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BIOCATALYSIS

Tsukamurella sp. E105 as a new biocatalyst for highly enantioselective hydrolysis of ethyl 2-(2-oxopyrrolidin-1-yl) butyrate

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Abstract A new bacterial strain, E105, has been introduced as a biocatalyst for the enantioselective hydrolysis of ethyl (*R,S*)-2-(2-oxopyrrolidin-1-yl) butyrate, (*R,S*)-**1**, to (*S*)-2-(2-oxopyrrolidin-1-yl) butyric acid, (*S*)-**2**. This strain was isolated from 60 soil samples using (*R,S*)-**1** as the sole carbon source. The isolate was identified as *Tsukamurella tyrosinosolvens* E105, based on its morphological characteristics, physiological tests, and 16S rDNA sequence analysis. The process of cell growth and hydrolase production for this strain was then investigated. The hydrolase activity reached its maximum after cultivation at 200 rpm and 30 °C for 36 h. Furthermore, the performance of the enantioselective hydrolysis of (*R,S*)-**1** was studied. The optimal reaction temperature, initial pH, substrate concentration, and concentration of suspended cells were 30 °C, 6.8, 10 and 30 g/l (DCW), respectively. Under these conditions, a high conversion (>45 %) of the product (*S*)-**2** with an excellent enantiomeric excess (*ee*) (>99 %), and a satisfied enantiomeric ratio (*E*) (>600) as well were obtained. This study showed that the bacterial isolate *T. tyrosinosolvens* E105 displayed a high enantioselectivity towards the hydrolysis of racemic ethyl 2-(2-oxopyrrolidin-1-yl) butyrate.

Keywords Isolation · Identification · *Tsukamurella tyrosinosolvens* · Enantioselective hydrolysis · (*S*)-2-(2-oxopyrrolidin-1-yl) Butyric acid

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Introduction

Levetiracetam, also known as (*S*)-2-(2-oxopyrrolidin-1-yl) butyramide, LEV or Keppra®, is one of the newly developed and leading adjunctive antiepileptic drugs (AEDs) with a broad spectrum of efficacy in epilepsy, and is an ethyl analogue of the nosotropic drug piracetam [14, 19, 23]. LEV offers several advantages over traditional therapy, owing to its unique mechanism of action [9]. Such advantages include the clinical efficacy, generally good tolerability, lack of interactions with other AEDs, and less adverse effects [8, 10, 13, 17]. Because of LEV's outstanding pharmacokinetic and pharmacological profile [18], it was rapidly approved as an antiepileptic drug by the FDA at the end of 1999, and as an add-on therapy for partial onset seizures in adults and children of 4 years and older with epilepsy in 2005 [3, 11].

With the rapidly growing demand for LEV, improved synthetic methods are necessary. Several chemical synthetic strategies for LEV, including chiral pool approaches, chemical resolution of etiracetam or its corresponding intermediates, and asymmetric reaction over several complexes, have been previously described [1, 12]. These processes require excess organic chemicals and/or heavy metal complexes, resulting in environmental pollution and loss of material. Biocatalysis is a developing and precise approach for improving a wide range of production processes, focused on reducing energy and raw material consumption, and generating less waste and toxic by-products [2]. Over the past few years, the use of enzymes as biocatalysts for the introduction of enantiopure active compounds has become an established manufacturing process in the specialty and pharmaceutical industries [15]. Hydrolases, more particularly lipases, are widely used enzymes with distinct advantages over other biocatalysts.

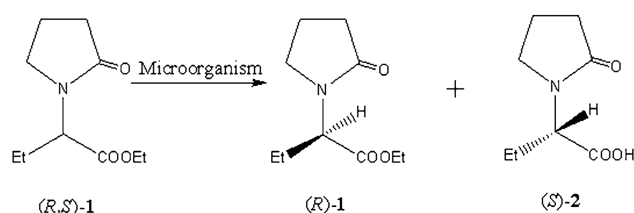

Fig. 1 Enantioselective hydrolysis of racemic ethyl 2-(2-oxopyrrolidin-1-yl) butyrate, (*R,S*)-**1**

They require no cofactors for their catalytic behavior and they are active not only in aqueous media but also in organic solvents, allowing the transformations of water-insoluble compounds. Furthermore, they commonly display low substrate specificity and high regio, chemo, and enantioselectivity [16]. Thus, these enzymes are an attractive tool for the synthesis of chiral building blocks and biologically active compounds in recent years [6, 20, 22].

The objective of the present study is to isolate an ester-hydrolase-producing strain, which can be applied to highly enantioselective hydrolysis of racemic ethyl 2-(2-oxopyrrolidin-1-yl)butyrate ((*R,S*)-**1**) to (*S*)-2-(2-oxopyrrolidin-1-yl) butyric acid ((*S*)-**2**), which is LEV's chiral intermediate (Fig. 1). The isolate was identified according to its physiological and biochemical characteristics, and 16S rDNA sequence analysis as well. Furthermore, the process of the cell growth and enzyme production for this strain was investigated and the biotransformation conditions were also optimized.

Materials and methods

Materials

Racemic ethyl 2-(2-oxopyrrolidin-1-yl) butyrate, (*R,S*)-**1**, (≥97 %, HPLC) and (*S*)-2-(2-oxopyrrolidin-1-yl) butyric acid, (*S*)-**2**, (chemical and optical purity ≥99 %, HPLC) were provided by Zhejiang Jiangbei Pharmaceutical Co., Ltd., Taizhou, PR China. The other chemicals used in this study were of analytical grade.

The enrichment medium consisted of (per liter): 2.0 g $(NH_4)_2SO_4$, 1.0 g KH_2PO_4, 2.0 g K_2HPO_4, 0.5 g NaCl, 0.5 g $MgSO_4{\cdot}7H_2O$ and 10 mmol of the substrate (*R,S*)-**1**. The initial pH of the medium was adjusted to 7.0. The liquid medium for enzyme production contained the following components (per liter): 10 g glucose, 5 g peptone, 2 g yeast extract, 2 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 2.0 g K_2HPO_4, 0.5 g NaCl and 0.2 g $MgSO_4{\cdot}7H_2O$. The initial pH of the medium was adjusted to 7.0 with 1 M NaOH.

Isolation of strains capable of enantioselectively hydrolyzing (R,S)-**1**

Soil samples were collected from different locations of Anhui, Hebei, Shandong, and Zhejiang Provinces in China. Each soil sample (10 g) was suspended in 100 ml of saline solution (0.85 %) and allowed to vibrate for 10 min, and then the suspension was diluted 10^3 and 10^4-fold. Subsequently, 1 ml of the diluent was transferred into 40 ml of the enrichment medium containing the carboxylic ester (*R,S*)-**1** as the sole carbon source. After cultivation at 30 °C and 200 rpm for 5 days, 1 ml of the solution was then transferred into the enrichment medium and cultured under the same conditions. This procedure was repeated four times, and then the enriched cultures were diluted and plated onto enrichment medium agar plates to isolate pure colonies. The grown colonies were inoculated individually into the liquid medium for enzyme production. After cultivation at 30 °C and 200 rpm for 48 h, cells were harvested by centrifugation and subjected to enantioselective hydrolysis of the carboxylic ester (*R,S*)-**1**. The isolate capable of hydrolyzing (*R,S*)-**1** to (*S*)-**2** with the best *ee* value was selected for further studies.

Identification of the isolate E105

Cell morphology of strain E105 was investigated by a light microscope (Leica DM4000 B, Wetzlar, Germany) and transmission electron microscope (JEOL JEM-1230, Tokyo, Japan). To assess basic physiology and biochemistry, carbon source utilization was determined using standardized Biolog microplates (GP2) searched within the Biolog system (Hayward, CA, USA).

The extraction of the chromosomal DNA of the isolate was carried out by TAKARA Biotechnology (Dalian) Co., Ltd., China. The 16S rDNA genes were amplified by PCR using a 16S rDNA Bacterial Identification PCR Kit (TAKARA Biotechnology). PCR reactions were carried out in a thermal cycler under the following conditions: 5 min at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 55 °C, 1.5 min at 72 °C and a final extension step of 5 min at 72 °C. The expected size of the fragment amplified from the 16S rDNA was approximately 1.5 Kb. The fragment was subsequently sequenced by TAKARA Biotechnology Co., Ltd. (Dalian, China). Related sequences with high similarities were selected from the GenBank database (National Center for Biotechnology Information, NCBI) using the BLAST search program. Multiple alignments of sequences, construction of a neighbor-joining phylogenetic tree with the nucleotide p-distance model and a bootstrap analysis for evolution of the phylogenetic topology were performed by ClustalX ver.2.0 and MEGA version 4.1, respectively.

Cultivation and enzyme production of *Tsukamurella* sp. E105

The cultivation medium for enzyme production was described in the Materials and methods section. The isolated strain, *Tsukamurella* sp. E105, was incubated aerobically in 250-ml Erlenmeyer flasks containing 80 ml of culture medium at 30 °C and 200 rpm for 48 h. At different time intervals, two flasks were withdrawn to determine ester-hydrolase activity, dry cell weight (DCW) and pH of the fermentation broth. Cells were harvested by centrifugation (4,000 × *g*, 4 °C, 15 min) and washed twice with phosphate buffer (pH 7.2), and then subjected to biocatalytic reaction.

Enzyme activity assay

The cells harvested from 80 ml of culture broth were washed with physiological saline solution, and resuspended in 10 ml phosphate buffer (0.1 M, pH 7.2). The mixture was pre-incubated in a 50-ml shaking flask capped with a septum for 10 min at 30 °C and 200 rpm. Then, 0.5 mmol of carboxylic ester (*R,S*)-**1** was added and the hydrolytic reaction was carried out under the conditions mentioned above. After 1 h of the reaction, the mixture was extracted with 10 ml of ethyl acetate. The organic phase was separated by centrifugation (4,000 × *g*, 4 °C) and dried with anhydrous Na_2SO_4. The concentration of formed (*S*)-**2** in the organic phase was determined by HPLC described below. One unit of hydrolytic activity was defined as the amount of enzyme capable of generating 1 μmol of (*S*)-**2** per minute under the experimental conditions described above.

Enantioselective hydrolysis of (R,S)-**1**

Resting cell (0.2 g, DCW) was suspended in 10 ml phosphate buffer (100 mM, pH 7.2). The reactions were started by adding 0.1 g of carboxylic ester (*R,S*)-**1**. The mixture was incubated at 30 °C and 200 rpm. Samples were withdrawn at regular intervals. The cells in the samples were removed by centrifugation (4,000 × *g*, 4 °C, 10 min) and the reaction was quenched. The concentrations and *ee* values of the substrate and the product were then determined. To investigate the effects of the reaction conditions on the enantioselective hydrolysis, different temperatures, initial pHs (6–8), substrate concentrations (5–30 g/l), and cell concentrations (10–35 g DCW/l) were evaluated. Triplicate runs were carried out for each trial.

Analytical methods

Mass spectra were performed on a ThermoFinnigan LCQ Advantage instrument (Waltham, MA, USA) with an ESI source. MS profiles of the substrate (*R,S*)-**1** and its corresponding product are shown in Fig. 2. The concentration of (*R,S*)-**1** and corresponding product were determined by HPLC (Agilent 1200, Santa Clara, CA, USA) using a reversed-phase Agilent C_{18} column (250 × 4.6 mm i.d., 5 μm, Agilent). The detection wavelength was 210 nm. The mobile phase was methanol/ammonium acetate solution (20 mM, pH 5.5) (20:80, v/v) and the flow rate was 1 ml/min.

The enantiomeric excess of substrate (*R,S*)-**1** (ee_s) and the product (*S*)-**2** (ee_p) were determined by GC analysis as described by Wang et al. [21].

The enantioselectivity was expressed as the enantiomeric ratio (*E*) calculated from the conversion (*x*), ee_s and ee_p [4, 24].

$$E = \frac{\ln[(1-x)(1-ee_s)]}{\ln[(1-x)(1+ee_s)]}$$

where

$$x = \frac{ee_s}{ee_s + ee_p}$$

Results and discussion

Screening of microorganisms

Sixty soil samples were collected and used for the isolation of strains. After an initial screening, various pure colonies exhibiting different morphologies were selected for further isolation by enriched culture. The ester-hydrolase activity and enantioselectivity of the isolates were then evaluated by HPLC and GC. Strain E105 turned out to be the most powerful and was thus selected for further studies.

Identification of strain E105

The colony morphology of isolate E105 on nutrient agar was irregular, light yellow colored, dry, less luster, and with a wavy edge. The morphological characteristics were observed by transmission electron microscope. As shown in Fig. 3a, it was found that cells were straight and rod-shaped with a size of 0.3–0.6 μm × 1.5–2.5 μm.

A detailed biochemical analysis of strain E105 was carried out by the Biolog system. An appropriate Biolog GP2 microplate was used to determine the relative capacity of carbon substrate utilization of strain E105. After incubation at 37 °C for 24 h, the microplate was read on a Biolog plate reader (Microlog 34.20.05). According to the data (not shown) from the Biolog system, the strain was preliminarily confirmed as *Tsukamurella* genus.

The partial 16S rDNA sequence of E105, with a length of 1,416 bp, was determined. The sequence was deposited

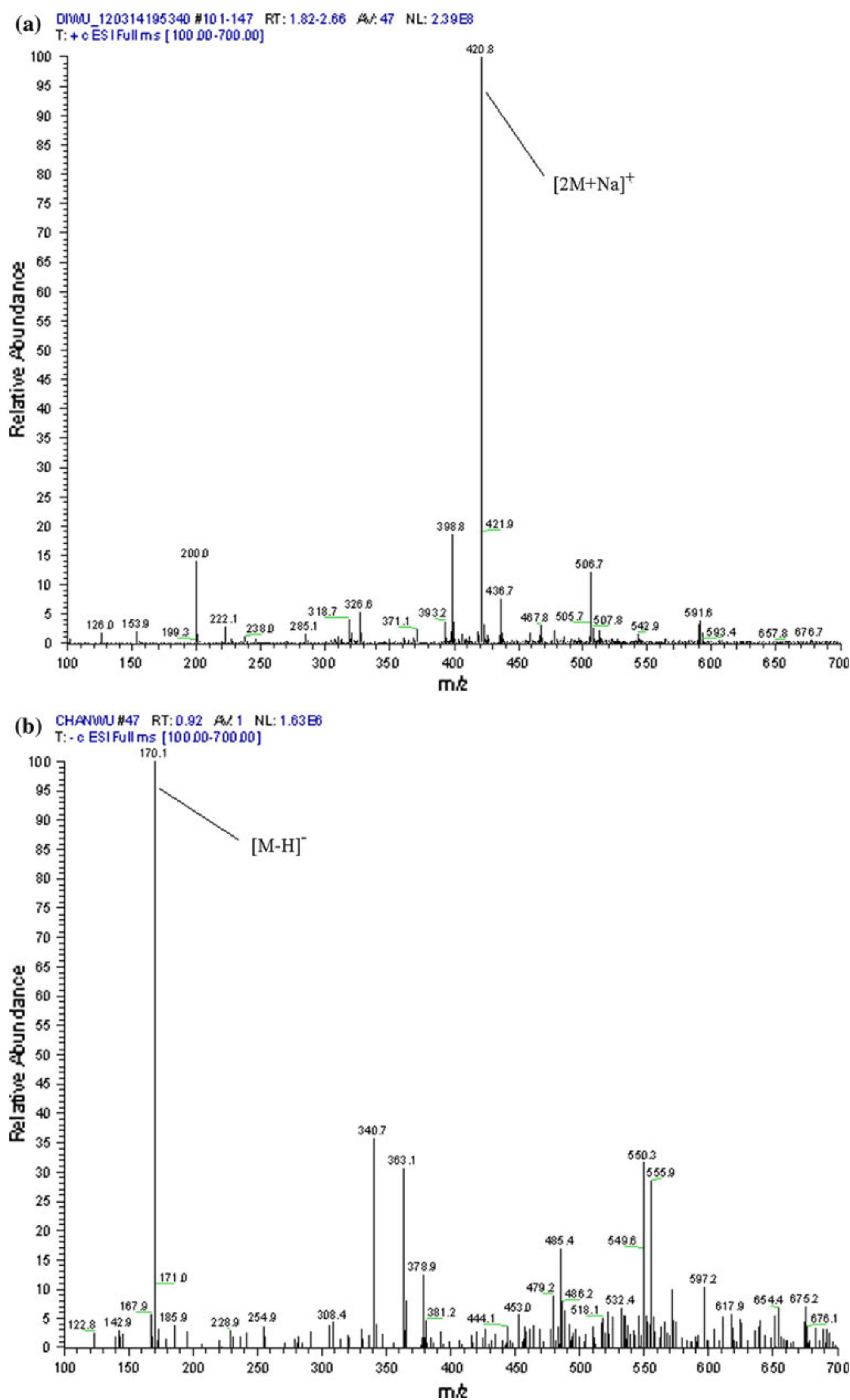

Fig. 2 MS (ESI) profiles of the substrate (*R*,*S*)-**1** (**a**) and its corresponding product (**b**)

in the GenBank database with accession No. GU318217. To elucidate the phylogenetic position of the isolate, sequence analysis of the 16S rDNA genes of this strain and related species were used to construct a phylogenetic dendrogram using the neighbor-joining method (Fig. 3b). It was observed that strain E105 shared a high similarity of

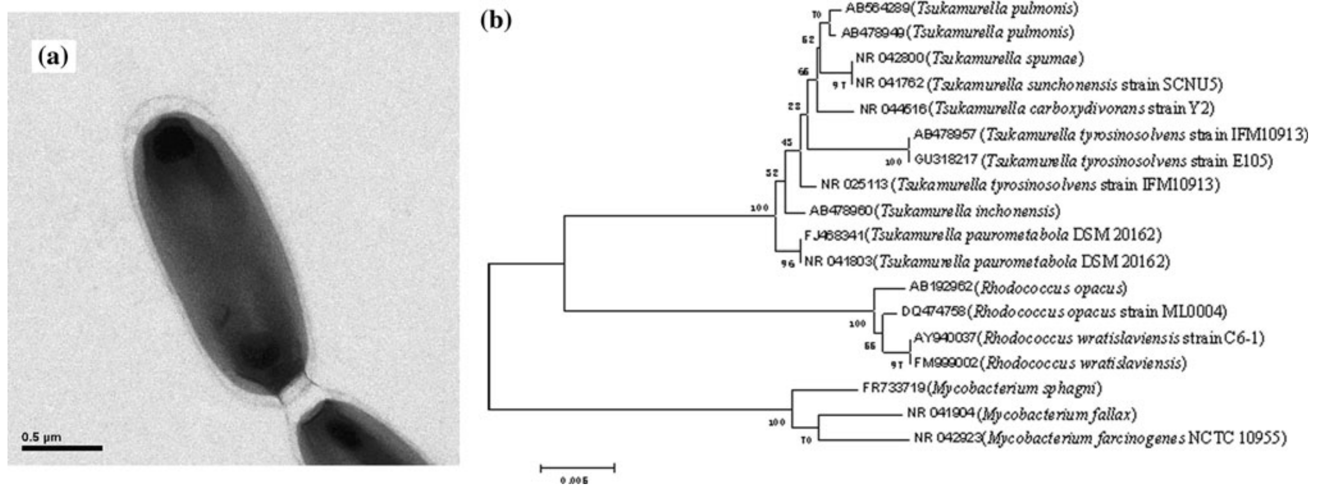

Fig. 3 Morphological characteristics and phylogenetic analysis of E105. **a** Electron micrograph; **b** phylogenetic dendrogram for isolate E105 and related strains based on 16S rDNA sequences. *Numbers in parentheses* are accession numbers of published sequences. Bootstrap values were based on 1,000 replicates

100 % with strain *Tsukamurella tyrosinosolvens* IFM10913 (GenBank accession No. AB478957). The results of this phylogenetic analysis were consistent with those of the Biolog GP phenotypic test. Therefore, based on the analyses of morphological characteristics, physiological tests and 16S rDNA sequence, the strain was identified as *T. tyrosinosolvens* and marked as *T. tyrosinosolvens* E105. This strain has been deposited in the China Center for Type Culture Collection (CCTCC) with accession number of CCTCC M 209306.

Cell growth and ester-hydrolase production of *T. tyrosinosolvens* E105

The time course of cell growth and ester-hydrolase production were tested by cultivating *T. tyrosinosolvens* E105. The culture conditions were described in the Materials and methods section. As shown in Fig. 4, the results show that the ester-hydrolase activity increased during the initial 36 h and reached its maximum value (5.84 U/l) at 36 h, and then decreased. The biomass had a similar trend with enzyme production during the initial 36 h, whereas it changed little during the next time phase (from 36 to 48 h). The pH of culture broth changed slightly throughout the cultivation. Consequently, the microorganism used in hydrolyzing carboxylic ester (*R,S*)-**1** to (*S*)-**2** was cultivated for 36 h in further studies.

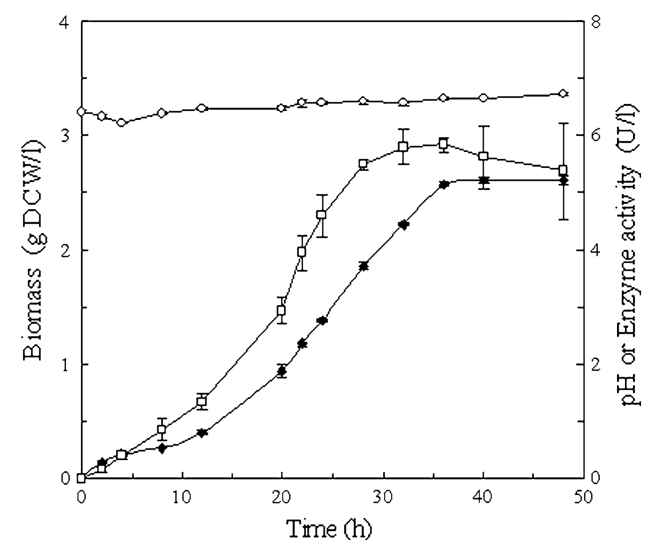

Fig. 4 Time course of cell growth and ester-hydrolase production of *T. tyrosinosolvens* E105. *Symbols* biomass (*filled triangle*); enzyme activity (*open square*); pH (*open circle*)

Effect of reaction conditions on the hydrolysis catalyzed by *T. tyrosinosolvens* E105

Since the enzymatic hydrolysis of carboxylic ester (*R,S*)-**1** is a kinetic resolution process, reaction temperature has an obvious effect on enantioselectivity. To find the optimal temperature for the hydrolysis, various temperatures ranging between 25 and 45 °C were tested. The results are shown in Fig. 5a. The extent of the conversion increased slowly with the temperature rise from 25 to 35 °C, and reached its maximum at 35 °C. The bioconversion decreased with the further rise in temperature due to enzyme deactivation. The reaction temperature also affects the enantiomeric excess of the product (ee_p). The result shows that the highest ee_p was obtained at 30 °C and the ee_p decreased slowly when the temperature was over 30 °C. In view of the conversion, the ee_p and the enantiomeric ratio (*E*), 30 °C was selected as the optimal temperature in this study.

In enzymatic hydrolysis, pH of the reaction medium might markedly influence the conversion and product *ee* [5, 25]. Thus, the effect of initial pH on the hydrolysis was investigated. The results are given in Fig. 5b. It is indicated that the initial pH considerably affected the degree of racemate conversion and ee_p value. The conversion increased in the pH range of 6–7.6 and then decreased, while ee_p value increased when the pH increased from 6 to 6.8, after which the ee_p decreased. Considering the highest ee_p value (97.1 %), highest *E*, and an acceptable conversion (45.3 %), an initial pH of 6.8 was considered to be optimal.

It is well known that substrate concentration is also an important parameter for biotransformation [7, 25]. Thus, the performance of the hydrolysis with different concentration of substrate (*R,S*)-**1** was evaluated (Fig. 5c). The results show that 10 g/l of the substrate was optimal for (*S*)-**1** production based on the enantiomeric ratio (*E*). At this concentration, ee_p reached its maximum (97.6 %) and a satisfied enantioselectivity ($E > 200$) was achieved. However, with higher substrate concentrations, the bioconversion declined.

To study the effect of cell concentration on the hydrolysis, the reaction was carried out under the different cell concentrations arranging between 10 and 35 g/l (DCW). As shown in Fig. 5d, the results show that the variation of the conversion and ee_p value was caused by application of different cell concentrations. When cell concentration increased from 10 to 30 g/l, the conversion and ee_p value accordingly increased and reached their maximum when the cell concentration was 30 g/l. It is indicated that higher cell concentrations provide larger amounts of enzyme, leading to a higher biocatalytic capacity. However, any further increase in cell concentration results in the decline of the conversion and ee_p. This suggests that excessively high cell concentration brings about the mass transfer limitation due to the high viscosity.

Time course of the hydrolysis under the optimized reaction conditions

Under the optimized conditions obtained above, the enantioselective hydrolysis of (*R,S*)-**1** by resting cells of *T. tyrosinosolvens* E105 was carried out. As shown in Fig. 6, the conversion increased rapidly in the first 24 h, while the ee_p value presents a slowly elevated trend. When the biotransformation was performed over 24 h, the conversion increased tardily, whereas the ee_p value declined. Consequently, the optimal reaction time was 24 h, since a high conversion (46.5 %) with the best ee_p value (≥99 %) was obtained, and an excellent enantioselectivity (data not shown in Fig. 6) with $E > 600$ as well.

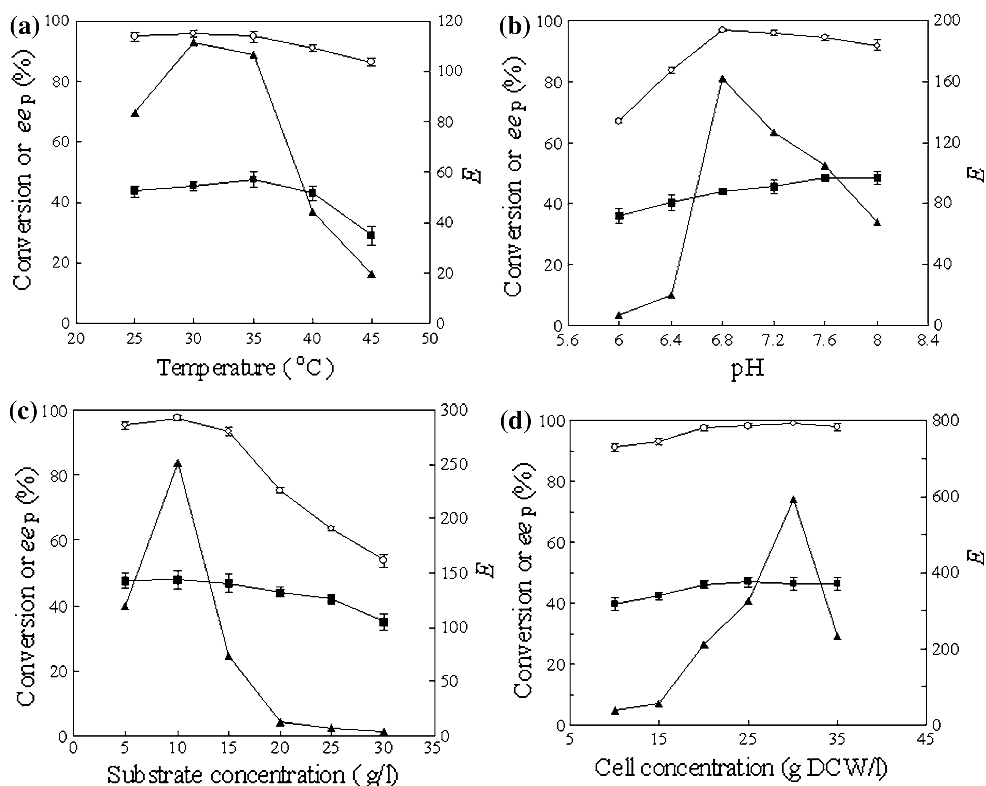

Fig. 5 Effects of reaction conditions on the hydrolysis catalyzed by *T. tyrosinosolvens* E105. **a** Effect of reaction temperature on the hydrolysis. The reaction time was 24 h. Other conditions were described in the "Materials and methods" section. **b** Effect of initial pH on the hydrolysis. **c** Effect of substrate concentration on the hydrolysis. The reaction was performed at pH 6.8 for 24 h. **d** Effect of concentration of suspended cells on the hydrolysis. The reaction was performed at pH 6.8 for 24 h. *Symbols E* (*filled triangle*); conversion (*filled square*); ee_p (*open circle*)

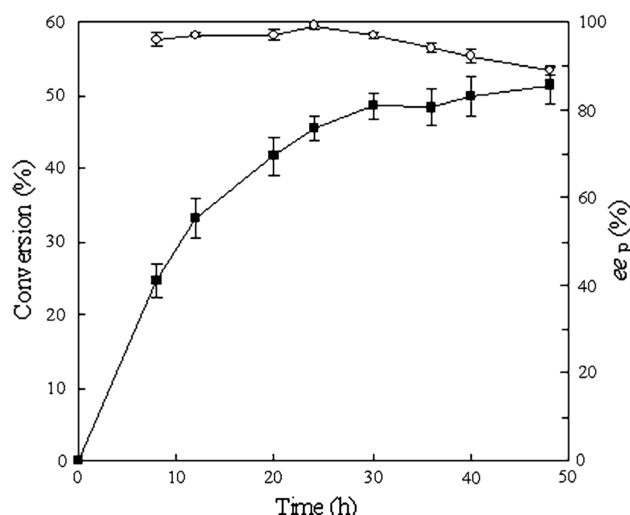

Fig. 6 Time course of the hydrolysis by *T. tyrosinosolvens* E105 under the optimized reaction conditions. The initial pH of reaction system was 6.8 and the cell concentration was 30 g/l (DCW). Other conditions are described in the "Materials and methods" section. *Symbols* conversion (*filled square*); ee_p (*open circle*)

Conclusions

A bacterial strain with high ester-hydrolase activity and enantioselectivity for the biotransformation of ester (*R,S*)-**1** to acid (*S*)-**2** was successfully obtained. According to its morphological characteristics, physiological properties and 16S rDNA sequence analysis, the newly isolated strain E105 was identified and designated *T. tyrosinosolvens* E105. The cells of *T. tyrosinosolvens* E105 with the maximum ester-hydrolase activity (5.84 U/l) and 2.58 g/l of the biomass (DCW) were harvested after cultivation in liquid medium at 30 °C and 200 rpm for 36 h. The investigation of the biotransformation performance showed that when the reaction temperature, buffer pH, concentration of substrate, and resting cells were 30 °C, 6.8, 10, and 30 g/l (DCW), respectively, excellent enantioselectivity ($E >$ 600) was achieved when the hydrolysis was carried out for 24 h. The data presented here suggest that the strain *T. tyrosinosolvens* E105 may have potential scientific and commercial applications.

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