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Kinetic Resolution of Racemic 1-phenyl-1,2-ethanediol Via *Gluconobacter oxydans*-catalyzed Asymmetric Bio-oxidation

Li Dai-Huan, Wei Dong-Zhi and Lin Jin-Ping*

State Key Laboratory of Bioreactor Engineering, NewWorld Institute of Biotechnology,
East China University of Science and Technology, Meilong Road 130, Shanghai 200237, China.

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Optically pure (S)-1-phenyl-1,2-ethanodiol(PED) is a versatile chiral building block for the synthesis of pharmaceuticals, pheromones and liquid crystals. An alternative and convenient biocatalytic process was developed for the preparation of (S)-PED by *Gluconobacter oxydans*-catalyzed optical resolution of the corresponding racemate. Whole cells of *G. oxydans* DSM2003 were found to catalyze the regio- and stereo-selective concurrent oxidation of racemic PED to give (S)-PED with the optical purity of 99% and yield of 38% in 10h, meanwhile (R)-mandelic acid with moderate ee value produced. This oxidation is carried out by two sets of enzymes, the one made of membrane-bound alcohol dehydrogenase is responsible for the PED oxidation to hydroxyl aldehyde intermediate and the other composed of membrane-bound aldehyde dehydrogenase and other enzymes is responsible for the mandelic acid production.

Key words: *Gluconobacter oxydans*, 1-phenyl-1,2-ethanodiol, Mandelic acid, Oxidative Resolution, Biocatalysis.

Gluconobacter oxydans (*G. oxydans*) is an obligate aerobic Gram-negative bacterium and known for its stereo- and regio-selective incomplete oxidation of a wide range of carbohydrates and alcohols to the corresponding aldehydes, ketones and organic acids which are secreted rapidly and almost completely into the medium. These features make *G. oxydans* as a valuable and versatile biocatalyst used in a wide variety of biotechnological processes for a long time [1-4]. The well-known examples include the production of vitamin from sorbitol [5] and of dihydroxyacetone (DHA) from glycerol[6, 7], the oxidation of glucose to gluconic acid which can be further oxidized to 2-keto-gluconic acid and 5-keto-gluconic acid[8, 9], the regioselective oxidation of N-formyl-1-amino-1-deoxy-D-sorbitol to N-formyl-6-amino-6-deoxy-L-sorbose, a precursor of the antidiabetic drug miglitol [5, 10]. For various poloys and sugar alcohols, the substrates are oxidized regioselectively by *G. oxydans* to the corresponding ketoses according to Bertrand and Hudson rule. *G. oxydans* has tendency for incomplete oxidative conversion of primary alcohols to acids. The 1,2-diols were proved to be oxidized to the corresponding hydroxyl acids with the whole cells of *G. oxydans*, due to the regioselective oxidation of primary hydroxyl group, including the production of glycolic acid from glycol [11], D-(-)-lactic acid from 1,2-propanediol [12] and (R)-2-hydroxybutyric acid from 1,2-butanediol [13].

Optically pure (S)-1-pheny-1,2-ethanodiol (PED) is an important and widely used chiral 1,2-diol which could be used in the synthesis of pharmaceuticals, agrochemicals, pheromones and liquid crystals, etc. [14]. Examples of chemical catalytic processes for the enantioselective synthesis of optical PED and other diols have been extensively reported. However, they usually require toxic metal catalysts and include quite a few steps[15, 16]. Compared with the chemical methods,

* To whom all correspondence should be addressed.
Tel.: +86 21 64253287; Fax: +86 21 64250068;
E-mail: jplin@ecust.edu.cn

biocatalysis is an alternative approach because of its high selectivity, mild reaction conditions and non-toxicity. A series of enzymatic methods have been explored in the synthesis of optically pure PED, including bio-reduction of α-hydroxy ketones [17-19], hydrolysis of racemic epoxide catalyzed by epoxide hydrolase [20-24] and kinetic resolution of the racemic PED or the corresponding hydroxyl monoesters followed by an additional hydrolysis step[14, 25, 26]. Unfortunately a lack of general methods is currently noticed because of their unsatisfied enantioselectivity or reactive efficiency.

On the basis of our previous experience in the region- and stereo-selective oxidation of racemic 1,2-propanediol to produce D-(-)-lactic acid, leaving (S)-1,2-propanediol as unreacted substrates in the reaction mixture, we decided to oxidize racemic PED with resting cells of *G. oxydans* DSM2003 to develop a synthetic pathway for the preparation of (S)-PED. Then, the reaction conditions were optimized and the reaction mechanism with the necessary oxidative enzymes was investigated.

MATERIALS AND METHODS

Chemicals

(R)/(S)-1-phenyl-1,2-ethanodiol and (R)/(S)-mandelic acid as standard samples were purchased from the Sigma-Aldrich Chemical Co (St. Louis, MO, USA). Racemic PED as substrate was purchased from YueyangYetop Fine Chemical Co.,Ltd (Yueyang, China). All other chemicals were of analytical grade and commercially available.

Microorganisms and culture conditions

The wild strain of *G. oxydans* DSM 2003and the two types of gene engineered strains *G. oxydans* DSM 2003 *adh*A::Km and DSM2003 *aldh*A::Km which were the disruptant of *adh*A gene in *G. oxydans* DSM 2003 with Km gene cassette and disruptant of *aldh*A gene in *G. oxydans* DSM 2003 with Km gene cassette respectively [27] were used in the study.

The *G. oxydans* DSM 2003 used in the asymmetry resolution was grown in a liquid medium containing: 80 g/l sorbitol, 20 g/l yeast extract, 1g/l KH_2PO_4, 0.5 g/l $MgSO_4 \cdot 7H_2O$ and 0.1g/l glutamine. All medium were sterilized by autoclaving at 115°C for 20 min. There was additional 50 mg/l kanamycin added in the liquid medium before inoculation for gene engineered strains. The seed cultures were inoculated from a Sorbitol Broth Agar plate and incubated in an orbital shaker (200 rpm, 30°C) until late exponential growth phase (about 22 to 24h). Then a 10% (v/v) inoculum of these cells was added to the liquid medium cultures and they were incubated at 30°C for about 22 to 24 h.

Analytical methods

To analyze the reaction products, the cells were removed from the reaction system by centrifugating. The concentrations of PED and mandelic acid were determined by HPLC (Agilent 1100) using a ZORBAX SB-AQ column (Agilent Technologies, USA) at 30°C, with an acidic aqueous solution (H_3PO_4, 0.1%) as the eluant at a flow rate of 1ml/min. When the absorbance of the eluate was monitored at 210 nm, PED and mandelic acid were observed at retention time of 16.0min and 19.2min respectively. The concentrations were calculated from the peak areas with the standard curves.

The optical purity of mandelic acid was determined by HPLC (Agilent 1100) using a Chirobiotic R (4.6×150mm,5μm) at 25°C, with 20% acetic acid-sodium acetate buffer (pH3.5) and 80% methanol as the eluant at a flow rate of 1ml/min. The (S)- and (R)-mandelic acid were detected at retention time of 3.0min and 6.4min respectively at 210nm and the ratio of the two peak areas was the ratio of their concentrations.

Then, (R)-PED and (S)-PED were extracted from the reaction solution without cells with ethyl acetate (1:1) by vigorous mixing. Then the optical purity of PED was determined by HPLC (Agilent 1100) using a Chiralcel OB-H column (4.6 mm× 250 mm; Daicel Chemical Ind., Ltd., Japan) with hexane and 2-propanol (9:1) as the mobile phase and 0.5 ml/min flow rate, detected at 210 nm. The (R)- and (S)-PED were observed at retention time of 15.4min and 18.5min respectively, and the ratio of the peak areas was the ratio of the concentrations of (R)- and (S)-PED.

To further confirm the structure of the acid product, the reaction product was isolated under unoptimized conditions, by extraction with organic solvent followed by silica gel column (C18) chromatography, to obtain R-mandelic acid for MS.

Resting-cell biotransformation

Unless stated otherwise, the reaction mixture in a 10 ml system comprised 0.1mol l^{-1}

potassium phosphate buffer, a certain concentration of (±)-PED and resting *G. oxydans* DSM2003 cells. The erlenmeyer flasks (50ml) with the reaction mixture were incubated in an orbital shaker (200rpm, 30°C) for a certain time. In the whole process of the reaction, the pH of the reaction mixture was adjusted using the solution of NaOH and HCl every some time.

RESULTS

The identification of products and the bioconversion process

To determine the products, the resting cells of *G. oxydans* DSM2003 at 50g/l was used as biocatalyst for the oxidative resolution of (±)-PED. The samples were taken at different time points (0h and 3h) and analyzed by HPLC and MS. As shown in Fig.1A, a compound that corresponded to the peak of authentic mandelic acid (19.2min) was produced, and the MS analysis signals of this compound were consistent with the molecular weight of mandelic acid. At the same time, the area of the peak corresponded to PED turned to smaller which reflected the consuming of PED. According to Fig.1B and C, the bioconversion of (R)-PED was faster than that of (S)-PED and correspondingly the production of (R)-mandelic acid was in the majority. This indicated that the *G. oxydans* cells showed different activities on the two isomerides, with the apparent Km of 516.9mmol/l for (S)-PED and of 44.8mmol/l for (R)-PED respectively.

The identification of the key-enzyme of the reaction

In our previous study, membrane-bound alcohol dehydrogenase (ADH) and membrane-

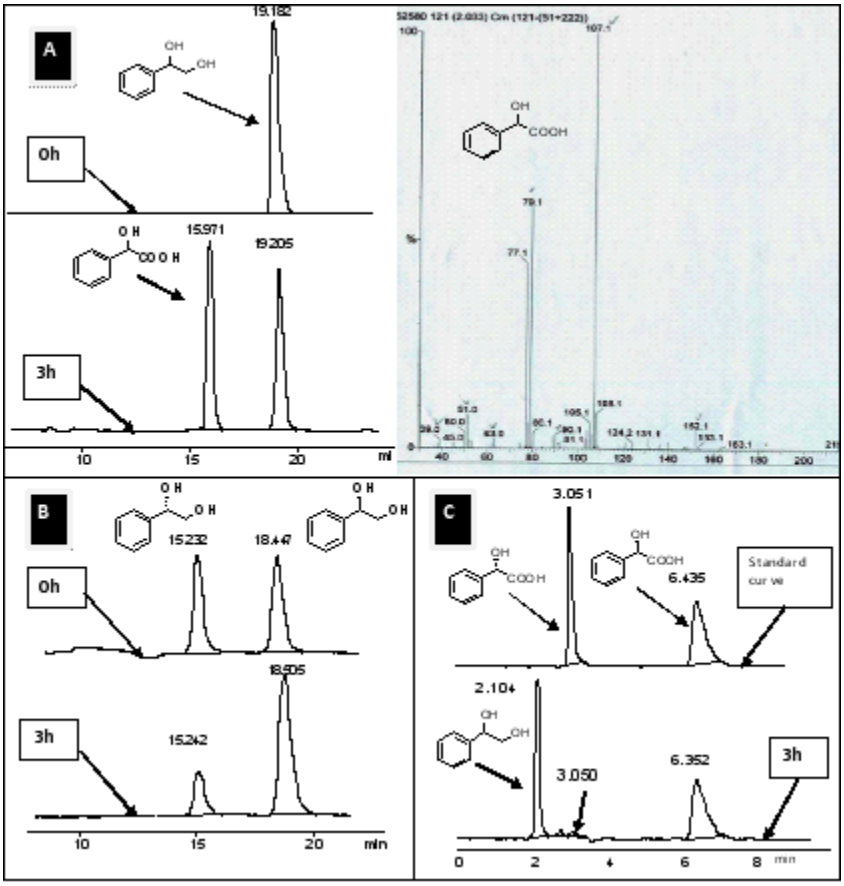


Fig. 1. HPLC and MS analysis of the samples taken at 0h and at 3h in the biotransformation of (±)-PED with resting cells of *G. oxydans* DSM2003. A was the HPLC prints of the sample taken at 0h and 3h and MS print of oxidized production. B and C indicated the changes of the reaction solution at 3h on (R)- and (S)-PED and on (R)- and (S)-mandelic acid, respectively

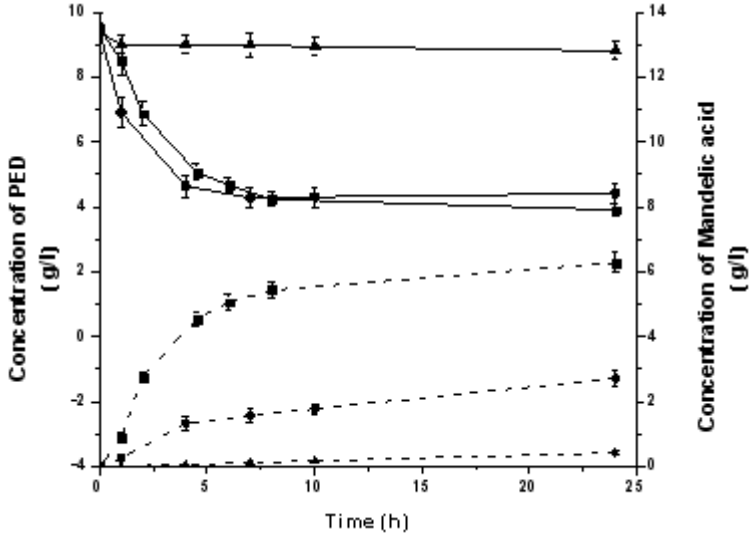


Fig. 2. The reaction courses with resting cells of the wild *G. oxydans* strain(■) plus the constructed ADH-knockout (▲) and ALDH-knockout (●) *G. oxydans* strains. The real lines and broken lines respectively represented the changes on concentrations of PED and mandelic acid

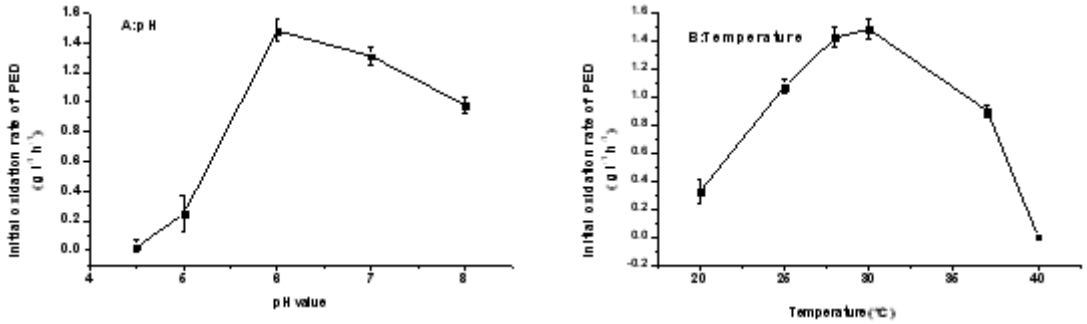


Fig. 3. The optimization of the reaction pH (A) and temperature (B)

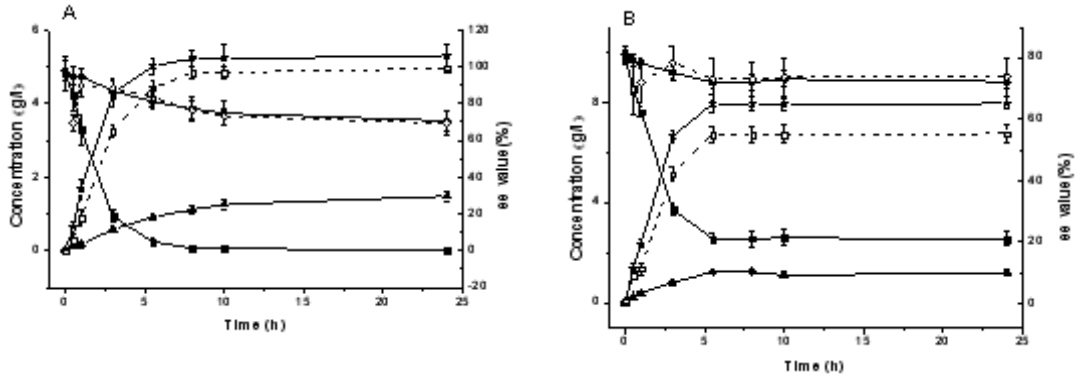


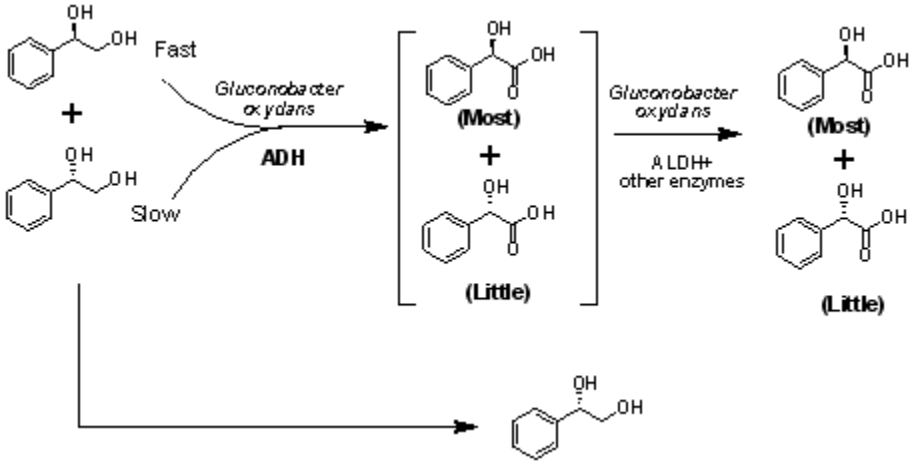
Fig. 4. The time curves of reaction at the concentration of substrate 10g/l (A) or 20g/l (B), respectively. The concentrations (real lines) of (R)-(■)/(S)-PED(●) and (R)-(✱)/(S)-mandelic acid(▲) were analyzed by HPLC. And then the ee value (broken lines) of (S)-PED(□) and (R)-mandelic acid (◇) were obtained as well. The reactions were carried out under 30°C and pH 6.0.

bound aldehyde dehydrogenase (ALDH) in *G.oxydans* were testified to be responsible for the conversion of α, ω-diols to ω-hydroxycarboxylic acids [27]. The constructed ADH-knockout and ALDH-knockout *G. oxydans* strains were also introduced to the bioconversion of PED in order to explore the possible mechanism of this biotransformation. The reaction courses were plotted in Fig.2. There were almost no production of mandelic acid and no reduction of PED when ADH was knocked off in *G. oxydans*. And if the ALDH was knocked off, there were few changes on the PED oxidation but only about half of mandelic acid produced compared with the results of wild strain reaction.

Optimization of (S)-PED production via whole cell-based oxidative resolution

The effects of the pH and temperature on the oxidative resolution of (±)-PED were evaluated firstly using 50g/l of *G. oxydans* resting cells and 10g/l racemic PED in 10ml 0.1mol/l phosphate buffer. As shown in Fig3A, the highest initial conversion rate of PED was detected at pH 6.0. The effect of the reaction temperature on the conversion was followed at temperature ranging from 20 to 40°C. And the optimum temperature for the bio-oxidation corresponded to 30°C (Fig.3B).

To further explore the catalytic potential of *G. oxydans* cells, the oxidation of (±)-PED was investigated with resting cells of *G. oxydans* at 50g/l under optimum pH 6.0 and 30°C of temperature at the substrate concentrations of 10g/l and 20g/l, respectively. Time-courses of selective oxidation of (±)-PED were given in Fig.4. Similar to what was shown in Figure 1(B) and 2, the concentration of (R)-PED decreased at a higher rate than that of (S)-PED, thus the $ee_{(S)}$ value of residual PED increased as the reaction went on. As shown in Fig.4(A), bioconversion of 10g/l of racemic PED gave (S)-PED in over 99% of ee value and 38% of yield in 10h when (R)-PED was almost converted to (R)-mandelic acid. At that time, the ee value of (R)-



Scheme 1. Regio- and Stereo-selective Concurrent Bio-oxidation of racemic PED with Resting Cells of *G. oxydans* DSM2003

mandelic acid was moderate due to a small quantity of (S)-mandelic acid produced from (S)-PED oxidation. When the initial concentration of substrate was up to 20g/l, (R)-PED would not be transformed completely because the reaction almost stopped at 6h, resulting in a very low enantioselectivity (about 56% of ee value) of (S)-PED obtained(Fig.4 B). At this time, the concentration of mandelic acid in the reaction mixture reached about 8g/l.

DISCUSSION

The resting cells of *G. oxydans* were able to catalyze regio- and stereoselective concurrent oxidation of terminal hydroxyl group of 1-phenyl-1, 2-ethanadiol to the corresponding hydroxyl acid, mandelic acid. The enzymes involved in this reaction were membrane-bound ADH and ALDH. ADH was indispensable for the reaction and in charge of the transformation of PED. And the

presence of ALDH had no effect on the transformation of PED, but was beneficial for the production of mandelic acid (Fig. 2). This also implied *G. oxydans* catalyzed the oxidation of 1-hydroxy group of (±)-PED to the hydroxyl aldehyde and then to the hydroxyl acid. Unfortunately, no aldehyde intermediate was observed in HPLC-MS during the reaction period. Additionally, hydroxyl aldehyde intermediate even if produced was quickly oxidized to hydroxyl acid. And according to the apparent Km of the resting cells, it was distinct that there were different oxidative activities of cells (ADH) to the two isomerides in racemic PED. Therefore, it was feasible to produce the optically pure (S)-PED and (R)-mandelic acid via the *G. oxydans*-catalyzed oxidation of racemic PED. The whole reaction process was summarized in Scheme 1.

Then, the optimized conditions of the reaction were obtained at 30°C and pH6.0 (Fig. 3). And moreover, for the exploitation of the catalytic potential of *G. oxydans* cells, the two reaction courses at the different substrate concentration, 10g/l and 20g/l, were explored and compared. The initial conversion rate of (R)-PED (almost representing the rate of the reaction) at the substrate concentration of 20g/l was faster than that at substrate concentration of 10g/l, but it was stopped before the (R)-PED was transferred completely when the substrate concentration was 20g/l (Fig. 4). This indicated that the substrate PED didn't have distinct inhibition on the reaction when it was below 20g/l. Therefore mandelic acid produced was speculated to be the main inhibitor to the biotransformation.

CONCLUSION

G. oxydans DSM2003 was explored for the preparation of optically pure (S)-PED by regio- and stereo-selective oxidative resolution of racemic PED in this study. In 10h, 10g/l of (±)-PED was biotransformed with whole cells of *G.oxydans* as catalyst to afford (S)-PED with over 99% of ee value and 38% of yield, and meanwhile (R)-mandelic acid was produced with moderate enantioselectivity. The membrane-bound alcohol dehydrogenase was accountable for the oxidation of PED in *G. oxydans* DSM2003. This bio-catalysis process provided a quick and effective method for simultaneous production of high optically pure (S)-PED and (R)-mandelic acid.

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