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Short Communication

Highly enantioselective oxidation of α-hydroxyacids bearing a substituent with an aryl group: Co-production of optically active α-hydroxyacids and α-ketoacids

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HIGHLIGHTS

- A high-throughput method was established to screen enantioselective α-HADH.
- *Sinorhizobium* sp. with high α-HADH activity and enantioselectivity was newly isolated.
- Co-obtaining of (R)-α-hydroxyacids and α-ketoacids was demonstrated.
- A novel methodology was provided to utilize α-hydroxyacids more efficiently.

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ABSTRACT

A novel and simple methodology for co-obtaining of enantiomerically pure α-hydroxyacids and α-ketoacids was developed by enantioselective oxidation of α-hydroxyacids bearing a substituent with an aryl group using α-hydroxyacid dehydrogenase (α-HADH). A high-throughput method was firstly established for screening of enantioselective α-HADHs. *Sinorhizobium* sp. ZJB1101 with high activity and excellent enantioselectivity of α-HADH for oxidation of α-hydroxyacids bearing a substituent with an aryl group was isolated and identified. This strain has potential for co-production of (*R*)-α-hydroxyacids and α-ketoacids in near theoretical yields, while no consecutive oxidation of α-ketoacids was observed. The green conversion system appears promising for potential applications in industry.

1. Introduction

Optically active α-hydroxyacids and α-ketoacids bearing a substituent with an aryl group, such as phenyllactic acid, phenylpyruvic acid, mandelic acid, benzoylformic acid and their derivatives, et al., play significant roles as synthons in the synthesis of natural and biologically active compounds (Bousquet and Musolino, 2003; Hashimoto et al., 1996; Xue et al., 2011). Although the chemical method is the most popular way to prepare chiral α-hydroxyacids and α-ketoacids in industry presently, it does not always satisfactorily work in terms of their enantioselectivity or yield in some cases. Utilization of chemical catalysts has also made the green production of α-hydroxyacids and α-ketoacids difficult to accomplish.

Recently, biocatalysis has emerged as a powerful strategy for the production of optically active α-hydroxyacids due to its remarkable stereoselectivity and high yield (Wohlgemuth, 2010). Biocatalytic oxidation could be an environmentally benign procedure as a substitute for traditional chemical reactions (Babiak et al., 2011; Matsumoto et al., 2011; Wu et al., 2010), and furthermore, enantioselective oxidation of racemic α-hydroxyacids by α-hydroxyacid dehydrogenases (α-HADHs) could produce the corresponding α-ketoacids and optically active α-hydroxyacids with high enantiomeric excess (ee) as remaining substrates (Gao et al., 2009, 2011). In the case of α-hydroxyacids bearing a substituent with an aryl group, several microorganisms including *Pseudomonas polycolor* IFO 3918 (Takahashi et al., 1995), *Pseudomonas putida* ECU1009 (Huang et al., 2005), *Brevibacterium* sp. CCSYU10011 (Zhang and Xu, 2006), and *Alcaligenes* sp. ECU0401 (He et al.,

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2008) have been reported to have the abilities to catalyze the asymmetric oxidation of racemic mandelic acid and/or its derivatives. However, the direct oxidation products (corresponding α-ketoacids), as quite active intermediates, cannot be accumulated in the reaction media because they may be degraded by the successive action of phenylglyoxylate decarboxylase and benzaldehyde dehydrogenase existed in those strains, which lowers the atom economy (Huang et al., 2005; Takahashi et al., 1995; Tsou et al., 1990; Xu and Mitra, 1999). Increasingly, stringent environmental legislation has generated a pressing need for cleaner methods of chemicals production by increasing the atom utilization (Noyori, 2009; Sheldon, 2007). Therefore, we should aim at synthesizing target compounds with 100% yield and 100% selectivity and avoid the production of waste.

Herein, to improve the atom economy, a novel and simple methodology for co-obtaining of enantiomerically pure α-hydroxyacids and α-ketoacids was developed by enantioselective oxidation of α-hydroxyacids bearing a substituent with an aryl group using biocatalyst, which could be screened based on an established high-throughput screening method.

2. Methods

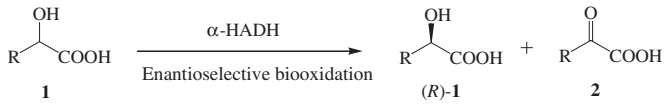
2.1. Chemicals

1a-1l, (*R*)-**1a-1l** and (*S*)-**1a-1l** (Table 1) were purchased from J&K Chemical Co., Ltd. (Shanghai, China). All other chemicals were of reagent grade and obtained commercially. $FeCl_3$ solution (Color indicator) for direct determination of **2a** was prepared by dissolving 1.85 mmol of $FeCl_3{\cdot}6H_2O$, 20 mL of acetic acid and 400 mL of DMSO in distilled water to make 1 L (pH 2.5).

2.2. Microbial cultures and cultivation conditions

The medium for the fermentation of α-HADH consisted of (g/L): glucose 10, yeast extract 10, K_2HPO_4 2.5, KH_2PO_4 2.5, $MgSO_4$ 0.2, $FeSO_4$ 0.03, (R,S)-**1a** 2. (pH 7.0). Potential enantioselective α-HADH-producing microorganism was cultivated in fermentation medium at 30 °C for 24 h in a rotary shaker. The culture medium (3 mL) was transferred to 50 mL of fermentation medium in a 250-mL flask and incubated at 30 °C for 48 h (150 rpm). Cells were harvested by centrifugation at 12,000*g* for 8 min at 4 °C. The cell pellets were washed thoroughly with 100 mM phosphate buffer (pH 8.0). Then, they were stored at 4 °C for further use.

Table 1
Apparent kinetic parameters of α-HADH in *Sinorhizobium* sp. ZJB1101 for different substrates.



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Substrate	*R*	K_m (mM)	V_m (mM/min)	V_m/K_m
1a	Benzyl	68.543	0.124	0.002
1b	Phenyl	64.979	0.385	0.006
1c	2-Chloro-phenyl	81.231	0.165	0.002
1d	3-Chloro-phenyl	4.954	0.039	0.008
1e	4-Chloro-phenyl	3.716	0.049	0.013
1f	2-Fluoro-phenyl	11.496	0.066	0.006
1g	4-Fluoro-phenyl	66.484	0.489	0.007
1h	4-Bromo-phenyl	3.631	0.054	0.015
1i	4-Methyl-phenyl	65.631	0.186	0.003
1j	4-Hydroxy-phenyl	73.523	0.153	0.002
1k	2,4-Difluoro-phenyl	15.197	0.147	0.010
1l	3,5-Difluoro-phenyl	8.575	0.089	0.010

2.3. Color-generating reaction

The color-generating reactions were performed in 96-well microplates at room temperature. Total reaction volume was 250 μL consisting of 240 μL $FeCl_3$ solution and 10 μL samples. UV–vis spectra of Fe^{3+}/**2a** complex and $FeCl_3$ solution were captured from 400 to 850 nm with a Beckman DU800 spectrophotometer (Beckman Coulter, USA). Parameters varied as follows for optimization of the reaction conditions: pH values of $FeCl_3$ solution were 1.0–6.0. Concentrations of $FeCl_3$ were 1.0–2.5 mM, reaction times were 10–60 min, and storage time of the colored Fe^{3+}/**2a** complex were 1–9 h. The samples were determined at 640 nm by an absorbance microplate reader (SpectraMAX puls 384, MD, USA).

2.4. Screening procedure

The enantioselective α-HADH-producing microorganisms were isolated through two rounds of screening: the first round of screening for α-HADH activity and the second round of screening for α-HADH enantioselectivity. Resting cells (0.2 g) of different microorganisms were resuspended in 10 mL phosphate buffer (100 mM, pH 8.0) containing 20 mM (*R,S*)-**1a**. The mixtures were shaken at 30 °C and 150 rpm for 4 h. Samples (10 μL) were taken at regular intervals and transformed to a 96-well microplate. 240 μL of $FeCl_3$ solution (1.85 mM, pH 2.5) was added to the plate for the derivatization with **2a** produced during the bioconversion. The colorimetric reactions were performed for 40 min at room temperature. A color change from light yellow to blue green was indicative of formation of **2a** and thus positive result. For the control, the same format was used without enzyme. After screening with (*R,S*)-**1a** as substrate, the positive candidates were checked for their enantioselectivity by comparing the rate of oxidation of (*R*)-**1a** with that of (*S*)-**1a** using the same system. The absorbance of the solution in the plate was determined at 640 nm by an absorbance microplate reader (SpectraMAX puls 384, MD, USA). The initial reaction rates for each enantiomer were calculated separately. The quotient of these rates gives the apparent enantioselectivity (E_{app}).

2.5. Enantioselective oxidation of different substrates with the newly isolated strain

Resting cells of *Sinorhizobium* sp. ZJB1101 were resuspended to a cell concentration of 50 g/L in 10 mL phosphate buffer (100 mM, pH 8.0) containing 20 mM **1a–1l**. The mixtures were shaken at 30 °C and 150 rpm in 50-mL flasks sealed with lids. Samples were taken at regular intervals and the reactions were terminated through centrifugation (12000*g*, 4 °C, 10 min). The yield and ee were determined by reversed-phase high-performance liquid chromatographic (RP-HPLC) method as described below. Apparent kinetic parameters of α-HADH in *Sinorhizobium* sp. ZJB1101 for different substrates were determined by using 8–10 substrate concentrations ranging from 0 to 20 mM. Data were calculated from Lineweaver-Bulk plots in origin 6.0 (Microcal Software, Inc.).

2.6. RP-HPLC analysis

Direct RP-HPLC method was developed for the separation of enantiomers of α-hydroxyacids and α-ketoacids bearing a substituent with an aryl group. The concentrations of (*R*)-**1a–1l**, (*S*)-**1a–1l** and **2a–2l** were assayed by RP-HPLC (Dionex UltiMate 3000, USA) equipped with a chiral column (Chirobiotic™ R