pollutants (Zhang *et al*., 2004). Another one, ZJF05, was *Rhizobium* sp. which was the species seldom to be studied before. By comparison with the data reported (Tian *et al*., 2002; Syakti *et al*., 2004), the four strains all exhibited excellent bioavailability of phenanthrene with such high concentration, and the strain ZJF08 was the best one (Table 1).

As temperature and pH were important factors influenced the biodegradation by microorganisms, their effects on ZJF08 were determined. The optimal condition for phenanthrene degradation by the strain was detected at 30 °C and pH 7.5 at the end of the degrading process (Fig. 1, 2). Temperature could influence by increasing both microbial activity and PAH solubility, but degradation rate was decreased above 30 °C, which indicated that the adverse effect of high temperature on cells was more important than the increase in substrate availability (Jacques *et al*., 2005).

The degradation of phenanthrene is a complicated process composed of several different catabolic pathways where various enzymes catalyse different reactions. PAH dioxygenase (PDO) and catechol-2,3-dioxygenase (C23O) are two enzymes during the process. PDO is responsible for the first step in the aerobic degradation of polyaromatic compounds, catalysing the hydroxylation of the substrate to the corresponding *cis*-dihydrodiol (Resnick *et al*., 1996). The maximal activity of PDO detected in strain ZJF08 was much higher than that from *P. mendocina* CGMCC 1.766 (0.0185 U) reported by Tian (2002). The catalytic meta-cleavage of catechol by C23O to produce 2-hydroxy-muconic semialdehyde seems to be the most common pathway in the biodegradation of phenanthrene (Kojima *et al*., 1961; Meyer, 1999). There were a lot of publications on C23O enzymic activities with respect to utilisation of naphthalene (Shamsuzzaman and Barnsley, 1974a, 1974b; Kiyohara and Nagao, 1978), while only a little information concerned its role in phenanthrene degradation. C23O activity varied greatly from bacteria to bacteria (Meyer *et al*., 1999) because of the differences in the enzyme gene regulation or structure. During the biodegradation of strain ZJF08, both PDO and C23O reached the maximum on the second day, and then there was a drastic drop on the 3rd day during the process (Fig. 3, 4). This could be caused by the fact that lots of intermediate metabolites had been accumulated after the degradation of phenanthrene for the first two days. As the intermediate metabolites were short-chain hydrocarbons and easier to be utilised than phenanthrene as the carbon source by bacteria, the expression of the key enzymes (PDO and C23O) could be inhibited and their activities could hardly be detected on the 3rd day. When the metabolites were almost exhausted and phenanthrene became the sole carbon source for the bacteria growth, the activities key enzymes started to increase slowly. In the previous experiments, these two enzymes activities were also detected in rich media without phenanthrene. There were no fluctuations in both enzymes activities during 7 days and the activities were much lower than those in MM with phenanthrene (0.03 U and 0.07 U of PDO and C23O respectively). The observation suggested that the two key enzymes might be inducible enzymes and it was consistent with the results from other reports (Tian *et al*., 2002; Tao *et al*., 2007).

The catechol-2,3-dioxygenase from *Pseudomonas* sp. strain ZJF08 was cloned, expressed and characterised in details. Despite the high degree of identity with its homologs in amino acid sequences, the enzyme possessed a notably higher thermostability. When the temperature was increased to 70 °C, a remarkable enzymatic activity could still be detected (Fig. 6). There was no report on C23O which maintained catalytic activity at such high temperature before. However, the optimal temperature for the wild strain ZJF08 degrading phenanthrene was 30 °C, which was notably different from the optimal temperature (40 °C) for the purified enzyme. This could be associated with the fact that multiple enzymes were employed in the process of degrading phenanthrene and the optimal temperature for the bacteria might be determined by the lowest optimal temperature for the members of the pathway. More studies are needed to clarify the relationship between the structure and the thermostability of the C23O from the *Pseudomonas* sp. strain ZJF08. Furthermore, the recombination of C23O genes from different strains will also be carried out to increase the activity and enhance the ability of the strains to utilise PAHs in natural environment.

Acknowledgements

This work was supported by the grants from the Ministry of Science and Technology, P.R.China (project No.: 2002AA2Z345A and No.: 2004AA2Z3801). We thanked Prof. J.J. Zhong for the constructive comments.

REFERENCES

Boopathy R. (2000). Factors limiting bioremediation technologies. Bioresource Technology, 75: 63-67.

Bradford M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254.

Chen S.H., Aitken M.D. (1999). Salicylate stimulates the degradation of high-molecular weight polycyclic aromatic hydrocarbons by *Pseudomonas saccharophila* P15. Environ. Sci. Technol., 33: 435-439.

Guieysse B., Viklund G., Toes A.C., Mattiasson B. (2004). Combined UV-biological degradation of PAHs. Chemosphere, 55: 1493-1499.

Hughes J.B., Beckles D.M., Chandra S.D., Ward C.H. (1997). Utilization of bioremediation processes for the treatment of PAH-contaminated sediments. J. Ind. Microbiol. Biotech., 18: 152-160.

Jacques R.J.S., Santos E.C., Bento F.M., Peralba M.C.R., Selbach P.A., Sá E.L.S., Camargo F.A.O. (2005). Anthracene biodegradation by *Pseudomonas* sp. isolated from a petrochemical sludge landfarming site. Int. Biodeter. Biodeg., 56: 143-150.

Johnsen A.R., Wick L.Y., Harms L. (2005). Principles of microbial PAH-degradation in soil. Environ. Pollut., 133: 71-84.

Kahng H.Y., Nam K., Kukor J.J., Yoon B.J., Lee D.H., Oh D.C., Kam S.K., Oh K.H. (2002). PAH utilization by *Pseudomonas rhodesiae* KK1 isolated from a former manufactured-gas plant site. Appl. Microbiol. Biotechnol., 60: 475-480.

Kanaly R.A., Haryama S. (2000). Biodegradation of high molecular-weight polycyclic aromatic hydrocarbons by bacteria. J. Bacteriol., 182: 2059-2067.

Kim D., Chae J-C., Jang J.Y., Zylstra G.J., Kim Y.M., Kang B.S., Kim E. (2005). Functional characterization and molecular modeling of methylcatechol 2,3-dioxygenase from o-xylene-degrading *Rhodococcus* sp. strain DK17. Biochem. Biophys. Res. Comm., 326: 880-886.

Kiyohara H., Nagao K. (1978). The catabolism of phenanthrene and naphthalene by bacteria. J. Gen. Microbiol., 105: 69-75.

Kojima Y., Itada N., Hayaishi O. (1961). Metapyrocatechase: a new catechol-cleaving enzyme. J. Biol. Chem., 236: 2223-2228.

Langworthy D.E., Stapleton R.D., Sayler G.S., Findlay R.H. (2002). Lipid analysis of the response of a sedimentary microbial community to polycyclic aromatic hydrocarbons. Microb. Ecol., 43: 189-198.

Meyer S., Moser R., Neef A., Stahl U., Kämpfer P. (1999). Differential detection of key enzymes of polyaromatic-hydrocarbon-degrading bacteria using PCR and gene probes. Microbiology, 145: 1731-1741.

Moffett B.F., Walsh K.A., Harris J.A., Hill T.C.J. (2000). Analysis of bacterial community structure using 16S rDNA analysis. Anaerobe, 6: 129-131.

Nozaki M., Iwaki M., Nakai C., Saeki Y., Horiiki K., Kagamiyama H., Nakazawa T., Ebina Y., Inoue S., Nakazawa A. (1982). In: Nozaki M., Yamamoto S., Ishimura Y., Coon M.J., Ernster L., Estabrook R.W., Eds, Oxygenases and Oxygen Metabolism, Academic Press, New York, pp. 15-26.

Resnick S.M., Lee K., Gibson D.T. (1996). Diverse reactions catalyzed by naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816. J. Ind. Microbiol., 17: 438-457.

Samanta S.K., Singh O.V., Jain R.K. (2002). Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation. Trends in Biotechnology, 20: 243-248.

Shamsuzzaman K.M., Barnsley E.A. (1974a). The regulation of naphthalene oxygenase in *Pseudomonads*. J. Gen. Microbiol., 83: 165-170.

Shamsuzzaman K.M., Barnsley E.A. (1974b). The regulation of naphthalene metabolism in *Pseudomonads*. Biochem. Biophys. Res. Comm., 60: 582-589.

Syakti A.D., Acquaviva M., Gliewicz M., Doumenq P., Bertrand J.C. (2004). Comparison of *n*-eicosane and phenanthrene removal by pure and mixed cultures of two marine bacteria. Environ. Res., 96: 228-234.

Tao X.Q., Lu G.N., Dang Z., Yang C., Yi X.Y. (2007). Isolation of phenanthrene-degrading bacteria and characterization of phenanthrene metabolites. World J. Microbiol. Biotechnol., 23: 647-654.

Tian L., Ma P., Zhong J.J. (2002). Kinetics and key enzyme activities of phenanthrene degradation by *Pseudomonas mendocina*. Process Biochemistry, 37: 1431-1437.

Wong J.W.C., Lai K.M., Wan C.K., Ma K.K., Fang M. (2002). Isolation and optimization of PAH degradative bacteria from contaminated soil for PAHs bioremediation. Water Air Soil Poll., 139: 1-3.

Zhang H., Kallimanis A., Koukkou A.I., Drainas C. (2004). Isolation and characterization of novel bacteria degrading polycyclic aromatic hydrocarbons from polluted Greek soils. Appl. Microbiol. Biotechnol., 65: 124-131.