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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, ^{1}H-NMR, ^{13}C-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
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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*, *Tricosporon* 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 (^{1}H-NMR, ^{13}C-NMR), mass spectrum (MS)

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and optical rotation analysis. Furthermore, the medium compositions and fermentation conditions were optimized in terms of cell growth and enzyme production.

2 Materials and methods

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 preps 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 3000^{plus} 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 KH_2PO_4, 2 g $K_2HPO_4{\cdot}3H_2O$, 0.5 g $MgSO_4{\cdot}7H_2O$, 3 g $FeSO_4{\cdot}7H_2O$ and 3 g $(NH_4)_2SO_4$. The fermentation medium consisted of 10 g disodium *cis*-epoxysuccinate, 10 g glucose, 3 g $(NH_4)_2SO_4$, 2 g yeast extract, 1 g $K_2HPO_4{\cdot}3H_2O$, 0.5 g $MgSO_4{\cdot}7H_2O$, and 0.01 g $FeSO_4{\cdot}7H_2O$ 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′-AGAGTTTGATCCTGGCTCAG-3′) and p16S- 1541 (5′-AAGGAGGTGATCCAGCCGCA-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 from 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 by 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-biotransfromed product was identified by IR, ^{1}H-NMR, ^{13}C-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-

ter 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.

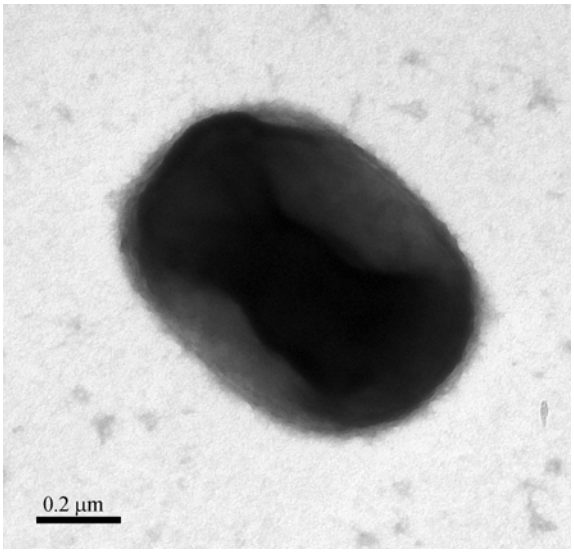

Fig. 1 Transmission electron micrograph of a single cell of strain BK-52 at 50000× magnification.

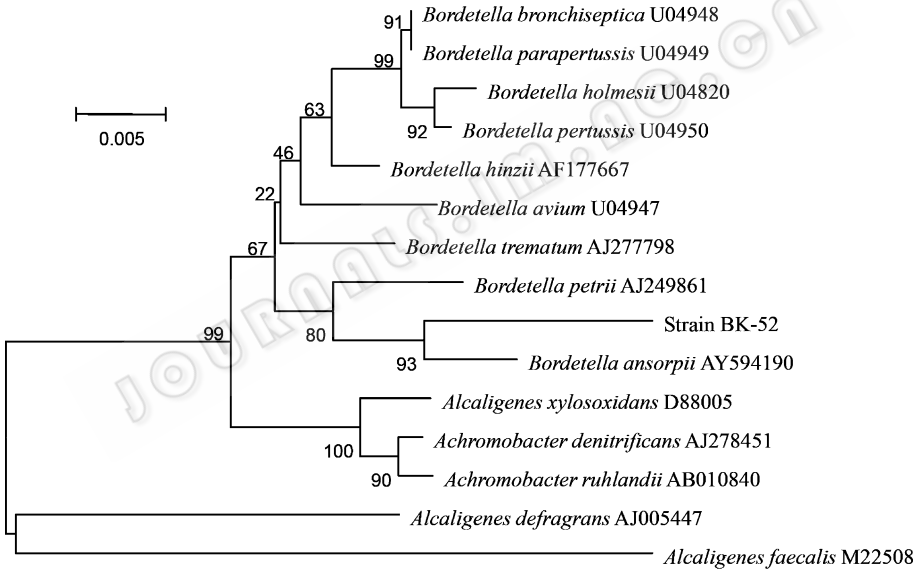

Fig. 2 Phylogenetic tree for isolate BK-52 and related strains based on the 16S rDNA sequence. The tree was constructed by the neighbor-joining approach. Bootstrap values (%) were indicated at the nodes.
Sequence accession numbers were following the validly italic species names.

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 biotransformated 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

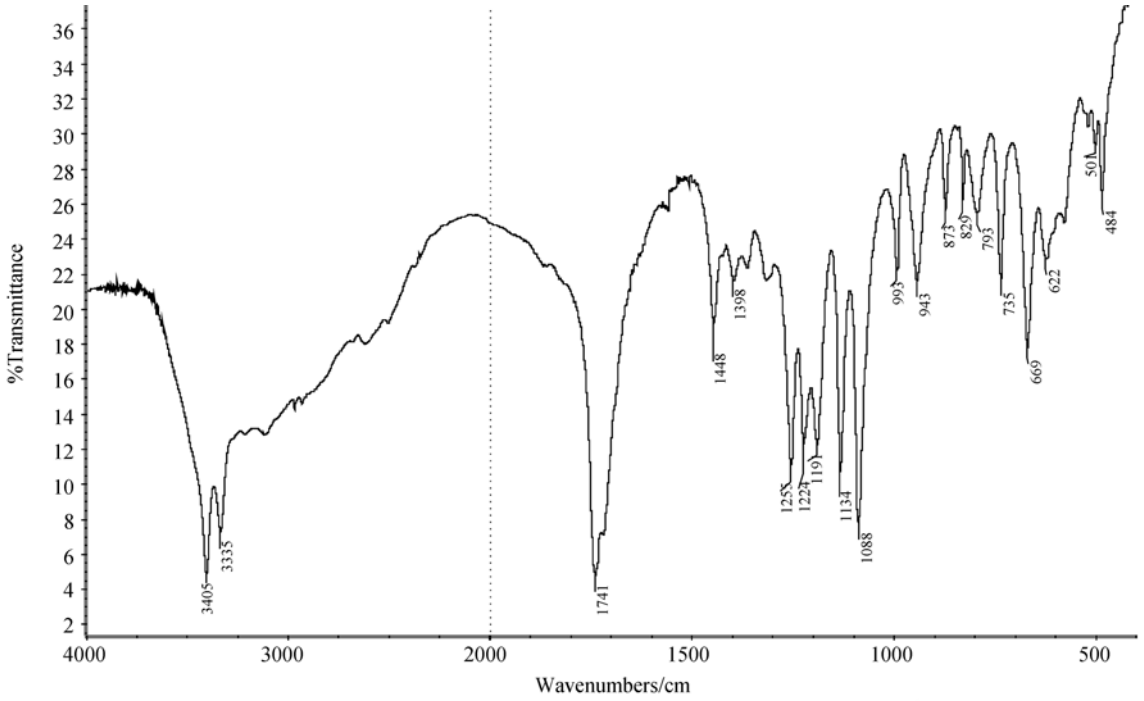

Fig. 3 KBr FT-IR spectra of the enzymatic-biotransformated product. One milligram of the purified sample was mixed thoroughly with 100 mg of KBr and a pellet was prepared using a press. Afterwards, the pellet was immediately put into the sample holder and infrared spectra were obtained using a Nicolet-Nexus 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.

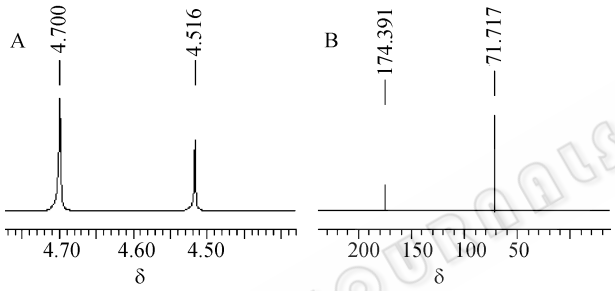

Fig. 4 ^{1}H-NMR (A) and ^{13}C-NMR (B) spectrum of the enzymatic-biotransformated product. About 5 mg purified sample was transferred to a NMR tube containing 0.5 mL deuterium oxide. ^{1}H- NMR and ^{13}C-NMR spectrum analysis was operated at room temperature (25°C) on a NMR spectrometer using a 5-mm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

product was 149.7 and this was consistent to the theoretical value of tartaric acid (150.09).

All of these indicated that the enzymatic-biotransformated product was tartaric acid. Then, polarimeter was used to identify its optical rotation. The optical rotation of the purified product and the standard D(-)-tartaric acid were determined to be −12.15 ($[\alpha]_D^{25}$) and −12.23 at 25°C by a digital polarimeter at 589 nm, respectively. The enantiomer excess (ee) value of the biotransformation product, which was used to express its optical purity, was calculated to be 99.35%. This indicated that the enzymatic product should be D(-)-tartaric acid but not L(+)-tartaric acid.

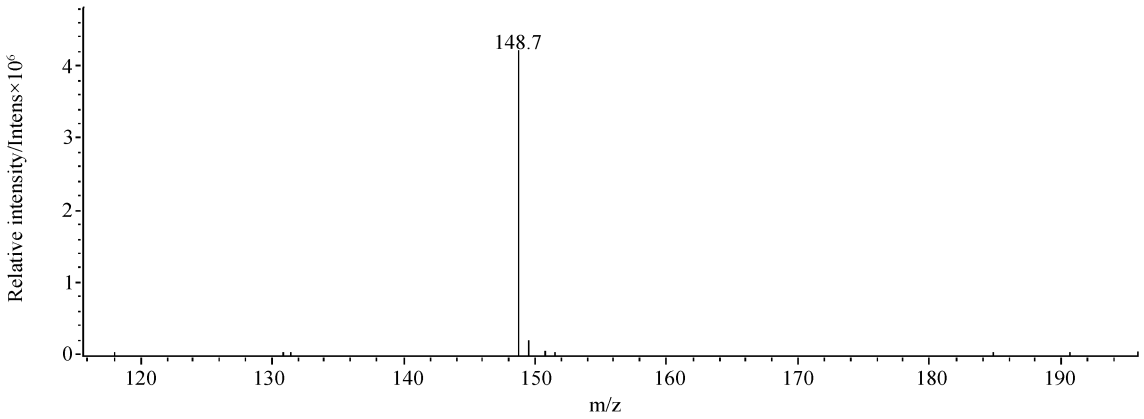

Fig. 5 Mass spectrum of the enzymatic-biotransformated product. The mass spectrum was acquired with mass spectrometer equipped with an electron spray ionization source and ion trap analyzer in the negative ionization mode acquired data in software 5.0. The sample was infused into the source chamber at 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 [15]. 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[16]. 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 NH_4NO_3, KNO_3, $(NH_4)_2SO_4$, NH_4Cl, and NH_4HCO_3 (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, $(NH_4)_2SO_4$ got highest enzyme activity (577 U/g biomass) and highest biomass concentration (OD_{600}= 4.5), whereas KNO_3 and NH_4HCO_3 remarkably inhibited the production of the enzyme. Accordingly, $(NH_4)_2SO_4$ was regarded as the optimal inorganic nitrogen source.

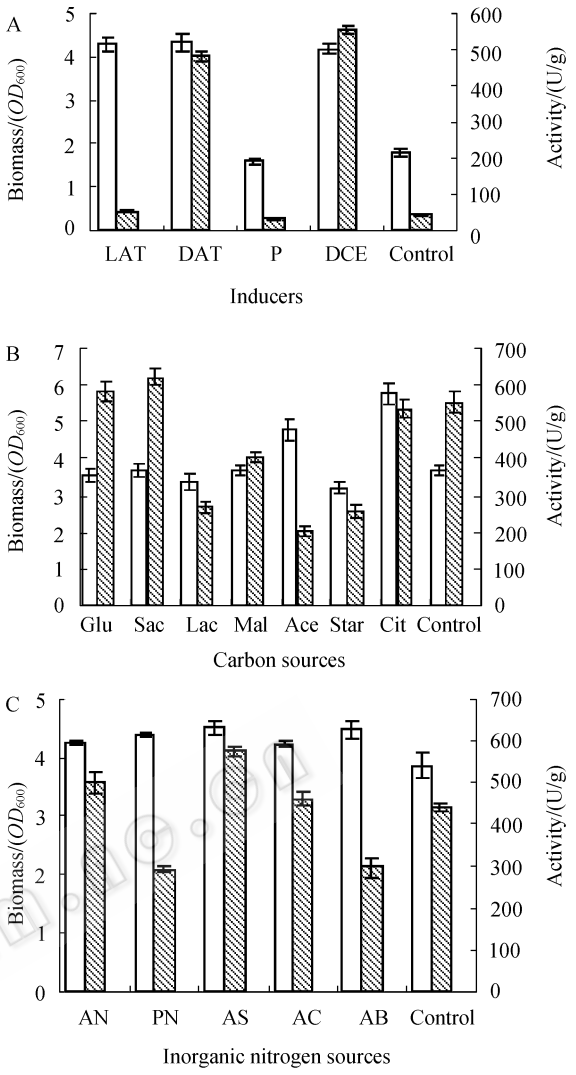

Fig. 6 Effects of inducers (A), carbon sources (B) and inorganic nitrogen sources (C) on biomass concentration (white bar) and enzyme production (hatched bar). Cultivation was carried out at 30°C, pH 7.0 for 36 h. A: Fermentation medium without disodium *cis*-epoxysuccinate with addition of different inducers (1%, W/V). B: Fermentation medium without glucose with addition of different carbon sources (1%, W/V). C: Fermentation medium replaced glucose with saccharose with addition of different inorganic nitrogen sources (with total inorganic nitrogen concentration of 45 mmol/L). Abbreviations: LAT, L(+)-tartaric acid; DAT, D(-)-tartaric acid; P, propanediol; DCE, disodium *cis*-epoxysuccinate; Glu, glucose; Sac, saccharose; Lac, lactose; Mal, maltose; Ace, Acetate; Star, starch; Cit, citrate; AN, ammonium nitrate; PN, potassium nitrate; AS, ammonium sulfate; AC, ammonium chloride; AB, ammonium bicarbonate.

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 $(NH_4)_2SO_4$, 2 g yeast extract, 1 g $K_2HPO_4 \cdot 3H_2O$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g/L $FeSO_4 \cdot 7H_2O$ 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.

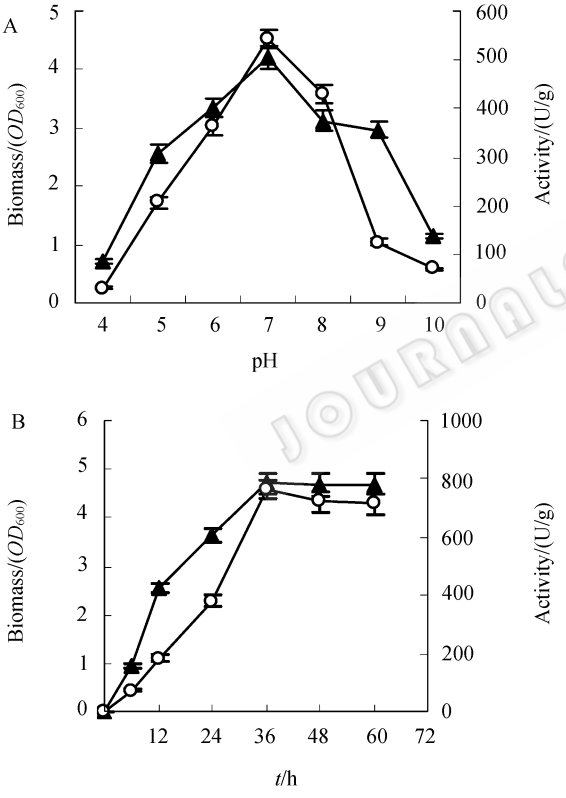

Fig. 7 Effect of initial pH on (A) and time course (B) of biomass concentration (▲) and enzyme production (○) under the optimized conditions.

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 laevorotatory 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 identificated 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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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:【目的】产顺式环氧琥珀酸水解酶（CESH）新型菌株的筛选、鉴定及其产酶条件优化。【方法】通过电镜、Biolog GN、(G+C)含量和 16S rDNA 序列研究，对筛选菌株进行分类鉴定。通过红外光谱、核磁共振氢谱和碳谱、质谱以及旋光度实验，鉴定纯化产物的结构并优化 CESH 产酶条件。【结果】本文筛选出一株产 CESH 的新型菌株，该菌株可将顺式琥珀酸盐转化为 D(-)-酒石酸，属于博德特氏菌属，并将其命名为博德特氏菌 BK-52。最佳发酵条件为：最佳温度 30°C，最佳 pH7.0，最佳发酵时间 36 h，最佳碳源蔗糖，最佳无机氮源硫酸铵，最佳酶诱导剂顺式环氧琥珀酸二钠。在此最佳条件下，CESH 酶活最高达 764 U/g 生物量。【结论】本文筛选的新菌株博德特氏菌 BK-52 为 D(-)-酒石酸的生产提供了一种新的方法。

关键词: 博德特氏菌；顺式环氧琥珀酸水解酶；D(-)-酒石酸；筛选；鉴定

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