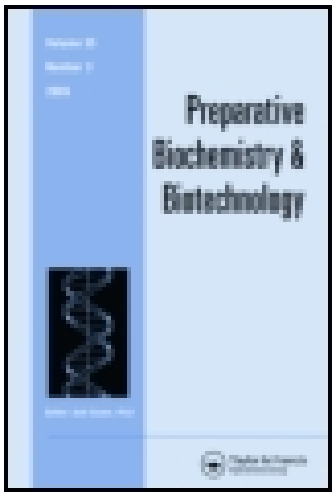

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Highly Selective Anti-Prelog Synthesis of Optically Active Aryl Alcohols by Recombinant Escherichia coli Expressing Stereospecific Alcohol Dehydrogenase

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Highly selective anti-Prelog synthesis of optically active aryl alcohols by recombinant *Escherichia coli* expressing stereospecific alcohol dehydrogenase

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Abstract

Biocatalytic asymmetric synthesis has been widely used for preparation of optically active chiral alcohol as the important intermediates and precursors of active pharmaceutical ingredients. However, the available whole-cell system involving anti-Prelog specific alcohol dehydrogenase is yet limited. Recombinant *Escherichia coli* system expressing anti-Prelog stereospecific alcohol dehydrogenase from *Candida parapsilosis* was established as a whole-cell system for catalyzing asymmetric reduction of aryl ketones to anti-Prelog configurated alcohols. Using 2-hydroxyacetophenone as the substrate, reaction factors including pH, cell status, and substrate concentration had obvious impacts on the outcome of whole-cell biocatalysis, and xylose was found to be an available auxiliary substrate for intracellular cofactor regeneration, by which (*S*)-1-phenyl-1,2-ethanediol was achieved with the optical purity of 97%e.e. and yield of

89% under the substrate concentration of 5 g/L. Additionally, the feasibility of the recombinant cells towards different aryl ketones was investigated, and most of the corresponding chiral alcohol products were obtained with the optical purity over 95%e.e. Therefore, the whole-cell system involving recombinant stereospecific alcohol dehydrogenase was constructed as an efficient biocatalyst for highly enantioselective anti-Prelog synthesis of optically active aryl alcohols and would be promising in pharmaceutical industry.

KEYWORDS: Alcohol dehydrogenase, anti-Prelog reduction, aryl ketone, asymmetric synthesis, optically active alcohol, active pharmaceutical ingredient

INTRODUCTION

Microbial oxidoreductive systems involving stereospecific oxidoreductases has gained increasing relevance in organic synthesis for the production of optically active alcohols,[1,2] such as 1-phenyl-1,2-ethanediol (PED) and its derivatives, the valuable and versatile chiral building blocks for synthesis of pharmaceuticals, agrochemicals, and pheromones, etc.[3,4] The asymmetric reduction of prochiral carbonyl compounds to their corresponding alcohols is potentially one of the most useful ways of introducing chirality in a molecule. For those chiral auxiliaries production from their corresponding prochiral ketones, alcohol dehydrogenases have the inherent advantages over chemo-catalysts in terms of their highly chemo-, enantio- and regioselectivity.[5,6] These features make

stereospecific alcohol dehydrogenases very interesting from both scientific and industrial perspectives.

Currently, however, most enzymes catalyzing reductions generally follow the Prelog's rule in the sense of the stereochemistry outcomes, and another type of biocatalysts with a complementary selectivity are yet limited.[7] Only several microorganisms were found to possess anti-Prelog selectivity.[8] Especially for optically active PED production, only *Geotrichum* sp. was reported to catalyze anti-Prelog reduction of 2-hydroxyacetophenone to (*S*)-PED (91%e.e., yield 62%) with substrate concentration of 2.72 g/L.[9] Therefore, the application of enzymes catalyzing anti-Prelog type reaction would be valuable for filling the demands of asymmetric synthesis to produce chiral alcohols in different configuration.

The key issue to biocatalytic process architecture defined in a particular case is whether the catalyst is to be used as a whole cell or an isolated enzyme. For cofactor-dependent alcohol dehydrogenases, the alternative whole-cell process will prove the preferred option.[10,11] Moreover, whole cell biocatalysts are usually more stable due to the protective cell matrix envelope for the enzyme.[12] Although whole cells typically contain multiple oxidoreductases, leading to mixed stereoselectivity and side reactions by other competing enzymes, the interference of other enzymes could be avoided by expressing

the desired enzyme in a recombinant system, and an easy way for cofactor regeneration can be afforded in organism cells.[13]

In this work, recombinant *Escherichia coli* system expressing the identified anti-Prelog stereospecific alcohol dehydrogenase from *Candida parapsilosis* (CPADH) was adopted to create a whole-cell system for catalyzing the reduction of prochiral carbonyl compounds to anti-Prelog configurated alcohols in high optical purity.[14,15] Additionally, the factors of the recombinant cell-mediated reaction system were optimized and the availability of the recombinant cells involving stereospecific alcohol dehydrogenase was investigated towards kinds of aryl ketones.

EXPERIMENTAL

Materials

2-Hydroxyacetophenone (1a) and its derivatives including *o*-chloro-2-hydroxyacetophenone (2a), *m*-chloro-2-hydroxyacetophenone (3a), *p*-chloro-2-hydroxyacetophenone (4a), *p*-methoxy-2-hydroxyacetophenone (5a), *p*-nitro-2-hydroxyacetophenone (6a), and acetophenone (7a), and the corresponding chiral alcohols (1b-7b) were purchased from ACROS Co. Ltd. or Sigma-Aldrich Chemical Co. Coenzymes including NAD(P)H and $NAD(P)^+$ were purchased from Sigma-Aldrich Chemical Co. All other chemicals used in this work were of analytical grade and commercially available.

Preparation Of Recombinant Cells For Synthesis

Recombinant *E. coli* BL21(DE3)(pETCPADH) expressing CPADH was constructed and cultivated as described previously.[15] After cultivation, the cells harvested by centrifugation at 10000 rpm for 30 min and washed twice with physiological saline solution were used as the wet cells. The lyophilized cells of recombinant *E. coli* were prepared by Freeze Dry System/Freezone 4.5 (Labconco Co.) and stored at 4 ºC for further use.

Synthesis Of Chiral Aryl Alcohols By Recombinant *E. Coli*

The reaction mixture in 2 mL was comprised of 0.1 M potassium phosphate buffer (pH 7.0), 2 mg substrate (1a-7a), and 0.2 g wet cells (equivalent to 0.03 g dry cell weight) or 0.05 g lyophilized cells of the recombinant *E. coli* transformant. For the substrates not completely soluble in potassium phosphate buffer, 100 μL of isopropanol was applied as co-solvent. To investigate the effects of auxiliary substrates, 10 mg of xylose, arabinose, glucose, fructose, lactose, sucrose, maltose, or 10 μL of isopropanol was added to the reaction mixture respectively. The reactions were carried out at 30 °C for 48 h with shaking at 200 rpm. The reaction mixture was extracted with 3 volumes of ethyl acetate and the organic layer was subjected to chiral HPLC analysis.

Analysis Of Chiral Aryl Alcohols Products

The reaction products were analyzed by chiral HPLC (HP 1100, Agilent, USA) equipped with Chiralcel OB-H column or OD-H column (4.6 mm × 250 mm; Daicel Chemical Ind. Ltd., Japan). Enantiomers were eluted with hexane and isopropanol at 0.5 mL/min. The effluent was monitored at 215 nm and the areas under each peak were integrated to calculate conversion, yield, and enantiomeric excess (e.e.) percentage values.[14,16] The detailed conditions for analysis of substrates and chiral alcohol products were shown in Table 1. All the values shown in this study were averaged from three replicates with standard deviations.

RESULTS AND DISCUSSION

Effects Of Reaction System Components And Conditions

The whole cells of recombinant *E. coli* harboring the overexpressed CPADH were applied for catalytic asymmetric reduction of 2-hydroxyacetophenone (1a). For biocatalytic systems involving stereospecific enzymes, reaction pH generally has an influence on the stereochemistry of enzymatic reactions.[17] Thus the effect of reaction pH on the reduction of 1a using recombinant *E. coli* cells was investigated with the pH values ranging from 4.0 to 9.0 (Figure 1). The stereospecificity of this CPADH-harboring whole-cell system was shown to be pH-dependent, which afforded higher optical purity (97%e.e.) and yield (83%) of (*S*)-1b at pH 7.0. However, the optimum pH of such whole-cell system catalyzing asymmetric reduction was different to that of isolated

CPADH,[15] which might be due to the difference of micro environmental pH between in vitro and in vivo in the reaction system with respect to the tuning of cell. Using whole cells as biocatalyst, the cell status including wet cells or lyophilized cells would have an influence on the biocatalytic performance and efficiency. It has been reported that lyophilization of cells might have an influence on the permeability of the cell wall or membrane and even the protein structure of intracellular enzymes.[18] Therefore, compared with wet cells, lyophilized cells were applied in the reaction system to investigate the effect of cell status on reaction efficiency by whole cells. Lyophilized cells of the recombinant *E. coli* only afforded the corresponding chiral alcohol product with optical purity of 72%e.e. and did not exhibit significant improvement of catalytic performance, compared with wet cells, which might be due to the change of steric structure and molecular rigidity of the stereospecific enzyme when processed by freeze drying.[19]

In asymmetric reduction of 1a, substrate inhibition occurred with the recombinant *E. coli* cell-mediated reaction. The apparent conversions and yields decreased at elevated substrate concentrations, while the space-time yield (expressed as mg of (*S*)-1b produced per liter of reaction solution per hour) increased, reaching a maximum at the substrate concentration of 3 g/L (Figure 2). This result indicated that the activity of the key enzyme in whole cells might be affected upon long-term exposure to reaction surroundings involving non-naturally organic compounds. However, the stereoselectivity of the

enzyme still remained at elevated substrate concentrations and optically active (*S*)-1b was obtained.

To improve the reaction efficiency at higher substrate concentrations, the amount of the biocatalyst was increased accordingly. Using 20% (w/v) recombinant *E. coli* cells, (*S*)-1b was obtained from 1a 3 g/L in a yield of 78.2%. Thus, applying more cells to the reaction would be helpful to facilitate the asymmetric reduction. Nevertheless, overloading of the cells in reaction would make the mixture slimy and breed negative influence to the system homogenization and substance transference in reaction. Besides, for cofactor-requiring oxidoreductions, the substrate inhibition might also result from insufficient cofactor regeneration.[20]

Co-Substrate-Coupled Cofactor Regeneration

From the cofactor requirement point of view, feasible cofactor regeneration would be necessary for improvement of the stereospecific oxidoreduction efficiency.[20] For whole-cell system using viable cells, cofactor-related cellular machinery and metabolic pathway in cell would be feasible for cofactor regeneration and thus co-substrate-coupled approach was further considered to take advantage of cellular machinery for cofactor regeneration. Compared with other sugars and alcohols commonly used for substrate-coupled cofactor regeneration, addition of xylose or arabinose significantly improved the reaction efficiency of asymmetric reduction of 1a to (*S*)-1b in an enhanced

yield over 85% (Figure 3). Then the effect of xylose concentration on the conversion by *E. coli* cells was further investigated. Addition of xylose more than 5 g/L would inhibit the reaction to some extent, which might result from substrate inhibition of xylose to the cellular metabolism. When 5 g/L xylose was used, (*S*)-1b was afforded with the optical purity of 97%e.e. in an increased yield of 89% with a higher substrate concentration of 5 g/L by 10% (w/v) recombinant *E. coli* wet cells. Concerning pentose metabolism in cells, the fact that xylose drove the equilibrium of NADPH-requiring reduction towards the desired product indicated that in vivo xylose metabolism would take place in the pentose phosphate pathway, and simultaneously NADPH, which facilitates the asymmetric reduction to produce (*S*)-1b, could be regenerated via pentose metabolism route and used for CPADH catalyzing asymmetric reduction of 1a.[21]

Asymmetric Synthesis Of Chiral Aryl Alcohols

For enzymatic reactions, the structural variety of substrates generally have a significant influence on the activity and stereoselectivity of enzymes by reason that the functional groups in substrate affect the substrate bonding and orientation in enzyme and the steric interactions between substrate and the active sites of enzyme.[22] Therefore, the feasibility of recombinant *E. coli* bearing CPADH was investigated concerning its substrate spectrum towards aryl ketones with different chemical structures (Table 2). For substituted 2-hydroxyacetophenones bearing chloro at various position of the phenyl ring (2a-4a), the *ortho*-chloro (2a) substitution was a somewhat poor substrate for the

recombinant *E. coli* cells, indicating that the substituent at ortho position might have a steric effect on the hydrogen attack from electron donator NADPH to the carbonyl group and significant influence on the reactivity of the functional enzyme. Another trend observed for substrates with a single substitution at para position on the phenyl ring was that electron-withdrawing groups provided for better efficiency than electron-donating groups. *p*-Chloro-substituted 4a and *p*-nitro-substituted 6a were more suitable for CPADH catalyzing asymmetric reduction than *p*-methoxy-substituted 2-hydroxyacetophenone (5a). These results indicated that the enzyme selectivity markedly depends on the nature, i.e. the electronic property and the position of the substituent group.[9] With anti-Prelog selectivity, CPADH expressed in recombinant *E. coli* catalyzed asymmetric reduction of various 2-hydroxy aryl ketones in anti-Prelog type, affording optical active aryl diols in (*S*)-configuration, whereas (*R*)-antipode for acetophenone (7a) due to the switch in CIP priority.

CONCLUSION

In this study, the recently cloned and overexpressed CPADH in *E. coli* was employed for the highly enantioselective reduction of a series of substituted 2-hydroxy aryl ketone derivatives to anti-Prelog configurated chiral alcohols. In addition, the enzyme was suggested to distinguish subtle steric and electronic differences in aryl ketone substrates, performing different enantioselectivities and catalytic activities to aryl ketones with various substituents. Besides the influences of reaction conditions, otherwise, available

pentoses were discovered as potent auxiliary substrates to improve the bioreduction efficiency. From these consequences, this whole-cell system involving recombinant stereospecific alcohol dehydrogenase was proposed as an efficient biocatalyst for enantioselective reduction and the symmetry breaking potential makes it attractive tool for synthesis of optically active aryl alcohols.

FUNDING

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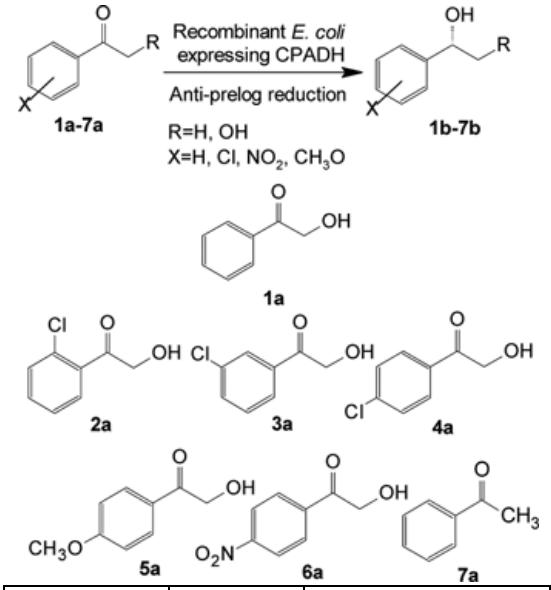
Table 1. Analysis of substrates and chiral alcohol products by HPLC

Condition	Retention time (min)
OB-H column, hexane/isopropanol (90:10, v/v)	15.0 (R-**1b**), 18.3 (S-**1b**), 27.1 (**1a**)
OD-H column, hexane/isopropanol (90:10, v/v)	12.8 (R-**2b**), 13.7 (S-**2b**), 22.5 (**2a**)
OD-H column, hexane/isopropanol (90:10, v/v)	14.1 (R-**3b**), 15.5 (S-**3b**), 24.1 (**3a**)
OD-H column, hexane/isopropanol (90:10, v/v)	14.3 (R-**4b**), 18.7 (S-**4b**), 22.0 (**4a**)
OD-H column, hexane/isopropanol (90:10, v/v)	12.5 (R-**5b**), 13.7 (S-**5b**), 23.7 (**5a**)
OD-H column, hexane/isopropanol (85:15, v/v)	14.2 (R-**6b**), 18.5 (S-**6b**), 23.3 (**6a**)
OB-H column, hexane/isopropanol (90:10, v/v)	16.4 (S-**7b**), 18.0 (R-**7b**), 19.4 (**7a**)

Table 2. Recombinant *E. coli*-Catalyzed Anti-Prelog Synthesis of Optically Active Aryl Alcohols



Substrate	Product	Conversion (%)	Optical purity (%e.e.)
1a	(*S*)-**1b**	83±2	97±1
2a	(*S*)-**2b**	58±3	81±2
3a	(*S*)-**3b**	61±1	95±3
4a	(*S*)-**4b**	81±2	96±2
5a	(*S*)-**5b**	69±1	91±3
6a	(*S*)-**6b**	78±3	95±4
7a	(*R*)-**7b**	82±2	97±2

Note. Reactions were carried out at 30 °C for 48 h in 2 mL 0.1 M potassium phosphate buffer (pH 7.0) comprising 2 mg substrate (1a-7a) and 0.2 g wet cells of the recombinant *E. coli* transformant. For the substrate of 7a, switch of stereo-configuration of the corresponding product was defined according to the CIP priority.

Figure 1. Effects of reaction pH on product optical purity (◆) and yield (□) of 1a reduction by recombinant *E. coli*. The reactions were carried out using recombinant *E. coli* wet cells (10%, w/v) with 1a 1 g/L in 2 mL 0.1 M acetic acid buffer (pH 4.0-6.0), potassium phosphate buffer (pH 6.0-8.0), or Tris-HCl buffer (pH 8.0-9.0) at 30 °C for 48 h.

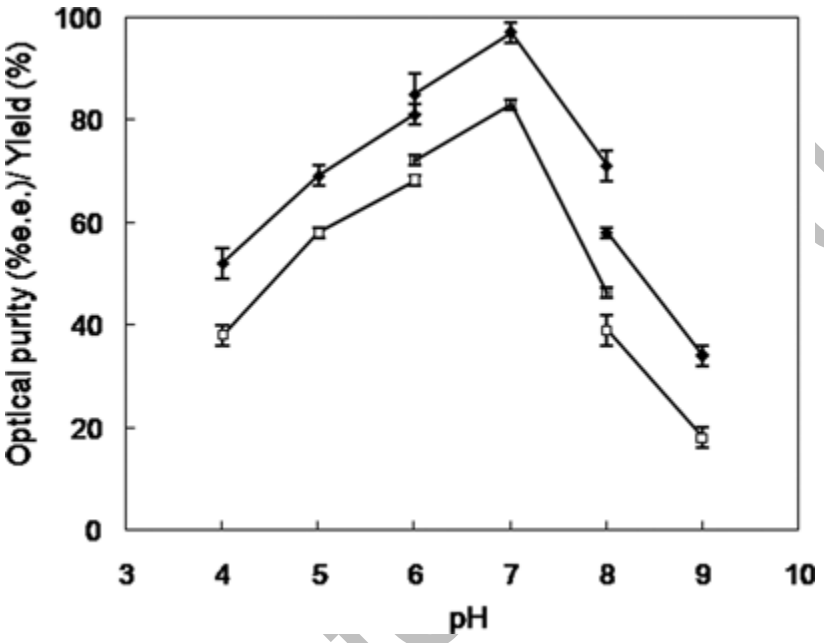


Figure 2. Effects of substrate concentration (1a) on conversion (◆), yield (▲), and space-time yield (■). The space-time yield was expressed as the amount of (*S*)-1b (mg) produced per liter of reaction solution per hour by recombinant *E. coli* wet cells (10%, w/v) in 2 mL 0.1 M potassium phosphate buffer (pH7.0) at 30 °C for 48 h.

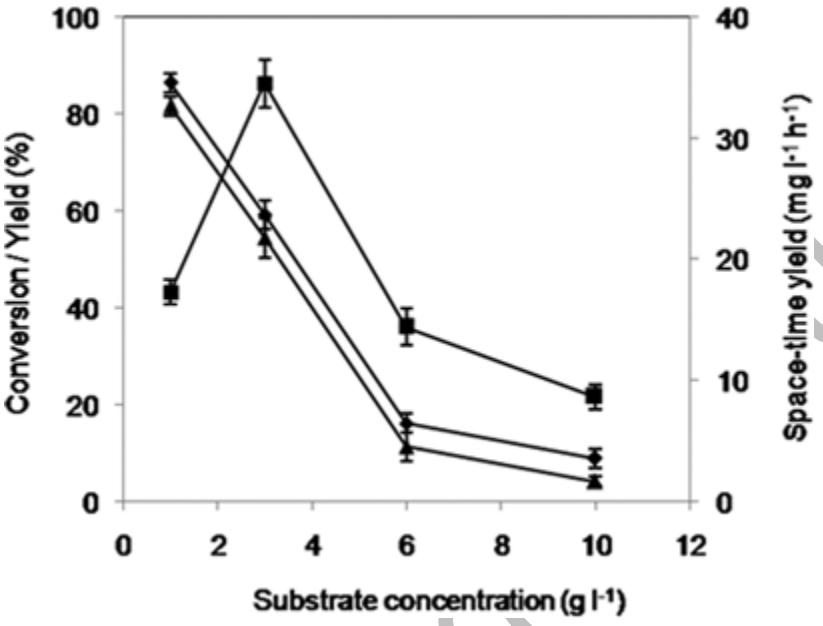


Figure 3. Effects of different sugars and alcohols as auxiliary substrates on product optical purity (□) and yield (■) of 1a reduction by recombinant *E. coli*. The reactions were carried out using recombinant *E. coli* wet cells (10%, w/v) with 1a 3 g/L in 2 mL 0.1 M potassium phosphate buffer (pH7.0) at 30 °C for 48 h.