

# Biodegradation of Phenanthrene by *Pseudomonas* sp. JPN2 and Structure-Based Degrading Mechanism Study

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**Abstract** The strain *Pseudomonas* sp. JPN2 had a high potential to degrade phenanthrene degrading 98.52% of the initial amount of 100 mg L<sup>-1</sup> after 10 days incubation. The analysis of metabolites demonstrated that the cleavage of phenanthrene started at the C<sub>9</sub> and C<sub>10</sub> positions on the aromatic ring by the dioxygenation reaction, and then further degraded via a phthalate pathway. To understand the interaction between phenanthrene and the amino acid residues in the active site of the target enzyme, a molecular docking simulation was performed. The results showed that the distances of C<sub>9</sub>-O<sub>1</sub> and C<sub>10</sub>-O<sub>2</sub> atoms were 3.47 and 3.67 Å, respectively. The C<sub>9</sub> and C<sub>10</sub> positions of the phenanthrene ring are much closer to the dioxygen molecule in the active site relative to the other atoms. Therefore, the C<sub>9</sub> and C<sub>10</sub> positions are vulnerable to attack in the initial oxygenation process.

**Keywords** Phenanthrene · Biodegradation · *Pseudomonas* sp. JPN2 · Molecular docking

Among persistent organic contaminants, many concerns have focused on polycyclic aromatic hydrocarbons (PAHs) owing to their toxicity, mutagenicity and carcinogenicity (Hadibarata and Kristanti 2014; Wang et al. 2007). They

are released to the environment via diverse anthropogenic and industrial activities, such as combustion of fossil fuels, distillation of crude oil, spills and leaks of storage tanks, and wood preservation (Wilson and Jones 1993). PAHs are recalcitrant to decomposition and persist in the natural environment due to their structural stability and hydrophobicity. Up to now, US Environmental Protection Agency (USEPA) has listed 16 PAHs as the priority contaminants that are being regulated (Wang et al. 2007; Wenzl et al. 2006; Wilson and Jones 1993). Phenanthrene, an aromatic hydrocarbon including three fused benzene rings in an angular fashion, is usually regarded as a model substrate for the metabolism studies of carcinogenic PAHs on account of the Bay-region and K-region on the phenanthrene structure (Mallick et al. 2011; Narro et al. 1992). For example, the Bay-region and K-region on the phenanthrene structure refer to the C<sub>4</sub>-C<sub>5</sub> and C<sub>9</sub>-C<sub>10</sub> positions, respectively. The epoxides on the two regions possess a highly reactive activity in chemistry and biology, and are commonly generated from the metabolic process of microorganisms (Samanta et al. 1999).

At present, the research efforts have focused on removing or detoxifying PAHs from the polluted sites. Compared to chemical oxidation, physical adsorption and photooxidation, bioremediation technology has acquired increasing attention, and it is considered to be a cost-effective and eco-friendly method for the removal of PAHs (Haritash and Kaushik 2009; Singh 2012). Naphthalene dioxygenase (NDO) is known for its relaxed substrate specificity, which is capable of catalyzing the initial degradation of a variety of aromatic substrates (Parales 2003). The study of Kim et al. (2005) demonstrated that the phenanthrene ring was firstly oxidized at the K-region by the strain *Mycobacterium vanbaalenii* PYR-1, and a metabolite, phenanthrene-9,10-dihydrodiol, was generated. Subsequently, this metabolite continued to be degraded and formed a dicarboxylic acid

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by ortho-ring cleavage dioxygenase. Zeinali et al. (2008) reported that a moderately thermophilic bacterium *Nocardia otitidiscaviarum* strain TSH1 could decompose phenanthrene at C<sub>3</sub>–C<sub>4</sub> or C<sub>9</sub>–C<sub>10</sub> positions by the dioxygenation reaction.

The aims of this work were to study the degradation potential of the strain *Pseudomonas* sp. JPN2 for phenanthrene and its degrading mechanism based on the homology modeling and molecular docking technologies. The percent degradation by strain JPN2 for phenanthrene was evaluated and the relevant metabolites were identified by GC-MS. The influence of phenanthrene concentration on the growth status of this strain was monitored by microcalorimetry method. In addition, we applied the molecular docking technology to study the interaction mechanism between phenanthrene and the active site of NDO in the strain JPN2.

## Materials and Methods

Phenanthrene (99%), dimethyl phthalate (99%) and 9-phenanthrol (98%) used in this study were purchased from Aladdin Industrial Inc. (Shanghai, China). All organic reagents were of analytical grade and purchased from Beijing Chemical Works (Beijing, China). The 9-methoxy phenanthrene was synthesized by the methylation of 9-phenanthrol (Narro et al. 1992).

The strain *Pseudomonas* sp. JPN2 (KT799677) was isolated from crude oil in our previous study (Jin et al. 2016). The biodegradation experiment of phenanthrene was carried out in 100 mL sterilized mineral salt medium (MSM) with 100 mg L<sup>-1</sup> phenanthrene. Subsequently, 1.0 mL bacterial suspension of the strain JPN2 (OD<sub>600nm</sub> = 0.20) was added into the medium, and then incubated at 30 °C and 150 rpm for 10 days. After every 2 days, 15 mL ethyl acetate was used to extract the remaining phenanthrene and its relevant metabolites from 5 mL culture solution, and this step was repeated for three times. The organic phase was separated and merged, and then it was dried by a column packed with anhydrous sodium sulfate. Finally, the extraction solution was concentrated by rotary evaporation. The extracted metabolites were methylated to avoid the loss of the active hydrogen atoms according to the method of Narro et al. (1992). The remaining phenanthrene and the methylated metabolites were identified by GC-MS (QP2010SE, Shimadzu, Japan) (Jin et al. 2015). The remaining amount of phenanthrene was quantified by an external calibration curve which was drawn by the peak areas and the corresponding concentrations of phenanthrene (0, 10, 25, 50, 100, 200 mg/L). The methylated metabolites 9-methoxy phenanthrene and dimethyl phthalate were identified by comparing the retention time and the data of mass spectrum with the corresponding standard substances. The control

samples were inoculated with 1.0 mL bacterial suspension pretreated by autoclaving at 121 °C and 0.1 MPa for 30 min. In addition, 3 mL culture solution was used to measure the growth activity of the strain JPN2 at OD<sub>600nm</sub> on a UV-1800 UV-vis absorption spectrophotometer (Shimadzu, Japan). All experiments were performed in triplicate. The experimental data displayed in the context was the average of three testing values.

To amplify the *nahAc* gene encoding the  $\alpha$  subunit of NDO, the strain JPN2 was cultured in MSM medium until the culture solution reached an optical density of 0.20. Subsequently, 2 mL culture solution was used to extract the genomic DNA by a Bacterial DNA Kit (Omega, USA). And then a pair of degenerate primers 5'-AGGGATCCCCANCCRTGRTAN SWRCA-3' and 5'-GGAATTCTG YMGNCAYMGNGG-3' were used to amplify the dioxygenase gene in the strain JPN2 (Kahng et al. 2002). The PCR amplification was performed on a GeneAmp<sup>®</sup> PCR System 9700 (Applied Biosystems, USA). The PCR reaction complex consisted of 25.0  $\mu$ L 2 $\times$  Taq MasterMix, 1.0  $\mu$ L genomic DNA, 20.0  $\mu$ L sterile ddH<sub>2</sub>O, and 2.0  $\mu$ L each primer, and then this reaction was performed with 30 cycles of denaturing (95 °C for 1 min), annealing (55 °C for 1 min, 72 °C for 1 min), and an additional 1 min of denaturation before the first cycle, as well as an additional 10 min of polymerization after the last cycle. The PCR products was purified using a Tiangel Midi Purification Kit (TIANGEN, China), and sequenced by Sangon Biotechnology Company (Beijing, China). Finally, the sequence alignment showed that the amplified gene in the strain JPN2 had an identity of 99% with the *nahAc* genes encoding  $\alpha$  subunit of NDO from the strains *P. stutzeri* NJ and *P. stutzeri* isolate 67. This gene sequence has been deposited at GenBank with an accession number KU365776.

To analyze the interaction between phenanthrene and the active site in NDO, the amplified gene sequence was translated to the corresponding amino acid sequence by DNASTar5.0 program. Based on the homology modeling method, the translated sequence of amino acid was used to build the 3D crystal structure of NDO in the strain JPN2 (hereinafter referred to as target enzyme). 1O7N, a crystal structure of NDO from the strain *Pseudomonas putida*, was obtained from Protein Data Bank (PDB) and acted as a template enzyme in the homology modeling of the crystal structure of the target enzyme. When the homology of the amino acid sequences between the target enzyme and the template enzyme is more than 30%, the template is suitable for the homology modeling of the target enzyme. At the same time, they will possess the similar backbone structures (Min et al. 2012). The homology modeling was performed based on the template and the modeled crystal structure was examined by the distribution positions of amino acid residues in Ramachandran plot (Porter and Rose 2011). The Surfex

molecular docking method in Sybyl7.3 was used to simulate the interaction between the active site of the target enzyme and phenanthrene. In the docking process, the phenanthrene structure in ‘mol2’ format as a substrate was docked into the active site, and the detailed interaction was analyzed. The docking parameters were set as defaults.

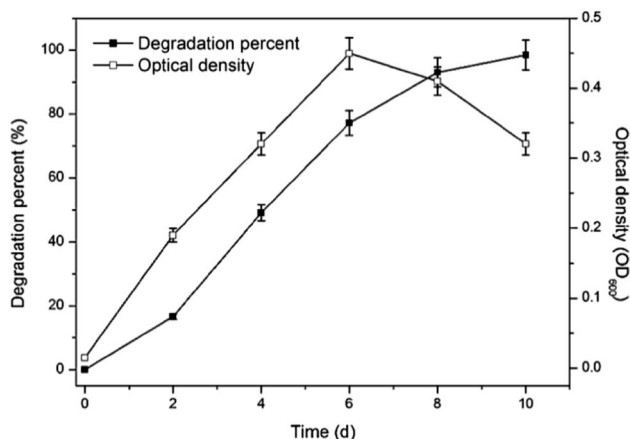
The influence of phenanthrene for the growth activity of the strain JPN2 was monitored by an isothermal micro-calorimeter TAM III (TA Instruments, USA) (Chen et al. 2014). The calorimetric experiment was carried out in 4-mL stainless steel ampoules sealed by stainless steel screw caps. Firstly, the phenanthrene resolved in ethyl acetate with various concentrations (0, 25, 100, 400, 1600, 3200 mg L<sup>-1</sup>) was added in the sterile ampoules, respectively. To volatilize the ethyl acetate, the ampoules were kept at room temperature for 15 min. Additionally, 2.0 mL LB medium was added, and the bacterium was inoculated into the ampoules via an inoculating needle. Finally, a series of thermodynamic

parameters including total heat ( $Q_t$ ), growth rate constant ( $k$ ), inhibitory rate ( $I$ ) for bacterial growth activity, highest thermal power ( $P_{max}$ ) and its corresponding time ( $t_{max}$ ) were measured.

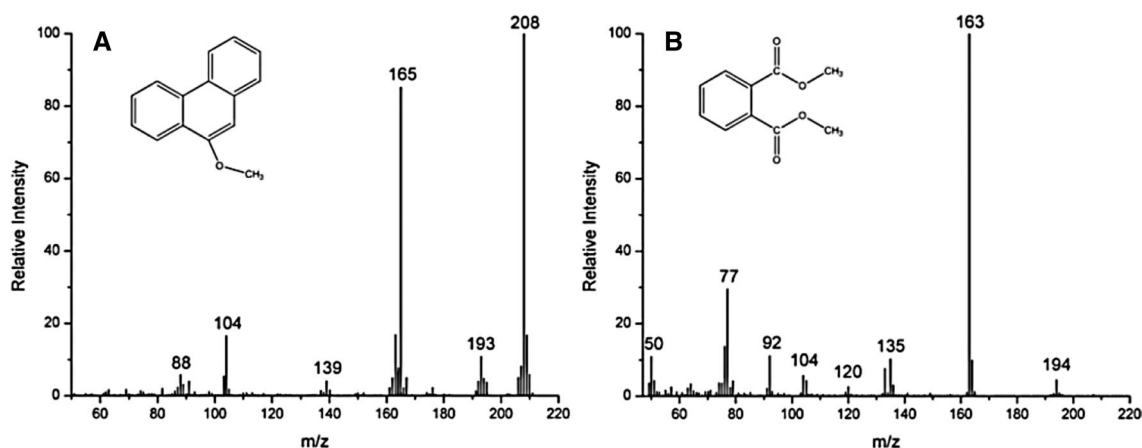
## Results and Discussion

The degradation of phenanthrene by strain JPN2 is shown in Fig. 1. After the first 2 days culture, the strain JPN2 degraded 16.58% of the initial amount of phenanthrene. Meanwhile, the growth activity of the bacterium was also relatively low with an optical density value of 0.19 at 600 nm. Subsequently, as the incubation time was extended, it was obvious that the growth activity of the strain JPN2 increased, and the amount of phenanthrene degradation gradually increased. After the 6th day, the optical density of the culture solution began to decline. After 10 days of incubation, the degradation percent reached to 98.52% of the total amount of phenanthrene added. In the control samples, the content analysis after every 2 days displayed that 99%–100% of phenanthrene remained in the culture solution. This indicated that phenanthrene was clearly degraded during the culture of JPN2 cells. The control results also demonstrated that the decrease of phenanthrene in the inoculated cultures was due to biodegradation by the strain JPN2 rather than the abiotic process. According to the conclusions of the biodegradation test, there are grounds to believe that the strain JPN2 has the potential to metabolize phenanthrene in the designated conditions.

To understand the degradation mechanism, it is essential to identify the relevant metabolites generated during the biodegradation of phenanthrene by the strain JPN2. In this study, two methylated metabolites were separated out from the culture solution. The mass spectrums of the two metabolites are displayed in Fig. 2.



**Fig. 1** Curves of the biodegradation percent and the growth activity of the strain JPN2 at OD<sub>600nm</sub> in the presence of 100 mg L<sup>-1</sup> phenanthrene



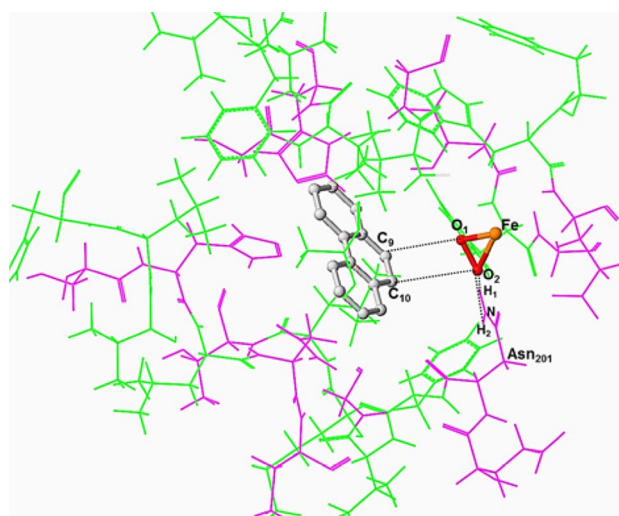
**Fig. 2** Mass spectrums of 9-methoxy phenanthrene (a) and dimethyl phthalate (b) generated from the biodegradation of phenanthrene by the strain JPN2

The methylated metabolite I (Fig. 2a) is detected at a retention time of 19.21 min. The mass spectrum in Fig. 2a demonstrates that the metabolite I had a molecular ion ( $M^+$ ) at  $m/z$  208, and fragmentation ions at  $m/z$  193 ( $M^+-15$ , loss of  $-CH_3$ ),  $m/z$  165 ( $M^+-43$ , loss of  $-CH_3$  and  $CO$ ) and  $m/z$  139. These data were identical with 9-methoxy phenanthrene. Therefore, the methylated metabolite I was identified as 9-phenanthrol which was derived from the rearrangement of phenanthrene 9,10-oxide or the dehydration of the phenanthrene trans-9,10-dihydrodiol according to the results of the previous studies (Narro et al. 1992; Sutherland et al. 1990). Therefore, we considered that the initial oxidation positions were located in the  $C_9$  and  $C_{10}$  atoms of the phenanthrene ring (i.e., K-region). Furthermore, it was the most common positions reacting with the diverse oxygenases in bacteria (Cerniglia 1993; Mallick et al. 2011).

The methylated metabolite II (Fig. 2b) has a retention time of 9.60 min. As shown in Fig. 2b, the metabolite II has an  $M^+$  at  $m/z$  194, and fragmentation ions at  $m/z$  163 ( $M^+-31$ , the loss of  $-OCH_3$ ),  $m/z$  135 ( $M^+-59$ , the loss of  $-COOCH_3$ ). The retention time and the fragmentation ions were in line with dimethyl phthalate. Accordingly, the methylated metabolite II was authenticated as phthalate which could be successively converted to protocatechuate by a series of enzymes including phthalate dioxygenase, cis-dihydroxy-dihydrophthalate dehydrogenase and dihydroxyphthalate decarboxylase. Afterwards, protocatechuate was further degraded from protocatechuate to pyruvate and oxaloacetate by protocatechuate-4,5-dioxygenase, 2-hydroxy-4-carboxymuconate semialdehyde dehydrogenase, 2-pyrone-4,6-dicarboxylate hydrolase, 4-oxalmeaconate hydratase and acyl transferase (Eaton 2001).

The interaction analysis between phenanthrene and the active site is necessary for understanding the degradation mechanism. The amplified *nahAc* gene sequence was translated to the amino acid sequence to build the crystal structure of the target enzyme by homology modeling. The crystal structure 1O7N from PDB database was used as a template enzyme in the homology modeling. The homology analysis demonstrated that the amino acid sequences of the target enzyme and the template enzyme had an identity of 91%. Therefore, 1O7N was suitable for acting as the template structure in the homology modeling of the target enzyme.

The interaction between the active site of the target enzyme and phenanthrene is described in Fig. 3. In the active site, the residues close to the phenanthrene ring were the hydrophobic amino acids displayed as green sticks. Moreover, the presence of the hydrophobic groups was conducive to the interaction between the hydrophobic phenanthrene and the active site of NDO. At the same time, the dioxygen molecule combined with the mononuclear iron atom, and thus a triangle structure had taken shape. The

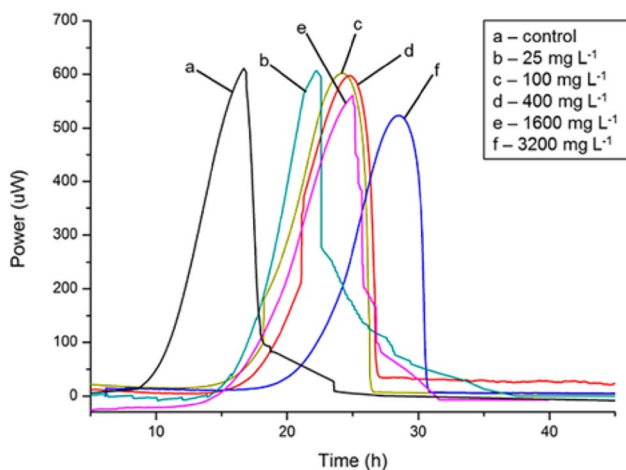


**Fig. 3** Interaction between phenanthrene and the active site of NDO in the strain JPN2. The hydrophobic and hydrophilic amino acids are shown in *stick formation* with green and magenta colors, respectively. The structures of phenanthrene and the dioxygen molecule are shown in *ball and stick formation*. The hydrogen bonds are shown in *black dashed line* between the residue Asn<sub>201</sub> and the oxygen atom

“O<sub>2</sub>” atom in Fig. 3 forms two hydrogen bonds with the terminal amino group of Asn<sub>201</sub>. The triangle structure and the non-covalent forces are beneficial for the structural stability of the dioxygen molecule in the active site. The distances of  $C_9-O_1$  and  $C_{10}-O_2$  are 3.47 and 3.67 Å, respectively. The two oxygen atoms and the mononuclear iron atom in the triangle motif are 5.17 and 4.98 Å apart, respectively. The torsion angle between phenanthrene and the triangle motif is 41.5°. These data indicated that the  $C_9$  and  $C_{10}$  positions are much closer to the dioxygen atoms relative to the other atoms on the phenanthrene ring, and thus the redox reaction was performed in the active site of the target enzyme. Consequently, the presence of the methylated metabolite I proved that the oxygenation reaction was initially occurred at the same positions. The result of the theoretical analysis is in accordance with that of the metabolite analysis.

To elucidate the influence of phenanthrene for the growth activity of the strain JPN2 in the experimental concentration, a series of thermodynamic curves representing the growth status of the strain JPN2 are measured in the presence of the various concentrations of phenanthrene as displayed in Fig. 4. Compared to the control, it was observed that the curves changed following the concentration increase of phenanthrene, and this indicated that the growth activity of the strain JPN2 was influenced in different degrees by phenanthrene. It is obvious from the Fig. 4 that the whole growth cycle of the samples containing various concentrations of phenanthrene were significantly postponed relative to the control.

The detailed thermodynamic data was displayed in Table 1. Under the low concentration conditions



**Fig. 4** Thermodynamic curves expressing the growth activity of the strain JPN2 with the various concentrations of phenanthrene

**Table 1** Thermodynamic parameters of the strain JPN2 obtained from the isothermal microcalorimetry

Conc. (mg L <sup>-1</sup> )	$Q_t$ (J)	$P_{max}$ (µW)	$t_{max}$ (h)	$k$ (h <sup>-1</sup> )	$I$ (%)
0	14.18	610.96	16.69	$0.6831 \pm 0.0031$	0
25	15.15	606.84	22.23	$0.6589 \pm 0.0052$	3.54
100	13.32	601.62	24.19	$0.6257 \pm 0.0019$	8.40
400	12.94	597.36	25.62	$0.5972 \pm 0.0306$	12.57
1600	11.88	560.79	26.25	$0.5743 \pm 0.0012$	15.92
3200	11.80	523.39	28.50	$0.5545 \pm 0.0183$	18.82

(25–400 mg L<sup>-1</sup>), the presence of phenanthrene during the growth process of this strain had indistinctive influence relative to the control sample, and  $Q_t$  value of the total heat decreased by -6.84% and 8.74% at 25 and 400 mg L<sup>-1</sup> phenanthrene, respectively. Meanwhile, the  $P_{max}$  values of the highest thermal power were changed from 606.84 to 597.36 µW in this concentration range. The inhibition percent was 3.54%, 8.40% and 12.57%, respectively. Significant differences were observed at 1600 mg L<sup>-1</sup> and 3200 mg L<sup>-1</sup>, and the toxicity of phenanthrene for the strain JPN2 was significantly increased. The  $Q_t$  values decreased by 16.22% and 16.78%, respectively. Meanwhile, the  $P_{max}$  values were also decreased to 560.79 and 523.39 µW, respectively. The inhibition percent were 15.92% and 18.82%, respectively. Taken together, the influence of phenanthrene for the growth activity of JPN2 was not significant under the experimental concentration (100 mg L<sup>-1</sup>). It also indicated that this strain had an excellent tolerance for phenanthrene and provided a chance to apply this strain for the bioremediation of phenanthrene and other PAHs contaminated environment.

In this study, the biodegradation potential of the strain JPN2 for phenanthrene was investigated. The metabolic

mechanism was discussed based on the analyses of the metabolites and the molecular docking. Taken together, the results displayed that the strain JPN2 had a high degrading ability for phenanthrene. Meanwhile, the theoretical analysis proved that the C<sub>9</sub> and C<sub>10</sub> positions on the phenanthrene ring were the initial oxidation sites. The microcalorimetry analysis showed that the strain JPN2 possessed an excellent tolerance for phenanthrene. Consequently, this study provided a potential PAH-degrading strain and a profile for comprehending the phenanthrene-degrading mechanism by a combined study method.

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