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SHORT PUBLICATION

Enzyme-catalyzed asymmetric synthesis of optically active (*R*)- and (*S*)-ethyl -4-phenyl-4-hydroxybutyrate with microbial cells

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Abstract

In efforts to obtain carbonyl reductases with high activity and enantioselectivity, forty microorganisms belonging to different taxonomical groups were investigated for the ability to catalyze the enantioselective reduction of ethyl 4-phenyl-4-oxobutyrate (EPOB) to the corresponding optically active ethyl 4-phenyl-4-hydroxybutyrate (EPHB). Highly enantioselective reduction of EPOB was achieved with *Candida magnoliae* CGMCC 2.1919 and *Saccharomyces cerevisiae* CGMCC 2.399 giving the corresponding (*R*)-EPHB and (*S*)-EPHB in 99% ee, respectively. The highly enantioselective bioreductions provide simple routes to optically active γ-hydroxyl acid esters that are useful pharmaceutical intermediates and versatile chiral building blocks.

Keywords: *Bioreduction, Candida magnoliae, Saccharomyces cerevisiae, Enantioselectivity*

Introduction

Enantioselective reduction provides direct access to chiral alcohols that are important building blocks for the synthesis of fine chemicals and pharmaceuticals (Matsuda et al. 2009). Optically active 4-aryl-4-hydroxybutanoic acid derivatives are valuable intermediates in the preparation of liquid crystalline compounds, agrochemicals and pharmaceuticals (Scheme 1), such as serotonin uptake inhibitors, fluoxetine and platelet activating factor (PAF) antagonists (Corey et al. 1987, 1988).

The asymmetric synthesis of optically active 4-aryl-4-hydroxybutanoic acid derivatives has been reported using chemical catalysts. For example, Noyori et al. (1992) described the enantioselective hydrogenation of γ-keto esters in the presence of chiral ruthenium complexes. Corey and coworkers reported the enantioselective synthesis of methyl (*R*)-4-(3, 4-dimethoxyphenyl)-4-hydroxybutyrate in 98% yield and 95% ee using chiral oxazaborolidine and borane reagents (Corey et al. 1987, 1988). Militzer et al. (2005) further developed the stereoselective reduction of 4-aryl-4-oxobutanoic acid derivatives with ruthenium-containing catalysts, and a series of chiral 4-aryl-4-hydroxybutanoic acid derivatives were obtained in 70–96% ee. In addition, Ramachandran et al. (2002) developed effective intramolecular asymmetric reduction of γ-keto acid ester with β-chlorodiisopinocampheylborane to produce (*R*)-4-phenyl-4-hydroxybutyrate in 94% ee. Recently, Militzer and coworkers also designed and developed a new chiral ruthenium catalyst, which was used for asymmetric reduction of γ-keto acid esters to their corresponding chiral hydroxyl acid esters (Ramachandran et al. 2002).

In comparison with chemical catalysts, the use of enzymes can provide benefits that cannot be obtained with traditional chemical treatments. These include mild reaction conditions, environmentally friendly reduction in waste and reduced energy consumption. Finding ideal biocatalysts is a critical part of enzyme-catalyzed asymmetric synthesis. (Chen et al. 2007, 2008, 2009, 2010). To the best of our knowledge, enzyme-catalyzed asymmetric reduction of 4-aryl-4-hydroxybutanoic acid esters has rarely been reported. Manzocchi et al. (1987) have previously

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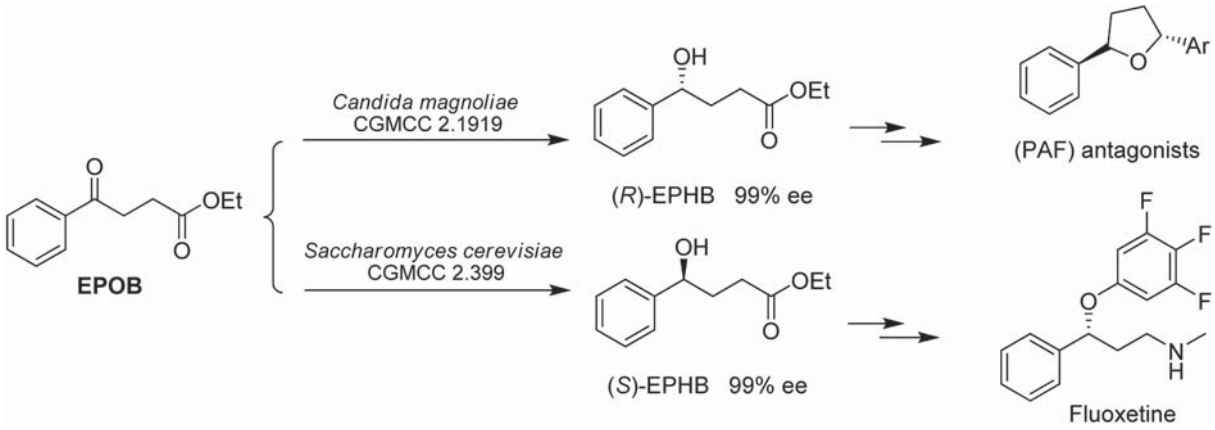


Scheme 1. Biocatalytic preparation and synthetic application of optically active (*R*) and (*S*)-EPHB.

reported that Baker's yeast can be used as a biocatalyst for the reduction of EPOB with unspecified enantioselectivity.

Materials and methods

General

All strains were obtained from our laboratory and registered at the China General Microbiological Culture Collection Center (CGMCC) and at our lab. All other reagents were purchased from commercial sources and were used without further purification. ^{1}H NMR spectra were recorded on a Bruker-300 (300/75 MHz) spectrometer using $CDCl_3$ as a solvent and TMS as an internal standard. TLC was performed on glass-backed silica plates. Column chromatography was performed by using silica gel (200–300 mesh) with ethyl acetate/petroleum ether as an eluent. Enantiomeric excess was determined by GC analysis using a Fuli GC9790 with a chiral column (CP-Chirasil-DEX CB, Varian, USA) and using a flame ionization detector, nitrogen was used as the carrier gas at 1.5 mL/min, the split ratio was 1:50 (v/v), the injector and detector temperatures were both set at 250°C, and the column temperature was programmed as 80°C for 3 min ramped to 220°C at a rate of 3°C/min. The configuration of the bioproduct **2** was assigned by using authentic samples of (*R*)-**2** and (*S*)-**2** as standards in chiral GC analysis.

General procedure for cell growth

Yeasts were grown in a medium containing 0.2% (w/v) glucose, 2% (w/v) peptone, and 1% (w/v) yeast extract solution (pH 6.8–7.0); bacteria were grown in a medium containing 0.1% (w/v) sodium chloride, 0.5% (w/v) beef extract, and 2% (w/v) peptone solution (pH 6.8); and strains were maintained on nutrient agar slants at 4°C. Erlenmeyer flasks of 250 mL containing 100 mL of the appropriate sterilized cultivation medium were inoculated with the test microorganism and incubated in an orbital shaker (180 rpm) at 27°C. After 48 h of growth (yeast, bacteria), the cells were harvested by centrifugation and washed twice with cool physiological saline (0.85%).

General procedure for enantioselective reduction with resting cells of microorganisms

To a 50 mL Erlenmeyer shaking-flask were added 10 mL potassium phosphate buffer (0.1 M, pH 7.0), 1.0 g (wet weight) freshly harvested cells, 50 mg glucose, and 10 mg EPOB, and the mixture was shaken for 24 h at 30°C. After 24 h, ethyl acetate (30 mL) was added to the reaction mixture, and the organic layer was dried and analyzed by GC to determine the yield and enantiomeric excesses.

Preparation of (R)-2 and (S)-2 with C. magnoliae CGMCC 2.1919 and S. cerevisiae CGMCC 2.399, respectively

To a 50 mL Erlenmeyer shaking-flask were added a suspension of *C. magnoliae* CGMCC 2.1919 or *S. cerevisiae* CGMCC 2.399 cells (5.0 g. wet weight) in 50 mL of potassium phosphate buffer (0.1 M, pH 6.8), substrate EPOB (50 mg), 5% glucose (w/v), and 10% ethanol (v/v), and the mixtures were shaken for 24 h at 30°C. After the reaction was complete, the mixture was centrifugated at 10,000 rpm for 10 min, the supernatant was then saturated with sodium chloride, and extracted with ethyl acetate (3×30 mL). The combined organic layers were

dried over anhydrous Na_2SO_4 and the solvent was removed under vacuum. The chemical yield and ee of the products were determined by GC analysis. The products were purified by silica gel column chromatography, and were identified by ^1H NMR analysis. The absolute configurations of the bioproducts (*R*)-EPHB and (*S*)-EPHB were assigned by comparison of the retention time in GC analysis.

(R)-Ethyl-4-phenyl-4-hydroxybutyrate 2

Colorless oil (38% yield): ^1H NMR (300 MHz, $CDCl_3$): δ7.25–7.35 (*m*, 5H, Ph), 4.68 (*m*, 1H, *CH*OH), 4.11(*q*, 2H, $J = 7.2$ Hz, OCH_2CH_3), 2.40 (*t*, $J = 7.8$ Hz, 2H, $-CH_2CO$), 2.36 (*s*, 1H, CH*OH*), 2.06 (*q*, 2H, $J = 7.2$ Hz, $CHCH_2$), 1.25 (*t*, 3H, $J = 7.2$ Hz, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ174, 144, 128, 127, 126, 74, 61, 34, 30, 14.

Results and discussion

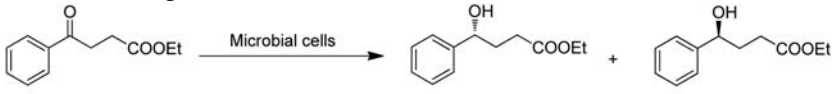
Forty strains were screened for the enantioselective reduction of EPOB to chiral EPHB, and the reaction progress was monitored by GC analysis. As shown in Table I. *T. variabilis* CGMCC 2.1570 and *G. candidum* CGMCC 2.616 gave especially good enantioselectivity of (*R*)-EPHB (81%–85% ee) (entries 1–2). The best results for the bioreduction were obtained with *S. cerevisiae* CGMCC 109 and *Candida magnoliae* CGMCC 2.1919, giving an ee for (*R*)-EPHB of 98% and 99%, respectively (entries 3–4). In contrast, the reduction of EPOB with other strains (*S. cerevisiae* CGMCC 2.396, *S. cerevisiae* CGMCC 2.25, *T. cutaneum* CGMCC 2.1795, and *T. cutaneum* CGMCC 2.570; entries 5–8) gave (*S*)-EPHB with moderate enantioselectivity of 53–77% ee. The highest (*S*)-enantioselectivity was obtained using *S. cerevisiae* CGMCC 2.399 as a biocatalyst.

Encouraged by these results, we investigated the reaction process to monitor whether byproducts were being formed and for process optimization. Figure 1 shows the time course of bioreduction of EPOB with resting cells of *C. magnoliae* CGMCC 2.1919. The reaction was very fast over the first 4 h, (*R*)-EPHB with 12% yield and the ee of (*R*)-EPHB was maintained over 99%. However, we found that a lot of keto acid appeared in this whole reaction process after 10 h. A 22% yield of (*R*)-EPHB with 78% residual 4-oxo-4-phenylbutanoic acid was obtained after extending the reaction time to 24 h. This indicates the presence of a competing ester hydrolytic activity but also that 4-oxo-4-phenylbutanoic acid is a poor substrate for the reductase and EPHB is not a substrate for the hydrolase, as 4-phenyl-4-hydroxybutyrate (PHB), the corresponding hydroxy acid to EPHB was not detected in this reaction process.

Reaction conditions such as reaction time, pH, glucose, and temperature were investigated for the biotransformation by *C. magnoliae* CGMCC 2.1919. These were shown to be pH 6.8–7.0, 30°C, and 5% glucose. Glucose was particularly important for cofactor regeneration in the biotransformation process as a very low yield (< 5.0%) was obtained without glucose in the reaction system. As shown in Figure 1, the highest yield was obtained when the reaction time was extended to 24 h, (*R*)-EPHB was obtained with 22% yield and > 99% ee, and extension of the reaction time had no effect on the enantioselectivity.

In order to increase the yield of product, a cosolvent system was investigated in this bioreduction process. Usually, a small amount of an organic

Table I. Screening of microbial strains for the bioreduction of EPOB to EPHB.



EPOB (*R*)-EPHB (*S*)-EPHB

Entry[a]	Strain	Time (h)	Yield (%)	ee (%)	Configuration[b]
1	*Trichosporon variabilis* CGMCC 2.1570	24	42	85	(*R*)
2	*Geotrichum candidum* CGMCC 2.616	24	51	81	(*R*)
3	*Saccharomyces cerevisiae* CGMCC 2.109	24	16	98	(*R*)
4	*Candida magnoliae* CGMCC 2.1919	24	22	99	(*R*)
5	*Trichosporon cutaneum* CGMCC 2.25	24	34	77	(*S*)
6	*Saccharomyces cerevisiae* CGMCC 2.396	24	29	59	(*S*)
7	*Trichosporon cutaneum* CGMCC 2.1795	24	51	53	(*S*)
8	*Trichosporon cutaneum* CGMCC 2.570	24	42	60	(*S*)
9	*Saccharomyces cerevisiae* CGMCC 2.399	24	40	99	(*S*)

[a]The reactions were run with 10 mg substrate EPOB in a 10-mL cell suspension (1 g fresh harvest wet cells) of microorganism's whole cells in 100 mM KH_2PO_4–K_2HPO_4 buffer (pH 6.8–7.0) containing 5% glucose (w/v) at 30°C and 160 rpm for 24 h.
[b]Determined by GC analysis.

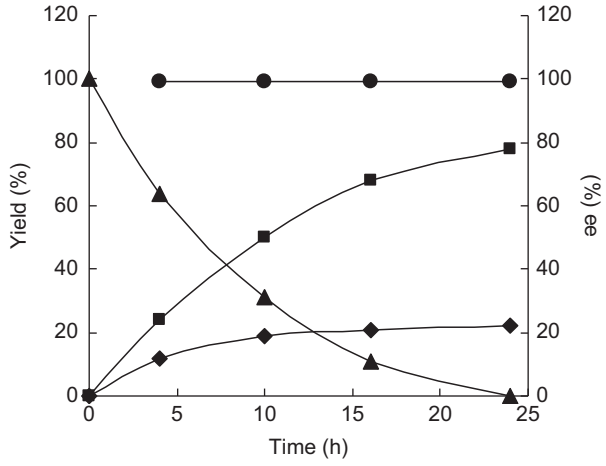


Figure 1. Time-course curve of bioreduction of EPOB in aqueous phase with *C. magnoliae* CGMCC 2.1919 (♦-yield of (*R*)-EPHB, •-ee of (*R*)-EPHB, ▲-Yield of EPOB, and ■-yield of 4-oxo-4-phenylbutanoic acid).

solvent is used to ensure sufficient solubility of keto ester substrates (Griebenow et al. 2001). We found that cosolvents played an important role in the bioreduction of EPOB to (*R*)-EPHB with *Candida magnoliae* CGMCC 2.1919. From the results shown in Table II, it can be noticed that addition of 10% (v/v) ethanol or dimethylformamide increased the yield but did not affect the enantioselectivity (entry 2 and entry 6). (*R*)-EPHB was obtained in 38% yield and 99% ee in the presence of 10% ethanol as a cosolvent. Addition of methyl *tert*-butyl ether, dimethyl sulfoxide, methanol, and hexane decreased both the activity and enantioselectivity. (entries 3–5, 7). A larger scale experiment was investigated under the optimum conditions (24 h, pH 6.8, 5% (w/v) glucose, 10% (v/v) ethanol, 30°C); 5 g wet cells suspended in 50 mL potassium phosphate buffer converted 50 mg EPOB to (*R*)-EPHB with 38% yield and 99% ee, confirming that the cosolvent system (10% ethanol v/v) was helpful for increasing the yield presumably by increasing the solubility of substrates. Moreover, 40% yield and 99% ee (*S*)-EPHB was also obtained under the optimum conditions (24 h, pH 6.8, 5% (w/v) glucose, 10% (v/v) ethanol, 30°C) with *S. cerevisiae* CGMCC 2.399.

Conclusion

In conclusion, *C. magnoliae* CGMCC 2.1919 and *S. cerevisiae* CGMCC 2.399 can be used as two enantiocomplimentary biocatalysts for highly enantioselective reductions of EPOB to produce (*R*)-EPHB and (*S*)-EPHB both in 99% ee, respectively. Bioreduction of EPOB using *S. cerevisiae* CGMCC 2.399 followed Prelog's rule giving (*S*)-EPHB in 99% ee with 40% yield. *C. magnoliae* CGMCC 2.1919 gave the corresponding (*R*)-EPHB in 99% ee with 38% yield under optimal reaction conditions. This provides a greener way and direct access to synthesize optically active γ-hydroxy acid esters that are useful pharmaceutical intermediates and versatile chiral building blocks. Moreover, the resting cells of *C. magnoliae* CGMCC 2.1919 and *S. cerevisiae* CGMCC 2.399 are easily available in large quantity and are easy to handle, thus being a routine biocatalyst for organic chemist.

Table II. Effect of cosolvents on the bioreduction of EPOB with *C. magnoliae* CGMCC 2.1919.

Entry[a]	Cosolvent (10% v/v)	Time (h)	Yield (%)[b]	ee (%) Configuration
1	None	24	22	99 (*R*)
2	Ethanol	24	38	99 (*R*)
3	Methanol	24	21	91 (*R*)
4	Hexane	24	19	89 (*R*)
5	Methyl Tert-Butyl Ether	24	19	97 (*R*)
6	Dimethylformamide	24	26	99 (*R*)
7	Dimethyl Sulfoxide	24	10	98 (*R*)

[a]The reactions were run with 10 mg substrate EPOB in a 10-mL cell suspension (1 g fresh harvest wet cells) of microorganism's whole cells in 100 mM KH_2PO_4–K_2HPO_4 buffer (pH 6.8–7.0) containing 5% glucose (w/v) and 10% cosolvent (v/v) at 30°C and 160 rpm for 24 h.
[b]Determined by GC analysis.

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