Molecular Cloning and Characterization of a New Cold-active Extradiol Dioxygenase from a Metagenomic Library Derived from Polychlorinated Biphenyl-contaminated Soil

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Abstract To find new extradiol dioxygenases(EDOs, EC 1.13.11.2), a metagenomics library was constructed from polychlorinated biphenyl-contaminated soil and was screened for some dioxygenase with aromatic ring cleavage activity. A novel EDO, designated as BphC_A, was identified and heterologously expressed in *Escherichia coli*. The deduced amino acid sequence of BphC_A exhibited a homology of less than 60% with other known EDOs. Phylogenetic analysis of BphC_A suggests that the protein is a novel member of the EDO family. The enzyme exhibits higher substrate affinity and catalytic efficiency toward 3-methylcatechol than toward 2,3-dihydroxybiphenyl or catechol, the preferred substrate of other known EDOs. The optimum activity of purified BphC_A occurred at pH=8.5 and 35 °C, and BphC_A showed more than 40% of its initial activity at 5 °C. The activity of purified BphC_A was significantly induced by Mn^{2+} and slightly reduced by Al^{3+}, Cu^{2+} and Zn^{2+}.

Keywords Extradiol dioxygenase; Metagenome; Cold-active enzyme; Gene cloning; Functional characterization

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1 Introduction

Aromatic compounds, such as polycyclic aromatic hydrocarbons(PAHs) and polychlorinated biphenyls(PCBs), are a major group of environmental pollutants[1,2]. Bioremediation, the microbial catabolic versatility responsible for degrading aromatic compounds, is a significant strategy because of its potential to destroy toxic and persistent pollutants[3]. The aerobic degradation of aromatic compounds by microorganisms often involves the conversion of the initial substrates into common intermediates, such as catechol or its derivatives. During biodegradation, catechol and its derivatives are cleaved into meta- or ortho-ring fission products, which are catalyzed by extradiol dioxygenases(EDOs) or intradiol dioxygenase[4].

EDOs catalyze the ring cleavage of catecholic compounds to produce meta-cleavage yellow compounds during the degradation of aromatic compounds. They are suitable markers for detecting the specificity of pathways that catabolize aromatic compounds and identified as the key enzymes for breaking down aromatic compounds[5]. Therefore, investigating the diversities of EDOs is important. To date, there have been numerous examples of extradiol dioxygenases described in the literature[6—8], which were obtained by the culturable techniques. However, more than 99% of the microorganisms in the environment are not readily culturable with currently available techniques[9]. The information about extradiol dioxygenases from uncultrue microorganism is relatively rare.

Recently, culture-independent approaches especially metagenomic techniques have been used to mine new and useful functional enzymes. Many metagenome-derived enzymes display novel characteristics and the method has been used successfully to clone genes from soil communities that encode enzymes that degrade xenobiotic compounds, such as dehalogenase[10], xylanase[11], esterases and lipases[12,13]. Thus, the construction of metagenomic libraries from contaminated environments to identify novel EDOs is beneficial for expanding our knowledge of genetic information and the characteristics of EDOs from different sources.

In the present study, a metagenomic library, constructed from PCBs-contaminated soil samples, was screened for some dioxygenase with aromatic ring cleavage activity. A novel extradiol dioxygenase-encoding gene, designated as *bphC*_A, was identified. The gene product shows only moderate sequence identity(≤60% identity) with other known EDOs. This novel EDO gene was successfully overexpressed and purified,

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and the characterization of the purified enzyme was investigated in detail.

2 Materials and Methods

2.1 Materials

All the biological reagents were sourced from Sigma (USA). Strain *Escherichia coli*(*E. coli*) DH5*α* and plasmid pBluscript SK(+) were obtained from Stratagene(USA). Mouse anti-6×His monoclonal antibody, horseradish peroxidase-conjugate goat anti-mouse IgG, and an improved HRP-DAB colorimetric Western blotting kit were purchased from Tiangen(Beijing, China). All the other chemicals were commercially available and of analytical grade.

Soil samples were collected from a transformer factory in Fushun City, Liaoning Province, China in October 2009. This area has been contaminated by PCBs for more than two decades. The soil was sampled between 10 cm and 20 cm in depth. The pH values of the soil samples were measured to be about 8.30 according to the method by Zhou *et al*.[14].

2.2 Metagenomic Library Construction and Activity Screening

The method used for soil metagenomic DNA isolation and purification was performed, as described by Brady[15] . The purified environmental DNA was partially digested with *Sau*3A I restriction enzymes. The resultant DNA fragments, ranging from 3000 bp to 8000 bp, were excised and concentrated in 1% agarose gel *via* a gel extraction kit(Omega, China). An 800 ng-size fraction was ligated into 100 ng of *Bam*H I-digested and dephosphorylated pBluescript SK(+) plasmid. After desalting, the products were electroporated at 1.8 kV into *E. coli* DH5*α* with the help of a micropulser. The transformed cells were plated onto Luria-Bertani(LB) agar plates containing 100 μg of ampicillin/mL. Incubation at 37 °C was performed until ampicillin-resistant colonies were formed. Functional screening of extradiol dioxygenases was performed by spraying the bacterium with 20 mg/mL 2,3-dihydroxybiphenyl solution (dissolved in acetone). The transformant colonies that formed a yellow halo within 30 s, indicating the cleavage of 2,3-dihydroxybiphenyl, were selected as positive clones. To exclude false positive clones, the plasmids isolated from all the positive clones in the preliminary screening were retransformed into *E. coli* DH5*α*. The ability of the resulting recombinants to cleave 2,3-dihydroxybiphenyl was confirmed again *via* the same procedure.

2.3 Sequence Analysis

The nucleotide sequences of the inserted DNA fragments from the positive plasmids were obtained by means of a primer walking method, which was performed by Sangon Biotech (Shanghai, China) Co., Ltd. Open reading frame(ORF) analysis was performed on an ORF Finder from National Center of Biotechnology Information of USA(NCBI, http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The predicted function of ORFs was annotated *via* BLASTX search of the NCBI non-redundant protein database. Amino acid sequence similarity searches and conserved domain comparisons for the predicted ORF were performed *via* BLASTP. The molecular weight(M_w) and isoelectric point(pI) of the mature protein were calculated by means of ProtParam. Multiple alignments between protein sequences were performed with the ClustalW2 program. The aligned sequences were from the following organisms(GenBank accession numbers are provided in parentheses): BphC_PS400 (X66122), 2,3-dihydroxybiphenyl dioxygenase, *Burkholderia xenovorans* LB400; TodE_PSF1(J04996), 3-methylcatechol 2,3-dioxygenase, *Pseudomonas putida* F1; BphC_PS102 (M26433), 2,3-dihydroxybiphenyl dioxygenase, *Pseudomonas sp*. strain KKS102; XylE_MT2(V01161), catechol 2,3-dioxygenase, *Pseudomonas putida* MT-2; CatE_PSHV3(L10655), catechol 2,3-dioxygenase, *Pseudomonas sp*. strain HV3; TfdC_CNJMP134(YP_298870), catechol 2,3-dioxygenase, *Cupriavidus necator* JMP134.

2.4 Subcloning, Expression and Purification

The putative extradiol dioxygenase gene(939 bp) was directly amplified by polymerase chain reaction(PCR) *via* Premix PrimeSTAR® HS high fidelity DNA polymerase from positive plasmids and the primer pairs *bphC*_AFP(5′-GCCAGAAATT-*CCATATG*AAAGAACTCAGAA-3′) and *bphC*ARP(5′-ATG-*GGATCC*TTATGCGAGCGCCGGC-3′), which contained restriction enzyme sites(in italics) for *Nde*I at the 5′-end and *Bam*HI at the 3′-end. The amplification products were digested with *Nde*I and *Bam*HI, and then cloned into an expression vector, pET-28a(Novagen, USA). The sequence of the inserted fragment was subsequently confirmed by sequencing. The positive vector, designated as pET28a:: *bphC*_A, was transformed into *E. coli* BL21(DE3) pLysS cells(Transgen, China) for the overexpression of the corresponding proteins.

When the cell density at visible light wavelength 600 nm reached around 0.5, 0.4 mmol of isopropyl *β-D*-1-thiogalactopyranoside(IPTG) was added to the culture system for induction. After 6 h of cultivation at 30 °C, the cells were harvested by centrifugation at 6000*g* for 20 min, washed with 50 mmol/L sodium phosphate buffer(pH=8.0), resuspended in the lysis buffer(20 mmol/L imidazole), and disrupted by sonication. The cell debris was removed by centrifugation at 20000*g* for 30 min at 4 °C. The resulting supernatant liquid was used in metal-chelating chromatography on a Ni-NTA column(Qiagen, Germany) and anion exchange chromatography according to Lu *et al*.[16], with slight modifications.

Protein concentrations were determined through the Bradford method[17] with bovine serum albumin(BSA) as the standard. Molecular weights were determined by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis(SDS-PAGE). Western blot analysis of the purified enzymes was performed as described by Sambrook *et al*.[18] with mouse anti-6×His monoclonal and HRP-conjugate goat anti-mouse IgG antibodies. Activity stains were performed according to the method by Levy[19] and determined by non-denaturing polyacrylamide gel electrophoresis(Native-PAGE) with 4% stacking gel and 7.5% separation gel. After electrophoresis, the gels

were soaked in 20 mL of dye solution buffer(pH=7.5), containing 50 mmol of sodium phosphate, 4 mg of nitrotetrazolium blue chloride, and 0.2 mmol of 2,3-dihydroxybiphenyl at 25 °C in a darkroom until the desired color intensity was obtained[20].

2.5 Enzyme Assay and Characterization

Enzyme activities were determined by the ring meta-cleavage of catecholic compounds. Absorbance, as a measure of enzymatic activity, was measured on a UV-2550 spectrophotometer(Shimadzu, Japan) equipped with a thermal controller(Shimadzu, Japan). Unless otherwise indicated, the standard enzyme activity assays were performed by incubating the purified enzyme with 50 μmol of catechol or each of related compounds in 50 mmol/L sodium phosphate buffer(pH=7.5) at 25 °C in a total volume of 1 mL. One unit of activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of the product per minute at 25 °C.

The relative ring cleavage activities of the enzyme were determined from the absorption coefficients of the ring cleavage product formed from the following substrates: catechol(5—1000 μmol/L), 375 nm(ε=33000 L·mol^{-1}cm^{-1}); 3-methylcatechol(10—2000 μmol/L), 388 nm(ε=13400 L·mol^{-1}·cm^{-1}); 4-methylcatechol(25—1000 μmol/L), 379 nm(ε=28100 L·mol^{-1}·cm^{-1}); 4-chlorocatechol(12.5—250 μmol/L), 379 nm (ε=36100 L·mol^{-1}·cm^{-1}); and 2,3-dihydroxybiphenyl(2.5—2000 μmol/L), 434 nm(ε=13200 L·mol^{-1}·cm^{-1})[21].

To determine the optimum temperature for enzyme activity, the reaction was carried out at various temperatures(5—60 °C) and at pH=7.5. The thermostability of the purified enzyme was determined by pre-incubating the enzyme for up to 30 min each at temperature intervals of 5 °C ranging between 5 and 60 °C, then measuring the remaining activity of it with the substrate added at 25 °C.

The effect of pH on enzyme activity was tested with a 50 mmol/L wide-range pH buffer(pH=4—11)[22]. The stability of the enzyme as a function of pH was evaluated by incubation for 30 min with different pH buffers at 25 °C. The residual activities were assayed under standard conditions.

To check the effects of different metal ions on the enzyme activity, the enzymes were incubated in 50 mmol/L phosphate buffer(pH=7.5) for 30 min at 25 °C with 1 mmol/L different metal ions, and the residual activity was determined.

2.6 Nucleotide Sequence Accession Numbers

The nucleotide sequence reported in the present paper has been submitted to GenBank under Accession No. GQ396266.1.

3 Results and Discussion

3.1 Identification of EDO Gene from Metagenomic Library

A metagenomic plasmid library was constructed from a PCB-contaminated soil sample. The EDO activities of the clones were screened on LB agar plates. One of the transformants that turned yellow was selected. Retransformation analysis confirms that the EDO activity of the transformant was derived from the inserted plasmid fragments. The positive clone was designated as pBphC_A.

Sequence analysis of the 3701 bp insert DNA shows the presence of one complete putative ORF that contains a nucleotide sequence of 939 bp and encodes a 312-amino acid protein with a predicted molecular weight of 34700 and a p*I* of 5.94. PSI-BLASTP analysis reveals that the deduced amino acid sequence of the ORF was highly similar to other metacleavage enzymes or biphenyl-2,3-diol-1,2-dioxygenases, commonly called extradiol dioxygenases. Most of these EDOs were discovered *via* whole genome sequencing, but few of them have been biochemically characterized. The predicted protein sequence of the ORF exhibited the highest identity(60%) with the putative catechol 2,3-dioxygenase(GenBank accession No. YP_004128522) of Alicycliphilus denitrificans BC, followed by an identity of 58% with the putative metapyrocatechase XylE(YP_004682700) of Cupriavidus necator N-1, and an identity of 47% with the 2,3-dihydroxybiphenyl dioxygenase (AAB58815) of Sphingobium xenophagum.

Based on the amino acid sequence alignment with six well-studied EDOs(Fig.1), the fingerprint region of the extradiol dioxygenase, PROSITE PS00082: [GNTIV]-x-H-x(5,7)-[LIVMF]-Y-x(2)-[DENTA]-P-x-[GP]-x(2,3)-E[23] was present in the ORF product at positions 248—269: VARHTLGSN-YFYYVRDPWGSFWE. The three important amino acids at the catalytic site, known to function in the binding of metal ions were also found at positions 161 and 218(histidines) and

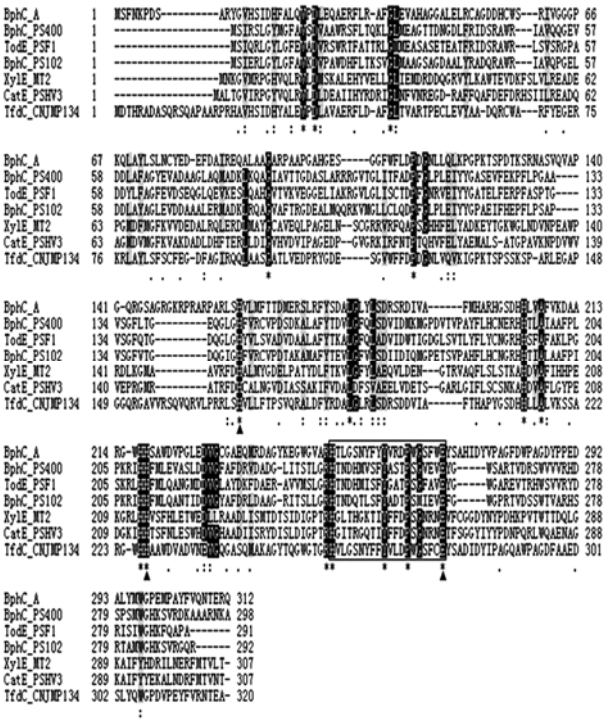

Fig.1 Amino acid sequence alignment of BphC_A with six other similar enzymes

Sequence alignment was performed *via* Clustal W2. The conserved and identical residues are highlighted in white on black and gray background, respectively. The three conserved active site residues that probably play a direct catalytic role are indicated by solid triangles(▲), and the putative fingerprint motif of the extradiol dioxygenases is boxed.

position 269(glutamate)[21]. This suggests that the catalytic center of the ORF product resembles those of the major class of extradiol dioxygenases. However, the ORF product shares an identity less than 60% with other extradiol dioxygenases; this indicates that although the enzymes may have similar quaternary structures, their primary structures are markedly different.

Based on similarities in their amino acid sequences, EDOs are classified into two evolutionary unrelated classes, designated as Type I and Type II, which have totally different primary sequences[23]. To further classify the ORF product, a phylogenetic tree(Fig.2) was constructed using 36 Type I extradiol dioxygenases representing five different families, and the results suggest that the ORF product is closely related to Family I.4, which has only two confirmed members to date: MPCII-AE22(P17296) and Q6W1M5 RHISN(Q6W1M5)[24]. Taken together, the protein appears to be an extradiol dioxygenase with a considerably novel sequence. This protein was designated as BphC_A.

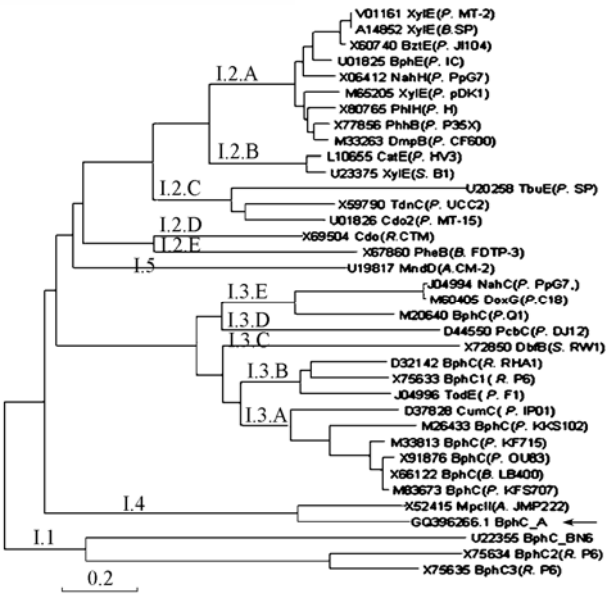

Fig.2 Phylogenetic tree of extradiol dioxygenase

The tree was constructed by analyzing the alignment of 36 extradiol dioxygenase sequences *via* the Clustal X program and the N-J method. The symbols at branch points, *e.g.,* I.2.A, designated as type(I), family(2), and subfamily(A). The names of enzymes are indicated after their GenBank database accession number. The names of strains are indicated in parentheses. A.: *Arthrobacter(A.*CM-2) or *Alcaligenes*(*A*. JMP222). B.: *Bacillus*(*B.* SP and *B.* FDTP-3) or *Burkholderia*(*B.*LB400); P.: *Pseudomonas*; R.: *Rhodococcus*; S.: *Sphingomonas.*

3.2 Purification and Electrophoresis Analysis of His-tagged BphC_A

To verify its activity and function, BphC_A was recombined with an expression vector pET28a, overexpressed in *E. coli* BL21(DE3) pLysS with a polyhistidine tag at the N-terminal, and then purified and immobilized *via* metal affinity chromatography. The crude purified enzyme was further processed with anion exchange chromatography. Finally, the purity of the enzyme prepared was more than 95%.

The purified enzyme migrated as a single band with a molecular weight of about 36800 on SDS-PAGE, close to its theoretical molecular weight of 37000 with the 6×His-tag(Fig.3, Lane 1). Western blot analysis shows a single band corresponding to 35000, which was observed in the PVDF membrane(Fig.3, Lane 2). To investigate its aromatic ring cleavage activity *in situ*, the purified BphC_A was run on native PAGE and activity staining was carried out with 2,3-dihydroxybiphenyl as the substrate. The clear bright yellow color was observed at the same band position of the purified enzyme(Fig.3, Lane 3), which verified that BphC_A catalytically oxidizes 2,3-dihydroxybiphenyl. These results illustrate that the purified His-tagged protein was the target recombinant protein. Thus, the recombinant protein was used for subsequent studies.

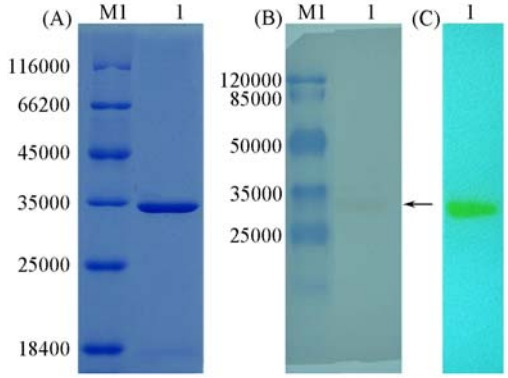

Fig.3 SDS-PAGE(A), Western blot(B) and native-PAGE (C) analysis of the purified His-tagged BphC_A

Lane 1: BphC_A. (A) Lanes M1 and 1 were stained with Coomassie brilliant blue R250 after SDS-PAGE. Lane M1 is the unstained protein molecular weight maker(Fermentas, China). (B) Lane M1 was stained with Coomassie brilliant blue R250 after SDS-PAGE and is the prestained protein molecular weight maker for Western blot(Fermentas, China). Western blot analysis was performed on Lane 1 after SDS-PAGE. (C) Lane 1 was obtained by activity staining after Native-PAGE. Each sample lane represents 5 μg of the protein. Molecular weights were determined with 12% SDS-PAGE.

3.3 Substrate Specificity of BphC_A

To determine the substrate specificity, the enzyme was tested for its ability to oxidize 2,3-dihydroxybiphenyl, catechol, 4-chlorocatechol, 3-methylcatechol, and 4-methylcatechol. The apparent kinetic constants of BphC_A are summarized in Table 1. The K_m for 3-methylcatechol was the lowest(22.69 μmol), whereas that for 4-methylcatechol was the highest(296.90 μmol). The highest k_{cat} was for 3-methylcatechol, and the largest k_{cat} reduction was for 4-methylcatechol.

Table 1 Apparent kinetic parameters of BphC_A*

Substrate	K_m/μmol	k_{cat}/s^{-1}	$(10^2 k_{cat}/K_m)/(s^{-1}\cdot\mu mol^{-1})$
3-Methylcatechol	22.69	48.73	215.00
2,3-Dihydroxybiphenyl	33.44	29.88	89.00
Catechol	79.91	11.03	13.80
4-Chlorocatechol	102.33	0.73	0.70
4-Methylcatechol	296.90	0.16	0.05

* The kinetic constants were calculated from the Lineweaver-Burk plots based on nonlinear regression with the help of GraphPad Prism 5(GraphPad, USA). All experiments were performed at least thrice.

BphC_A showed higher catalytic activity for 3-methylcatechol, different from the most EDOs that preferentially cleave catechol or 2,3-dihydroxybiphenyl. BphC_A had better

catalytic efficiency for catechol and 2,3-dihydroxybiphenyl (k_{cat}/K_m), corresponding to 3/50 and 21/50 that of BphC_A for 3-methylcatechol, respectively. In contrast, 4-chlorocatechol and 4-methylcatechol were poor substrates for BphC_A. For 4-chlorocatechol, BphC_A showed catalytic efficiency with three orders of magnitude lower than that observed with 3-methylcatechol as substrate. Similarly, 4-methylcatechol, compared with 4-chlorocatechol, was very inefficiently transformed by BphC_A, with k_{cat}/K_m values that are four orders of magnitude lower than the corresponding values with 3-methylcatechol. According to the specificity constant(k_{cat}/K_m, Table 1), BphC_A cleaved the dihydroxylated substrates in the following order of specificity: 3-methylcatechol>2,3-dihydroxybiphenyl>catechol>4-chlorocatechol>4-methylcatechol. This implies that 3-methylcatechol is the best one catalyzed by the enzyme in the given substrates.

3.4 Effects of Metal Ions on BphC_A Activity

The effects of metal ions on the enzyme activity were tested with 3-methylcatechol as the substrate. As shown in Table 2, 1 mmol/L $CoCl_2$ weakly stimulated the enzyme activity, whereas $CuCl_2$ and $ZnCl_2$ slightly reduced the activity. The enzyme activity was significantly inhibited by Al^{3+}(1 mmol/L caused 96% inhibition) and Mn^{2+} stimulated the activity by 166%. These results are similar to those of biphenyl-2,3-diol-1,2-dioxygenases, BphC1 and BphC2 from *Rhodococcus* sp. R04[25].

Table 2 Effects of metal ions on the activity of BphC_A[a]

Metal ion	Relative activity(%)[b]	Metal ion	Relative activity(%)[b]
No addition	100.0	$Co^{2+}(CoCl_2)$	102.9±6.7
$K^+(KCl)$	88.7±5.7	$Ca^{2+}(CaCl_2)$	67.8±4.3
$Al^{3+}(AlCl_3)$	3.6±0.1	$Fe^{2+}(FeCl_2)$	83.6±5.4
$Cu^{2+}(CuCl_2)$	43.1±0.2	$Zn^{2+}(ZnCl_2)$	38.6±2.5
$Mg^{2+}(MgCl_2)$	67.8±2.9	$Fe^{3+}(FeCl_3)$	60.8±3.9
$Mn^{2+}(MnCl_2)$	166.9±10.9		

a. The concentration of each of additional metal ions was 1 mmol/L; *b*. the extent of inhibition or activation of enzyme activity was described as percentages of the ratio of residual activity to complete enzyme activity in the control sample(without the addition of metal ions).

3.5 Effect of pH Value on BphC_A Activity and Stability

The effect of pH value on enzyme activity was tested at various pH values(4—11)[Fig.4(A)]. The optimum pH value was 8.5 at 25 °C with 3-methylcatechol as the substrate. The enzyme retained substantial activity at pH value up to 9.0 and very little activity(<20%) at pH value lower than 7.0. The enzyme was stable in a narrow pH value range from 7.5 to 8, where almost 80% of the initial activity remained. This result suggests that BphC_A requires weak alkaline conditions during the catalytic process.

3.6 Effect of Temperature on BphC_A Activity and Stability

The temperature *vs.* activity profile of BphC_A was examined over temperatures ranging from 5 °C to 60 °C under assay conditions, with 3-methylcatechol as the substrate. Activity was significantly increased at temperatures ranging from 5 °C to 35 °C, declined beyond 35 °C, and was completely inactivated at 60 °C. The enzyme had an maximal activity at 35 °C, but the enzyme retained more than 42% of its activity at 5 °C[Fig.4(B)]. Regarding the effect of temperature on enzyme stability, BphC_A was highly stable at 5—30 °C, with a residual activity greater than 95% of the initial activity. However, incubation beyond 35 °C caused the activity to decline sharply with practically no activity after incubation for 30 min at 45 °C. According to the temperature activity experiments, BphC_A could exhibit relatively high catalytic activity at low temperatures and was very thermo sensitive, which suggests cold-adapted property[26]. We determined the activation energy for the 3-methylcatechol catalyzed by BphC_A. The activation energy of the cold-adapted enzymes is usually lower than those from their mesophilic counterparts[27]. As expected, the activation energy of BphC_A was about 59.021 kJ/mol in a range of 5 °C to 25 °C, but the enzyme activity at temperatures above 40 °C declined very rapidly with an inactivation energy of 812.055 kJ/mol(data not shown).

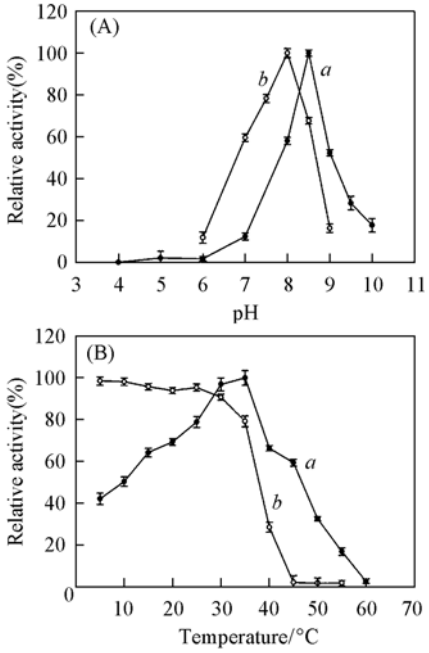

Fig.4 Effects of pH(A) and temperature(B) on the activity and stability of BphC_A

(A) *a.* activity; *b.* pH stability; (B) *a.* activity; *b.* thermal stability. Relative activity is expressed as a percentage of the maximum of enzyme activity of BphC_A.Values are means of three different experiments. Error bars indicate the standard deviation(SD).

A low optimized temperature or high activity at low temperatures is a prerequisite for an enzyme to be categorized as cold active one[28]. The high catalytic activity at 5 °C suggests that BphC_A satisfies the criteria as a cold-active enzyme. However the low temperature optimum for any other extradiol dioxygenases has never been reported. The optimum low temperature of BphC_A is similar to those of other cold adapted enzymes *e.g.*, the low-temperature esterase whose optimum temperature was 50 °C[29] and cold active amylase which shows

a relatively high activity from 25 °C to 55 °C[30]. Taken together, BphC_A was a cold-adapted enzyme.

4 Conclusions

An extradiol dioxygenases, termed BphC_A, from a metagenomic library was purified from recombinant *E. coli* and characterized. The protein shares less than 60% amino acid sequence identity with other known EDOs. In addition, the purified BphC_A has a preference for 3-methylcatechol and exhibits high activity at low temperatures. These characteristics indicate that BphC_A is a novel member of the EDOs family. The present functional screening for extradiol dioxygenases from a metagenomic library is a remarkably effective way to exploit the great gene pool constructed from contaminated samples. Furthermore, the cloning and overexpression of BphC_A provide a basis for further kinetic and structural studies, and the eventual identification of the underlying structure-function correlation and the substrate specificity of this new EDO. Ultimately, these studies may help engineer a BphC_A to be used as a potential bioremediation enzyme.

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