Isolation and identification of a novel cis-epoxysuccinate hydrolase-producing Bordetella sp. BK-52 and optimization of enzyme production

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Abstract: [Objective] To isolate and identify a novel strain with cis-epoxysuccinate hydrolase (CESH) activity and to optimize its enzyme production. [Methods] The isolated strain was identified by electron microscopy, Biolog gram negative (GN) test, G+C (guanine plus cytosine) content measurement and 16S rDNA sequence. The purified enzymatic biotransformed product was identified by IR, ¹H-NMR, ¹³C-NMR, MS and optical rotation analysis. Then the fermentation conditions for CESH production were optimized. [Results] A novel CESH-producing strain was isolated for biotransforming cis-epoxysuccinate to D(-)-tartaric acid. It was assigned to genus Bordetella and named Bordetella sp. BK-52. The optimal conditions were found to be 30°C, pH 7.0, fermentation time 36 h, carbon source of saccharose, inorganic nitrogen source of ammonium sulfate and enzyme inducer of disodium cis-epoxysuccinate. Under these conditions, the maximum CESH activity reached 764 U/g biomass. [Conclusion] The isolated Bordetella sp. BK-52 provided a new alternative for biosynthesis of D(-)-tartaric acid from cis-epoxysuccinate.

Keywords: Bordetella; cis-epoxysuccinate hydrolase; D(-)-tartaric acid; Isolation; Identification


1 INTRODUCTION

D(-)-tartaric acid (2S,3S-2,3-dihydroxy-1,4-butanedioic acid, CAS number: 147-71-7), hardly existing as a natural resource[1], is mainly used as chiral auxiliaries, resolving agents and building blocks in the pharmaceutical industry[2-4]. In addition, D(-)-tartaric acid has also been widely used in synthesizing and manufacturing many kinds of chiral food additives and nutrient additives. Because of its important roles, the demand of D(-)-tartaric acid is increasing progressively year by year.

Traditionally, D(-)-tartaric acid is mainly prepared through chemical resolution of DL-tartaric acid[5] which is easily obtained by reacting maleic acid or anhydride with a peroxide and hydrolyzing the resulting oxide product. Another alternative for D(-)-tartaric acid production is biochemical resolution from DL-tartaric acid by selectively assimilating its dextrorotatory optical isomer, L(+)-tartaric acid, with special microorganisms such as genus Pseudomonas, Cryptococcus, Tricocspor and Klebsiella[6]. As commonly accepted, biocatalytical preparations of organic acids have superceded chemical synthesis due to their ecological and economical advantages. Therefore, a novel microbially approach for D(-)-tartaric acid production from cis-epoxysuccinate by some microorganisms, such as genus Pseudomonas and Alcaligenes[7,8], has become overwhelming. In these microorganisms, the enzyme cis-epoxysuccinate hydrolase (CESH), an epoxide hydrolase (EC 3.3.2.3), is expressed, which is a versatile biocatalyst for the asymmetric hydrolysis of epoxides requiring neither cofactors, prosthetic groups nor metal ions for its activity. For either research or industrial purpose, it’s a prerequisite to obtain a CESH-producing strain.

This study aimed to isolate a wild-type strain capable of producing CESH for generating D(-)-tartaric acid from cis-epoxysuccinate. The strain was then identified according to its morphological, biochemical characteristics, and 16S rDNA gene sequence. The enzymatic-biotransformed product was identified by infrared spectrum (IR), nuclear magnetic resonance spectrum (¹H-NMR, ¹³C-NMR), mass spectrum (MS)
2.1 Chemicals and instruments

The standard D(-)-tartaric acid was purchased from RichJoint Chemical Reagents CO., Ltd. (China). EZ-10 spin column genomic DNA isolation kit was purchased from Bio Basic Inc. (Canada). Wizard PCR prep kit was purchased from Promega (USA). pMD18-T vector was purchased from TaKaRa (Japan). Other chemicals used were analytical grade. Instruments included JEM-1230 transmission electron microscopy (JEOL, Japan), UV-2550 spectrophotometer (Shimadzu, Japan), Nicolet-Nexus 670 Fourier Transform Infrared spectrometer (FT-IR), Bruker Avance DMX500 NMR spectrometer and Bruker Esquire 3000plus mass spectrometer (Bruker, Bremen, Germany).

2.2 Medium

One litre of the screening medium comprised 10 g disodium cis-epoxysuccinate, 1 g yeast extract, 0.5 g KH₂PO₄, 2 g K₂HPO₄·3H₂O, 0.5 g MgSO₄·7H₂O, 3 g FeSO₄·7H₂O and 3 g (NH₄)₂SO₄. The fermentation medium consisted of 10 g disodium cis-epoxysuccinate, 10 g glucose, 3 g (NH₄)₂SO₄, 2 g yeast extract, 1 g K₂HPO₄·3H₂O, 0.5 g MgSO₄·7H₂O, and 0.01 g FeSO₄·7H₂O in 1000 mL distilled water. The pH of each medium was adjusted to 7.0 before autoclaved at 121°C for 20 min.

2.3 Isolation of strains

About 1 g of the soil sample collected from a vegetable field in Hangzhou, China, was incubated aerobically at 30°C for 4 days in a 250 mL flask with 25 mL screening medium. A loop of culture was streaked on a petri dish with the screening medium. After incubation at 30°C for 3 days, the colonies were inoculated into a 250 mL flask with 50 mL fermentation medium and cultivated at 30°C for 36 h. The cells were harvested by centrifugation for further enzyme assays.

2.4 Identification of strain

The identification of the strain was based on its morphological, biochemical properties and 16S rDNA gene sequence. The morphology of isolates was observed using electron microscopy according to the method described by Odenyo et al [9]. Biolog GN test was used to determine the ability of the strain to oxidize various carbon sources according to the method reported by He et al [10].

For (G+C) content measurement and 16S rDNA sequence analysis, genomic DNA was extracted. The (G+C) content of the genomic DNA was determined with the thermal denaturation method by a spectrophotometer equipped with a water cycle heating system. Using p16S-8 (5'-AGAGTTTGATCCTGGCTCA-3') and p16S-1541 (5'-AAGGAGGTGATCACC GCA-3') as the primers, 16S rDNA was PCR-amplified under the following conditions: 5 min at 95°C, 30 cycles of 40 s at 95°C, 60 s at 53°C, 2 min at 72°C and one final step of 10 min at 72°C. The PCR products were extracted and purified with the agarose gel and ligated with pMD18-T by using the T/A cloning procedure. The constructed vectors were transformed into the competent Escherichia coli JM109 cells according to the method described by Chung et al [11] and a positive clone was selected. DNA was sequenced and compared with sequences in the GenBank databases using BLAST program. To construct the phylogenetic tree, the sequences were retrieved from the NCBI database individually, aligned by CLUSTALX 1.8.1 with default settings and the phylogenetic tree was constructed using MEGA 3.0 based on the neighbor-joining method with bootstrapping 1000 times.

2.5 Purification and analysis of the enzymatic-biotransformed product

Purification of tartaric acid was performed according to the method described by Huang and Qian [12]. The enzymatic-biotransformed product was identified by IR, 1H-NMR, 13C-NMR, MC and optical rotation analysis.

2.6 Enzyme assay

About 1.0 g of wet cells were washed twice with physiological saline and resuspended in 10 mL of 1 mol/L disodium cis-epoxysuccinate solution (pH 8.0) containing 0.1% (W/V) sodium deoxycholate. The bioconversion was carried out with constant stirring at 37°C for 60 min. The content of tartaric acid was determined by the Ammonium Meta Vanadate method [13] and the specific activity of enzyme was consequently calculated according to the definition of one unit of enzyme as the amount of enzyme in 1.0 g wet cells capable of generating 1 μmol tartaric acid per hour under the experimental conditions described above.

3 Results

3.1 Strain isolation

Disodium cis-epoxysuccinate was the sole carbon source in the screening medium, so that the microorganisms capable of producing CESH must be able to utilize it as a carbon source and transform it to tartaric acid. In the culture supplemented with disodium cis-epoxysuccinate and inoculated with soil samples collected from vegetable fields, a strain named BK-52 capable of producing CESH was obtained, which was selected and deposited in China General Microbiological Culture Collection Center (CGMCC No. 2075, Beijing, China) for further studies.

3.2 Characterization and identification

On the screening medium plate, strain BK-52 formed a circular, convex, smooth, lustrous, pearly and white colony with approximately 1 mm in diame-
After 2 days incubation at 30°C. Furthermore, it was nonsporeforming, Gram-negative and ovoid-rod-shaped (1.7 μm long, 1.2 μm wide), and no motile or flagellum was observed (Fig.1). Biolog GN test showed that strain BK-52 could oxidize 37 kinds of substrates, and the highest similarity to the species of Bordetella was observed.

The (G+C) content of strain BK-52 was 63.9%. A 16S rDNA fragment of strain BK-52 with 1450 bp in length was deposited in the GenBank database under accession number EU000383. Multiple alignments revealed that its 16S rDNA sequence was closely related to that of *Bordetella avium* (AF177666, 97% of similarity), *B. hinzii* (AF177667, 97% of similarity) and *B. ansorpii* (AY594190, 97% of similarity). To identify the phylogeny of strain BK-52, strains from different genera were chosen to construct the phylogenetic tree based on 16S rDNA sequences. Fig.2 indicated that the strain BK-52 clustered closely with *B. ansorpii* (AY594190). By examining morphological, biochemistry characteristics and comparing its 16S rDNA sequence, strain BK-52 was assigned to genus *Bordetella* and named *Bordetella* sp. BK-52.

### 3.3 Identification of the enzymatic-biotransformed product

After fermentation, about 10 g disodium cis-epoxysuccinate were added into the fermentation broth and the bioconversion was carried out at 37°C. After purification and crystallization of the biotransformed product, about 6.3 g of colorless and scentless crystals were obtained. Then the product was identified by IR, $^1$H-NMR, $^{13}$C-NMR, MC and optical rotation analysis. The IR spectra of the biotransformed product were shown in the Fig.3 and the results were identical to the reported spectra of tartaric acid.$^{[14]}$

In addition, $^1$H-NMR spectrum (Fig.4-A) showed that there were only two signals: the singlets at δ 4.516 and δ 4.700 that corresponded to the CH of tartaric acid and deuterium oxide, respectively. The proton signals of OH and COOH of tartaric acid were lost due to the solvent which contained active deuterium. Because of its symmetric structure, only two singlets at δ 71.717 (-CHOH-) and δ 174.391 (-COOH) were detected by $^{13}$C-NMR (Fig.4-B). Furthermore, MS spectrum (Fig.5) showed that the molecular weight of the
Fig. 3  KBr FT-IR spectra of the enzymatic-biotransformed product. One milligram of the purified sample was mixed thoroughly with 100 mg of KBr and a pellet was prepared using a press. Afterwards, the pellet was immediately put into the sample holder and infrared spectra were obtained using a Nicolet-Nexus 670 Fourier Transform Infrared spectrometer (FT-IR). FT-IR scanning was conducted in ambient conditions. The resolution was set to 4 cycle per cm and the operating range was 400 to 4000 cycle per cm.

Fig. 4  $^1$H-NMR (A) and $^{13}$C-NMR (B) spectrum of the enzymatic-biotransformed product. About 5 mg purified sample was transferred to a NMR tube containing 0.5 mL deuterium oxide. $^1$H-NMR and $^{13}$C-NMR spectrum analysis was operated at room temperature (25°C) on a NMR spectrometer using a 5-mm QNP probe.

Fig. 5  Mass spectrum of the enzymatic-biotransformed product. The mass spectrum was acquired with mass spectrometer equipped with an electron spray ionization source and ion trap analyzer in the negative ionization mode acquired data in software 5.0. The sample was infused into the source chamber at a flow rate of 3 μL/min and nitrogen was used as the nebulization gas at a pressure of 10 pound/square inch and the dry gas at a flow rate of 5 L/min. The capillary voltage was set at 4 kV, and ion source temperature was at 250°C.
3.4 Effect of different inducers on biomass concentration and enzyme production

Most of the epoxide hydrolases from microorganisms were induced enzyme. In this study, four different inducers (1%, W/V) were added at the beginning of cultivation and their effects on biomass concentration and enzyme production were investigated. The fermentation medium without disodium cis-epoxysuccinate was considered as the control. As shown in Fig.6-A, the highest enzyme activity was obtained with disodium cis-epoxysuccinate (558 U/g biomass) as the inducer, followed by D(-)-tartaric acid (481 U/g biomass), whereas L(+)-tartaric acid and propanediol could hardly induce enzyme production. These indicated that CESH was an induced enzyme which was coincident with the reported microorganisms. Because of its enantioselective property, it’s not surprising that L(+)-tartaric acid couldn’t induce enzyme production. It’s also seen that almost the same concentration of biomass was obtained when using disodium cis-epoxysuccinate, L(+)-tartaric acid and D(-)-tartaric acid as inducers, respectively. Obviously from the above analysis results, cis-epoxysuccinate was the best inducer and thus chosen for further studies.

3.5 Effect of carbon source on biomass concentration and enzyme production

Carbon source is the most important element at the period of the growing and metabolic processes of the strains. Effect of seven kinds of carbon sources (1%, W/V) on biomass concentration and enzyme production was examined. The fermentation medium without glucose was considered as the control. The results shown in Fig.6-B demonstrated that all these carbon sources had little promotional effect on growth except acetate and citrate. Furthermore, the use of saccharose resulted in a slightly higher enzyme production (624 U/g biomass) as compared with the control (554 U/g biomass), whereas lactose, maltose, starch and acetate remarkably inhibited the production of the enzyme. Therefore, saccharose was selected as the carbon source in the following studies.

3.6 Effect of inorganic nitrogen source on biomass concentration and enzyme production

The effect of NH₄NO₃, KNO₃, (NH₄)₂SO₄, NH₄Cl, and NH₄HCO₃ (with total inorganic nitrogen concentration of 45 mmol/L) on biomass concentration and enzyme production was investigated. Glucose in the fermentation medium was replaced with saccharose, and this was considered as the control. From the results shown in Fig.6-C, all of the tested inorganic nitrogen sources showed a slightly positive effect on growth. Among these, (NH₄)₂SO₄ got highest enzyme activity (577 U/g biomass) and highest biomass concentration (OD₆₀₀=4.5), whereas KNO₃ and NH₄HCO₃ remarkably inhibited the production of the enzyme. Accordingly, (NH₄)₂SO₄ was regarded as the optimal inorganic nitrogen source.

3.7 Effect of temperature and initial pH on biomass concentration and enzyme production

The effect of fermentation temperature and initial pH on the biomass concentration and enzyme production was also investigated. An optimization test indicated that the optimal temperature for growth and enzyme production were both 30°C. Fig.7-A indicated that the initial pH value significantly influenced the growth and enzyme production, and the optimum pH value for growth and enzyme production were both 7.0.
Therefore, 30°C and pH7.0 were adopted as the temperature and pH for cultivation of cells and enzyme production, respectively.

### 3.8 Time course of biomass concentration and enzyme production

Based on the analysis of the above results, the optimum fermentation medium comprised of 10 g disodium cis-epoxysuccinate, 10 g saccharose, 3 g (NH4)2SO4, 2 g yeast extract, 1 g K2HPO4·3H2O, 0.5 g MgSO4·7H2O, 0.01 g/L FeSO4·7H2O in 1000 mL distilled water. The optimal initial pH value was 7.0 and the optimal fermentation temperature was 30°C. Under the above optimized conditions, the time course of biomass concentration and enzyme production during the fermentation process of *Bordetella* sp. BK-52 was shown in Fig.7-B. It’s seen that the biomass concentration increased rapidly within 24 h and reached the highest at 36 h ($OD_{600} = 4.71$). During the fermentation process, the enzyme activity constantly increased, arrived in the maximum point (764 U/g biomass) at 36 h and slowly declined afterward.

![Fig. 7](image)

**Fig. 7** Effect of initial pH on (A) and time course (B) of biomass concentration (▲) and enzyme production (○) under the optimized conditions.

### 4 Discussion

Enantioselective biotransformations are increasingly being considered to manufacture a wide range of chiral intermediates and products\([17]\). L(+)-tartaric acid and D(-)-tartaric acid are enantiomers, but they have different optical properties and applications. L(+)-tartaric acid has dextrorotatory optical activity and is widely used as food additives, chemical resolving agent and pharmaceutical intermediate. Because it exists as a natural resource, many species were reported on the production of L(+)-tartaric acid by biotransformation, such as *Aspergillus glaucus*, *Gluconobacter suboxydans*, *Gluconobacter suboxydans* and *Gluconobacter oxydans*.

D(-)-tartaric acid has laevo rotatory optical activity and plays an important role in the pharmaceutical industry. However, there were few reports on the production of D(-)-tartaric acid by biotransformation\([7,8]\). In this study, the isolated strain *Bordetella* sp. BK-52 could transform cis-epoxysuccinate into D(-)-tartaric acid but not L(+)-tartaric acid. Therefore, this research provided a new method to produce D(-)-tartaric acid using the new isolated bacterium. In addition, cis-epoxysuccinic acid or its derivatives (such as sodium, potassium, calcium and ammonium salts of cis-epoxysuccinic acid) can be easily obtained, for example, by epoxidizing maleic anhydride, maleic acid or a derivative of any of these with hydrogen peroxide. Therefore, the relative cheap raw materials make a practical potential for production.

In conclusion, for the first time, we isolated and identified a novel *Bordetella* sp. BK-52 with cis-epoxysuccinate hydrolase activity for biotransforming cis-epoxysuccinate into D(-)-tartaric acid. However its enzyme activity was much lower than those microorganisms producing L(+)-tartaric acid (about 10000 U/g biomass), such as *Rhodococcus opacus*. Therefore, the wild-type *Bordetella* sp. BK-52 should be mutagenized to screen high-yield CESH-producing mutants. Cloning and over expression of CESH gene is another way to obtain high-yield CESH strains.

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**REFERENCES**


and L-allo-configured glycaro-1,5-lactams from tartaric acid.


产顺式环氧琥珀酸水解酶的博德特氏菌 BK-52 的筛选、鉴定及其产酶条件优化

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摘要：本文通过Biolog GN (G+C) 16S rDNA 检测和aBiolog GN (G+C) 16S rDNA 检测和a
的筛选、鉴定及其产酶条件优化

关键词：BK-52, D(-)-7epoxysuccinic acid hydrolase, Pseudomonas, screening,

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