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Molecular Cloning and Characterization of a *cis*-Epoxysuccinate Hydrolase from *Bordetella* sp. BK-52

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A *cis*-epoxysuccinate hydrolase (CESH) from *Bordetella* sp. BK-52 was purified 51.4-fold with a yield of 27.1% using ammonium sulfate precipitation, ionic exchange, hydrophobic interaction, molecular sieve chromatography and an additional anion-exchange chromatography. The CESH was stable in a broad range of temperature (up to 50°C) and pH (4.0–10.0) with optima of 40°C and pH 6.5, respectively. It could be partially inhibited by EDTA-Na_2, Ag^+, SDS, and DTT, and slightly enhanced by Ba^{2+} and Ca^{2+}. The enzyme exhibited high stereospecificity in D(–)-tartaric acid (enantiomeric excess value higher than 99%) with K_m and V_{max} values of 18.67 mM and 94.34 μM/min/mg for disodium *cis*-epoxysuccinate, respectively. The *Bordetella* sp. BK-52 CESH gene, which contained 885 nucleotides (open reading frame) encoding 294 amino acids with a molecular mass of about 32 kDa, was successfully overexpressed in *Escherichia coli* using a T7/lac promoter vector and the enzyme activity was increased 42-times compared with the original strain. It may be an industrial biocatalyst for the preparation of D(–)-tartaric acid.

Keywords: *cis*-Epoxysuccinate hydrolase, *Bordetella*, D(–)-tartaric acid, cloning, purification, characterization

Epoxide hydrolases (E.C. 3.3.2.3) have gained considerable attention in recent years owing to their role in cellular detoxification processes and the metabolism of a number of biologically important compounds. From a biotechnological perspective, epoxide hydrolases of high enantioselectivity are useful biocatalysts for the production of optically active epoxides and vicinal diols [19]. *cis*-Epoxysuccinate hydrolase (CESH), an epoxide hydrolase, is a versatile biocatalyst for the asymmetric hydrolysis of *cis*-epoxysuccinate to the corresponding tartrate requiring neither cofactors, and prosthetic groups nor metal ions for its activity [20].

D(–)-Tartaric acid, hardly existing as a natural resource, can be used as chiral auxiliaries and resolving agents [4]. Traditionally, D(–)-tartaric acid is prepared through chemical or biological separation of DL-tartaric acid [5]. Recently, a biotransformation approach for production of D(–)-tartaric acid with CESH from genera *Pseudomonas*, *Alcaligenes* (M. Ikuta *et al*. 2000. JP 2000-014391), and *Bordetella* [16] has become popula owing to its higher enantiomeric excess (ee), higher yield, and environmentally friendlier process without the need of heavy-metal-based catalysts in contrast to the chemical approach. At present, only a gene encoding *Alcaligenes* sp. MCI 3611 CESH for production of D(–)-tartaric acid was reported (Y. Asai *et al*. 2000. JP 2000-295992).

Recently, we have isolated and identified a *Bordetella* sp. BK-52, which has the ability to produce CESH that can convert inexpensive and readily available *cis*-epoxysuccinate to D(–)-tartaric acid. However, its enzyme activity was low (764 U/g biomass) owing to the low enzyme productivity [16]. Therefore, in this study, the CESH from *Bordetella* sp. BK-52 was purified and some characteristics of purified CESH were investigated. Then, the CESH gene was cloned and expressed in *Escherichia coli* in order to improve the enzyme productivity.

Materials and Methods

Strains, Plasmids, and Growth Conditions

Bordetella sp. BK-52 was deposited in the China General Microbiological Culture Collection Center (CGMCC No. 2075, Beijing, China) and cultivation was followed according to the previous work [16]. *E. coli* DH5α and BL21 (DE3) were used as a transformation and expression host. The plasmids pUC_m-T (Sangon) and pET22b(+) (Novagen) were used for cloning and expression, respectively. *E. coli* strains were cultivated at 37°C and 250 rpm in

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Luria–Bertani medium. Ampicillin, when added, was used at a final concentration of 0.05 g/l.

Activity Assay

Throughout the purification of the enzyme, CESH activity was assayed at 37°C for 20 min in 100 mM potassium phosphate (pH 8.0) with 200 mM disodium *cis*-epoxysuccinate. The content of tartaric acid was determined by the ammonium metavanadate method [10]. One unit of enzyme was defined as the amount of enzyme that generated 1 μmole of tartaric acid per minute under the above-mentioned reaction conditions and the number of such units per milligram of protein was defined as specific activity.

SDS–PAGE and Protein Quantification

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was conducted in slab gels consisting of a 12% acrylamide resolving gel and a 5% acrylamide stacking gel [9]. The gel was stained with Coomassie Brilliant blue R-250. Protein quantitative analysis was determined by the Bradford method with bovine serum albumin as a standard [2].

Purification of CESH

Purification of CESH from *Bordetella* sp. BK-52 was performed according to the method by Kotik and Kyslik [7]. Briefly, *Bordetella* sp. BK-52 was suspended in buffer A (100 mM potassium phosphate, pH 8.0), ruptured by sonication, and the supernatant was considered as crude enzyme. The precipitate was collected from 40% to 65% saturation of ammonium sulfate, dissolved, dialyzed against buffer A, applied to a 120 cm^3 DEAE-Sepharose Fast Flow column (Pharmacia Biotech), and eluted with a linear gradient of 0 to 0.6 M KCl. Active fractions were pooled, dialyzed against buffer B (25 mM potassium phosphate containing 1 M KCl, pH 8.0), loaded onto a 50 cm^3 Phenyl-Sepharose CL-4B column (Pharmacia Biotech), and eluted with a linear gradient of 1 to 0 M KCl and 0 to 50% ethylene glycol. Active fractions were pooled, dialyzed against buffer A, loaded onto a 80 cm^3 Sephadex G75 column (Pharmacia Biotech), and eluted with buffer A. Active fractions were pooled, loaded onto a 30 cm^3 Q-Sepharaose column (Pharmacia Biotech), and eluted with a linear gradient of 0 to 0.5 M KCl. The resulting CESH preparation was stored at −20°C in buffer A containing 50% glycerol.

Effects of Temperature and pH

The optimum temperature was determined by varying the assay temperature in the reaction mixture, and thermal stability was obtained after pre-incubation of purified CESH at different temperatures for 30 min. The residual activity (%) that assayed under standard reaction was taken as 100%. Each experiment involved three independent events.

The optimum pH was obtained by various pH buffers at 37°C. pH stability was investigated after pre-incubation of CESH in different pH buffers at room temperature (25°C) for 30 min, followed by rapid recovery to the assay pH (8.0). The residual activity (%) that assayed under standard reaction was taken as 100%. In all cases, overlaps were obtained when buffers were changed so that correction could be made for spurious buffer effects. Each experiment involved three independent events.

Effects of Metal Ions and Other Reagents

The effects of different metal ions and other reagents on CESH activity were assayed by incubating the enzyme in the presence of reagents under standard reaction in three independent experiments. The residual activity (%) was calculated in relation to values obtained upon incubation of enzyme without any reagent.

Kinetic Parameters

The Michaelis–Menten kinetic parameters, K_m and V_{max}, of CESH were determined with 22 μg of purified enzyme at increasing substrate concentration ranging from 12 mM to 200 mM in 100 mM phosphate buffer (pH6.5) at 40°C. The Lineweaver–Burk plot was used to determine K_m and V_{max} values.

Enantioselectivity Assay

The enzymatic reaction was performed under standard conditions and stopped by diluting the mixture 200 times with H_2O acidified to pH 2.2. For determination of enantioselectivity, the ee value was determined by HPLC (Agilent 1200) at 30°C on a chiral column Astec CLC (150 mm×4.6 mm) with a sample volume of 20 μl. The mobile phase was 3 mM $CuSO_4$ (pH 3.2) with a flow rate of 1 ml/min and the detection was done at 254 nm.

Determination of CESH N-Terminal Amino Acid Sequences

Samples of purified CESH were separated by SDS–PAGE and transferred to PVDF membranes by Western blotting. The PVDF membranes were stained with Coomassie Brilliant Blue R-250 and the band corresponding to CESH was excised and sequenced using an Applied Biosystems Procise 492 cLC protein sequencer.

Genomic DNA Extraction and PCR Amplification

Genomic DNA of *Bordetella* sp. BK-52 was extracted using an EZ-10 spin column genomic DNA isolation kit (Bio Basic Inc). The CESH gene was amplified with primers P1: 5'-ATGACNYGN(or R)ACNAARXTN(or R)-3' (Y represents A or C, R represents A or G, X represents T or C, N represents A, C, G, or T) and P2: 5'-CTAGTTGCTAATACCCAG-3', which were designed based on the sequences of the NH_2-terminus of CESH of *Bordetella* sp. BK-52 and the sequence (GenBank Accession No. E50984) reported by the National Center for Biotechnology Information (NCBI), respectively. PCR was carried out in a 50-μl volume under the following conditions: 5 min at 94°C, 30 cycles of 50 s at 94°C, 30 s at 40°C, 1 min at 72°C, and one final step of 10 min at 72°C. The PCR products were purified from the agarose gel using a Wizard PCR preps kit (Promega), ligated with pUC_m-T by using the T/A cloning procedure, and transformed into *E. coli* DH5α cells [3]. A positive clone was selected and sequenced by Takara Biotechnology Co., Ltd., China.

Cloning and Expression of CESH in *E. coli*

According to the result of sequencing, a pair of primers, P3 and P4, were designed to amplify the ORF fragment of CESH, where the underlined parts below were the *Nde*I and *Bam*HI sites, respectively.

P3: 5'-GCCATATGATGACTCGAACCAAGTTGATACT-3'

P4: 5'-CCGGATCCTTAGTTGCTAATACCCAGAATTT-3'

PCR was carried out with *Bordetella* sp. BK-52 genomic DNA as template under the following conditions: 5 min at 94°C, 30 cycles of 50 s at 94°C, 30 s at 57°C, 1 min at 72°C, and one final step of 10 min at 72°C. The ORF fragments and the vector pET22b(+) were digested with *Nde*I and *Bam*HI, ligated by T_4 DNA ligase, and transformed into *E. coli* BL21. Recombinant *E. coli*/pET22b-CESH was precultured overnight at 37°C in 25 ml of LB medium. The culture solution was then completed to 500 ml of LB medium at

Table 1. Summary of purification of CESH from *Bordetella* sp. BK-52.

Step	Activity (U)	Protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Crude enzyme	888.2	493.5	1.8	100	1
Ammonium sulfate	791.1	313.7	2.5	89.1	1.4
DEAE-Sepharose	666.5	29.7	22.4	75	12.4
Phenyl-Sepharose	469.5	12.2	38.5	52.9	21.4
Sephadex G75	382.9	5.3	72.2	43.1	40.1
Q-Sepharose	240.7	2.6	92.6	27.1	51.4

37°C. When cell growth in the culture reached 0.6 optical density at 600 nm, isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM to initiate overexpression for an additional 20 h at 25°C.

Nucleotide Sequence Accession Number

The nucleotide sequence of CESH from *Bordetella* sp. BK-52 was submitted to the GenBank nucleotide sequence database under Accession No. EU053208.

RESULTS

Purification of CESH

The CESH from *Bordetella* sp. BK-52 was purified with a 27.1% yield by ammonium sulfate precipitation and four chromatographic steps (Table 1). The specific activity increased from 1.8 to 92.6 U/mg, indicating a 51.4-fold purification of the CESH. SDS–PAGE revealed that the CESH isolated was essentially pure with only very faint bands of contaminating protein being visible, and the molecular mass of CESH was between 25 kDa and 35 kDa (Fig. 1).

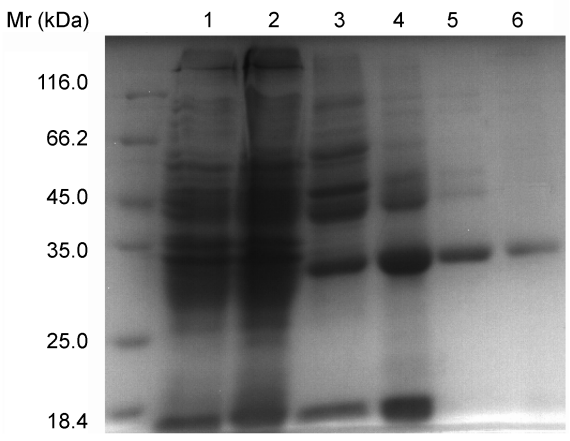

Fig. 1. Purification of CESH from *Bordetella* sp. BK-52.
Samples were taken at different stages of CESH purification (Table 1) and subjected to 12% SDS–PAGE. Lanes: 1, Crude extract; 2, 40–65% saturated ammonium sulfate precipitate; 3, pooled fractions after the DEAE-Sepharose step; 4, pooled fractions after the Phenyl-Sepharose step; 5, pooled fractions after the Sephadex G75 step; 6, pooled fractions after the Q-Sepharose step.

Characterization of CESH

As shown in Fig. 2, CESH was most active between 37°C and 45°C with an optimum of 40°C but was strongly decreased at temperatures above 55°C. The enzyme could maintain nearly 74% activity when incubated at 50°C for 30 min. As shown in Fig. 3A, CESH showed high activity in a pH range between 6.0 and 8.0 with an optimum pH of 6.5 and showed high stability in a broad pH range between 4.6 and 9.0 (Fig. 3B). There was 60% residual activity at pH 10.0 or 4.0.

As shown in Table 2, CESH was partially inhibited by metal chelator EDTA-Na_2, Ag^+, SDS, and dithiothreitol (DTT) with DTT being the strongest, and slightly inhibited by Fe^{2+}. It was slightly enhanced by Ba^{2+} and Ca^{2+}, which indicated that they had a stabilizing effect on CESH. Other metal ions (Zn^{2+}, Mn^{2+}, Mg^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Cs^{2+} with concentration of 1 mM), ethanol [1% (v/v)], and Triton X-100 [1% (v/v)] could weakly affect enzyme activity (data not shown).

Lineweaver–Burk plot analysis calculated the values of K_m and V_{max} to be 18.67 mM and 94.34 μM/min/mg. HPLC analysis revealed that CESH could enantioselectively hydrolyze *cis*-epoxysuccinate to D(–)-tartaric acid with an ee value higher than 99%.

CESH Gene Amplification

The sequences of the NH_2-terminus of *Bordetella* sp. BK-52 CESH were shown as follows: Met Thr Arg Thr Lys

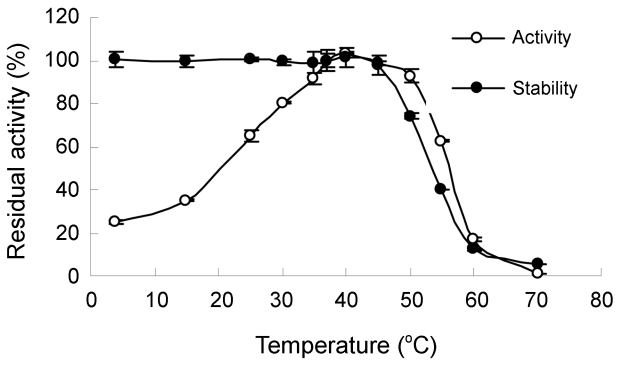

Fig. 2. Effect of temperature on the activity and stability of purified CESH.

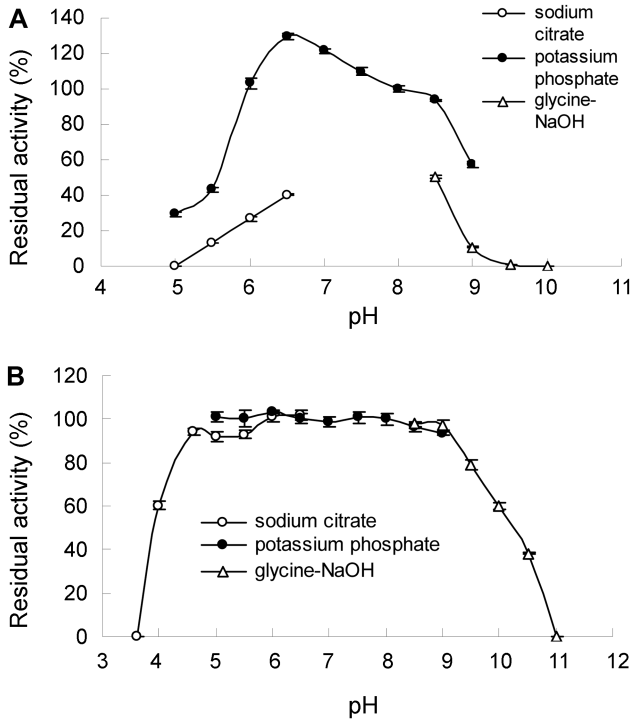

Fig. 3. Effect of pH on the activity (**A**) and stability (**B**) of purified CESH.
Buffers including sodium citrate (pH 3.6–6.5), potassium phosphate (pH 5.0–9.0), and glycine-NaOH (pH 8.5–11.0) with concentrations of 100 mM each were used.

Leu Ile Leu Glu Ala Arg Ile. Therefore, the degenerate primer P1 was designed as the upstream primer of PCR. N-Terminal sequence alignment was performed using BLAST supplied by the NCBI, and the translated nucleotide sequence of CESH from *Alcaligenes* sp. MCI 3611 (GenBank Accession No. E50984) showed 100% identity.

Table 2. Effects of metal ions and other reagents on CESH activity.

Reagent	Concentration	Relative activity (%)
None	-	100±0.059
EDTA-Na_2	10 mM	77.06±2.50
Ca^{2+}	1 mM	108.82±0.01
Fe^{2+}	1 mM	92.06±2.50
Ag^{+}	1 mM	52.94±2.18
Ba^{2+}	1 mM	109.41±1.24
DTT	10 mM	34.71±0.88
SDS	1% (w/v)	47.06±1.03

Therefore the downstream primer P2 was designed according to the sequence E50984. Then, the primers P1 and P2 were used to amplify the CESH gene and a sequence of about 0.9 kb was achieved. The sequence analysis using software DNAMEN (Version 4.0, Lynnon Biosoft) indicated that the fragment contained an ORF of 885 bp encoding 294 amino acids beginning with the ATG methionine codon (GenBank Accession No. EU053208). The calculated molecular mass of 32.48 kDa was in agreement with the molecular mass observed in the purification procedure. This was the first report on the sequence of gene encoding *Bordetella* sp. CESH.

Cloning and Expression of CESH in *E. coli*

The DNA encoding CESH was amplified and introduced into pET22b. The recombinant plasmid pET22b-CESH map is shown in Fig. 4A. Overexpression of CESH would facilitate better understanding of its characteristics and could result in improving enzyme productivity. Therefore, CESH was designed to express without any vector sequence, such as tag or signal peptide, in order to obtain its natural structure. Expression of CESH was induced by addition of

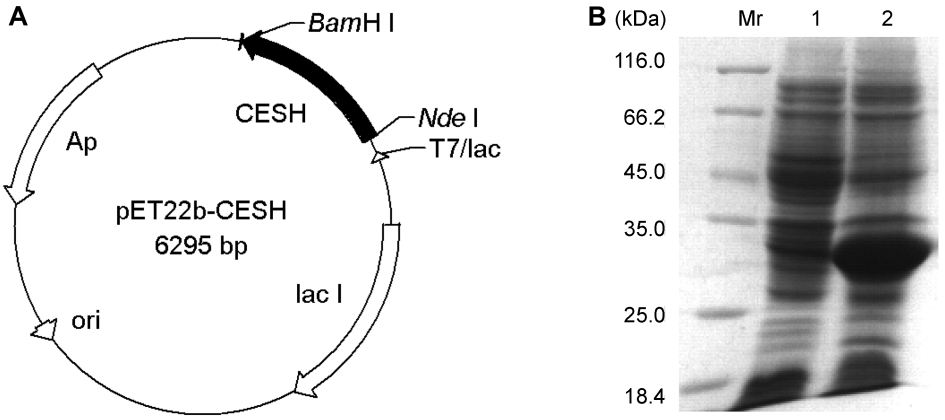

Fig. 4. Schematic diagram of recombinant plasmid construct (**A**) and identification of the successfulness of CESH expression by SDS–PAGE (**B**).
Lanes: 1, Total protein of recombinant bacteria *E. coli*/pET22b-CESH without induction; 2, total protein of recombinant bacteria *E. coli*/pET22b-CESH induced by 0.4 mM IPTG.

IPTG at low temperature (25°C) to obtain a higher ratio of soluble active enzyme and a lower ratio of insoluble inclusion bodies [17]. The recombinant *E. coli* showed low background expression of CESH without IPTG induction, whereas high-level expression of CESH induced by IPTG (Fig. 4B). All these results showed that the CESH gene from *Bordetella* sp. BK-52 was successfully expressed in *E. coli*. According to the method described by our previous work [16], the enzyme activity of engineering bacteria could achieve 32,418 U/g biomass, which was about 42 times as much as that of the original strain (764 U/g biomass). This established a beneficial foundation for industrial production of D(−)-tartaric acid.

DISCUSSION

In recent years, bacterial epoxide hydrolases, as a promising biocatalyst, were widely put into industrial applications for the preparation of enantiopure pharmaceuticals and other fine chemicals. The industrial synthesis of L(+)- and meso-tartaric acids was the first application of an epoxide-hydrolase-catalyzed epoxide hydrolysis [1]. Various bacterial epoxide hydrolases were isolated and purified from different strains such as *Nocardia* sp. EH1 [8], *Rhodococcus* sp. NCIMB 11216 [13], *Pseudomonas* sp. strain AD1 [6], and *Corynebacterium* sp. strain N-1074 [14]. Genes encoding epoxide hydrolases were also reported [11, 12, 18].

However, these epoxide hydrolases described above showed stereospecificity for L(+)-tartaric acid, but not for D(−)-tartaric acid. So far, only the gene encoding *Alcaligenes* sp. MCI 3611 CESH was reported to produce D(−)-tartaric acid. This paper described the discovery, cloning, expression, and characterization of a highly enantioselective CESH for producing D(−)-tartaric acid from *Bordetella* sp. BK-52. Interestingly, its deduced amino acid sequence was absolutely identical to the deduced sequence of the β-subunit of CESH from *Alcaligenes* sp. MCI3611. The CESH from *Alcaligenes* sp. MCI3611 (molecular mass of about 80 kDa) comprised two hetero subunits including an α-subunit (1,164 nucleotides encoding 387 amino acids with a molecular mass of about 40 kDa) and a β-subunit (885 nucleotides encoding 294 amino acids with a molecular mass of about 33 kDa) (Y. Asai *et al*. 2000. JP 2000-295992). No more information was reported on the function of each subunit and enzyme characteristics. However, as Fig. 1 shows, there was no α-subunit in *Bordetella* sp. BK-52 CESH, indicating that the α-subunit of *Alcaligenes* sp. MCI3611 CESH may play no essential functions in the reaction.

Nucleotide sequences of *Bordetella* sp. BK-52 CESH and the β-subunit of *Alcaligenes* sp. MCI3611 CESH had some differences. Eight same sense mutation sites of the CESH gene were found between genera *Bordetella* and *Alcaligenes*, including the termination codon. This revealed that the CESH for producing D(−)-tartaric acid possessed extremely high conservatism during species evolution. However, the CESH for producing L(+)-tartaric acid possessed relatively low conservatism. For example, epoxide hydrolase sequences comparison between *Agrobacterium radiobacter* AD1 and *Corynebacterium* sp. C12 using CLUSTALX 2.0 shared 27% identity, whereas *A. radiobacter* AD1 and *Pseudomonas stutzeri* A1501 shared 20% identity and *Corynebacterium* sp. C12 and *P. stutzeri* A1501 shared 18% identity.

Although the CESH for production of D(−)-tartaric acid and L(+)-tartaric acid had the same function (hydrolysis of *cis*-epoxysuccinate into tartaric acid) except for stereoselectivity, sequence comparison showed that the CESH for production of D(−)-tartaric acid (*Bordetella* sp. BK-52 and *Alcaligenes* sp. MCI3611) had extremely low similarity to the CESH for production of L(+)-tartaric acid from *A. radiobacter* AD1 (2%), *Corynebacterium* sp. C12 (5%), and *P. stutzeri* A1501 (2%). Considering the hydrophobicity of both CESH, moreover, the hydrophobic amino acids (Ala, Ile, Leu, Phe, Trp, and Val) by frequency in *Bordetella* sp. BK-52 CESH and *Alcaligenes* sp. MCI3611 CESH (34.7%) were obviously lower than that in *A. radiobacter* (36.7%), *Corynebacterium* sp. (38.1%), and *P. stutzeri* (39.6%). *A. radiobacter* AD1 epoxide hydrolase provided the scaffolding for the catalytic triad residues, Asp 107, Asp 246, and His 275 [15]. Multiple sequence alignment using CLUSTALX 2.0 (Fig. 5) showed that Asp 107 and His 275 were conserved in CESH for production of L(+)-tartaric acid, whereas *Bordetella* sp. BK-52 CESH and *Alcaligenes* sp. MCI3611 CESH were not. Furthermore, CESH for production of D(−)-tartaric acid showed extremely low similarity to the conserved domains of CESH for production of L(+)-tartaric acid (marked with fuscous or tink shading in Fig. 5), which indicated a different catalysis mechanism and may provide the clue for further study. In addition, our study indicated that *Bordetella* sp. BK-52 CESH was stable with lower V_{max} (94.34 μM/min/mg), whereas the CESH from *Rhodococcus opacus* for production of L(+)-tartaric acid was sensitive to heat with relatively higher V_{max} (2,240 μM/min/mg) [11]. Therefore, further studies are needed to uncover the underlying structure and catalysis mechanism to describe these differences between these two types of CESH.

Investigating the enzymatic properties of CESH is necessary to identify, develop, and optimize enzymes for use in industrial processes. Our results revealed that *Bordetella* sp. BK-52 CESH showed high activity in a broad temperature and pH range, had high temperature and pH stabilities, was active in the presence of most metal ions, and showed a high stereospecificity in D(−)-tartaric acid, which indicated that the recombinant CESH has potential for use in industrial application. In addition, the enzyme activity of engineering bacteria could achieve

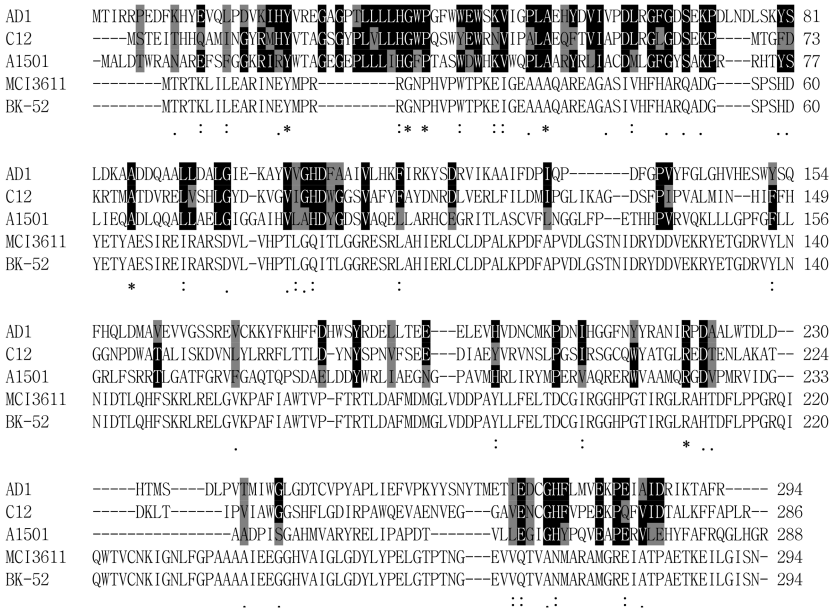

Fig. 5. Sequence alignments on the desired amino acid sequence.
The amino acid sequences used for the alignment using the multiple alignment program CLUSTALX 2.0 are shown as follows: *Agrobacterium radiobacter* AD1 epoxide hydrolase (AD1, gi: 2292731); *Corynebacterium* sp. C12 epoxide hydrolase (C12, gi: 2897599); *Pseudomonas stutzeri* A1501 epoxide hydrolase (A1501, gi: 146280756); *Alcaligenes* sp. MCI3611 epoxide hydrolase (MCI3611, gi: 18622161); *Bordetella* sp. BK-52 epoxide hydrolase (BK-52, gi: 155090567). "*", ":", and "." denote identical residues, conserved substitution residues, and semiconserved substitution residues in all sequences, respectively. The conserved residues of epoxide hydrolase (AD1, C12, and A1501) are marked with fuscous or tink shading.

32,418 U/g biomass, which established a beneficial foundation for industrial production.

In conclusion, the CESH was purified from *Bordetella* sp. BK-52, some characteristics were investigated, and its gene was successfully cloned and expressed in *E. coli* in this study. Overexpression of CESH would facilitate better understanding of its structure and the recombinant *Bordetella* sp. BK-52 CESH may be an industrial biocatalyst for the preparation of D(−)-tartaric acid.

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