Organic Process Research & Development **2004**, *8*, 246–251

Highly Enantioselective Conversion of Racemic 1-Phenyl-1,2-ethanediol by Stereoinversion Involving a Novel Cofactor-Dependent Oxidoreduction System of *Candida parapsilosis* CCTCC M203011

Yao Nie, Yan Xu,* and Xiao Qing Mu

Key Laboratory of Industrial Biotechnology of Ministry of Education and School of Biotechnology, Southern Yangtze University, Wuxi 214036, P. R. China

Abstract:

An economical and convenient biocatalytic process was developed for the preparation of (*S*)-1-phenyl-1,2-ethanediol (PED), which is a valuable chiral building block for pharmaceuticals and liquid crystals, by stereoselective microbial conversion from the corresponding racemate. As a result of screening bacteria, yeasts, and molds, the enantioselective conversion of racemic PED by *Candida parapsilosis* CCTCC M203011 was found to be the most efficient process to produce (*S*)-PED with high optical purity of 98% ee and yield of 92%. By detecting the intermediate produced in the reaction by GC−MS, it was suggested that (*S*)-enantiomer was produced from the intermediate identified as *β*-hydroxyacetophenone by asymmetric reduction after stereoselective oxidation of (*R*)-enantiomer to *β*-hydroxyacetophenone. After investigating the cofactor requirement and stereospecificity of the reaction catalyzed by the cell-free extract from *C. parapsilosis* CCTCC M203011, it was found that the stereoselective conversion involved the oxidation of (*R*)-PED to the intermediate with $NADP^+$ as the cofactor and the reduction reaction that formed the product used NADH as the cofactor, which was catalyzed by a novel cofactor-dependent oxidoreduction system. The $NADP^+$-dependent (*R*)-specific alcohol dehydrogenase involved in stereoinversion was purified from *C. parapsilosis* CCTCC M203011, which has a relative molecular mass of 45kD.

Introduction

Optically active 1-phenyl-1,2-ethanediol (PED) is a valuable and versatile chiral building block for the synthesis of pharmaceuticals, agrochemicals, pheromones, and liquid crystals, etc. Of the racemate, (*S*)-enantiomer can further be used as precursor for the production of chiral biphosphines and chiral initiator for stereoselective polymerization.[1]

Several bio-methods for preparation of optically active PED and other 1,2-diols have been developed, including of stereospecific dihydroxylation of styrene catalyzed by naphthalene dioxygenase (NDO),[2] optical resolution of racemic PED by lipase-catalyzed transesterification,[3] enantioselective oxidation catalyzed by glycerol dehydrogenase (GDH),[4] and microbial stereoinversion.[5] Among various methods of producing optically active PED, microbial stereoinversion with a whole cell system can be used to avoid coenzyme addition or regeneration system and obtain products with high optical purity and yield.[6−10] Various microorganisms, including *Candida parapsilosis*, *C. maltosa*, *C. boidinii*, *Loddero-myces elongisporus*, *Pichia bovis*, *Nocardia fusca*, *Arthrobacter viscosus*, *Lactobacillus kefir*, etc., have been found to possess the same ability of preparing optically active alcohols from the racemates.[11−14] However, the stereoselective reactions catalyzed by alcohol dehydrogenases from these strains were different not only in the efficiency of enantioselective conversion, such as ee value and yield of products, but also in the coenzyme requirement and the reaction mechanism. Among these microorganisms, *C. parapsilosis* IFO0708 has been reported to prepare optically active 1,2-diols from the corresponding racemates through stereoinversion.[5] In this procedure, (*S*)-PED with the optical purity of 100% ee and the yield of 100% was produced from the corresponding racemate with the substrate concentration of 5 g/L, and the stereoinversion involved the oxidation of (*R*)-diol to 2-keto-1-alcohol by an NAD^+-linked (*R*)-specific alcohol dehydrogenase and the reduction of 2-keto-1-alcohol to (*S*)-diol by an NADPH-linked (*S*)-specific reductase. Although (*S*)-PED with high optical purity and yield was prepared by this means, it has not been considered to be suitable for large-scale production and industrial application due to the low concentration of substrate in the reaction. A highly enantioselective and productive conversion system for racemic PED is essential to prepare optically pure enantiomer for potential large-scale application.

Using whole cells for preparative conversions, the coenzyme requirement of the enzyme involved in the reaction

* Author for correspondence.Telephone: +86-510-5864735. Fax: +86-510-5864112. E-mail: yxu@sytu.edu.cn (Y. Xu).

(1) Iwasaki, F.; Maki, T.; Nakashima, W.; Onomura, O.; Matsumura, Y. *Org. Lett.* **1999**, *1*, 969.
(2) Lee, K.; Gibson, D. T. *J. Bacteriol.* **1996**, *178*, 3353.
(3) Bosetti, A.; Bianchi, D.; Cesti, P.; Golini, P.; Spezia, S. *J. Chem. Soc., Perkin Trans.* **1992**, 2395.
(4) Liese, A.; Karutz, M.; Kamphuis, J.; Wandrey, C.; Kragl, U. *Biotechnol. Bioeng.* **1996**, *51*, 544.
(5) Hasegawa, J.; Ogura, M.; Tsuda, S.; Maemoto, S.; Kutsuki, H.; Ohashi, T. *Agric. Biol. Chem.* **1990**, *54*, 1819.
(6) Schmid, A.; Dordick, J. S.; Hauer, B.; Kiener, A.; Wubbolts, M.; Witholt, B. *Nature* **2001**, *409*, 258.
(7) Carnell, A. J. *Adv. Biochem. Eng. Biotechnol.* **1999**, *63*, 57.
(8) Matsuyama, A.; Yamamoto, H.; Kawada, N.; Kobayashi, Y. *J. Mol. Catal. B: Enzym.* **2001**, *11*, 513.
(9) Panke, S.; Wubbolts, M. G. *Curr. Opin. Biotechnol.* **2002**, *13*, 111.
(10) Chadha, A.; Baskar, B. *Tetrahedron: Asymmetry* **2002**, *13*, 1461.
(11) Nakamura, K. *J. Mol. Catal. B: Enzym.* **1998**, *5*, 129.
(12) Ogawa, J.; Xie, S. X.; Shimizu, S. *Biotechnol. Lett.* **1999**, *21*, 331.
(13) Azerad, R.; Buisson, D. *Curr. Opin. Biotechnol.* **2000**, *11*, 565.
(14) Wendhausen, R., Jr.; Moran, P. J. S.; Joekes, I.; Rodrigues, J. A. R. *J. Mol. Catal. B: Enzym.* **1998**, *5*, 69.

Published on Web 02/18/2004
10.1021/op0341519 CCC: $27.50 © 2004 American Chemical Society

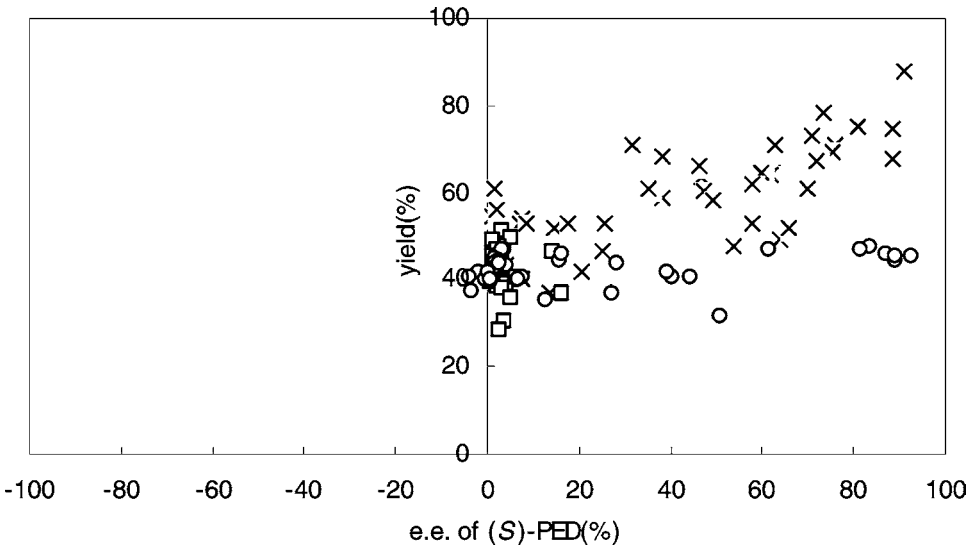


Figure 1. **Screening of microorganisms producing optically active PED from the racemate. (×) yeasts; (□) molds; (○) bacteria.**

and the regeneration of the coenzyme are important for the conversions by cofactor-dependent enzymes. Various enantioselective oxidoreductases, such as alcohol dehydrogenase, aryl-alcohol dehydrogenase, carbonyl reductase, phenylacetaldehyde reductase, etc., have been found to require cofactors specially to catalyze the asymmetric reaction.[15−18] Most of these oxidoreductases are highly selective for their cofactor, the specific nature of the enzyme and its cofactor determines its stereoselectivity, and the coenzyme specificity could even be different from the similar reactions catalyzed by the enzymes from the strains of the same species. Therefore, the discovery of enzymes of novel cofactor requirement can provide valuable information on the diversity of microbial enzymes, especially related enzymes from microorganisms of the same species. Furthermore, the information on novel cofactor dependence of enzymes can be useful for the alteration of the coenzyme requirement by the approach of site-directed mutagenesis to change or extend the coenzyme specificity of enzymes and make the enzymes more available.[19,20]

The main objectives of this study were to develop an efficient procedure to obtain optically active PED from the corresponding racemate with high optical purity and yield, to analyze the reaction mechanism, and to investigate the cofactor requirement and stereospecificity of the suitable catalyst in the asymmetric reaction.

Results and Discussion

Screening of Microorganisms Producing (*S*)-PED from the Racemate. More than 100 strains, including bacteria, yeasts, and molds, were tested for screening suitable strains in preparation of optically active PED from the racemate (Figure 1).

Among microorganisms found to have the ability of converting racemic PED to optically pure product, some strains, such as *C. parapsilosis*, *C. boidinii*, *Saccharomyces cerevisiae*, *Brevibacterium protophormiae*, *B. flavum*, *Bacillus polymyxa* and *B. sphaericus*, showed better conversion yield. Among them, only the (*S*)-isomer was obtained, and no microorganisms were found to produce (*R*)-PED from the racemate by stereoselective conversion. Furthermore, most of the molds did not show the capability of producing optically pure PED, and several bacteria produced (*S*)-PED with high optical purity but in the yields of below 50%, and only a couple of yeasts converted the racemate to (*S*)-isomer with high optical purity and yield of 98% ee and 92%, respectively. These results suggested that the production of (*S*)-PED from the racemate was through stereoselective degradation by some bacteria, and some yeasts were able to convert (*R*)-PED to the (*S*)-enantiomer through stereoinversion.

From the results of screening, the species of *C. parapsilosis* generally showed notable ability of producing (*S*)-PED from the racemate. The strains of *C. parapsilosis* from different sources were then compared in catalyzing stereoselective conversion in detail (Table 1). Although most of *C. parapsilosis* was found to have the capability to convert (*RS*)-PED to the (*S*)-isomer, the efficiency of stereoinversion by the strains from various sources were different with the span of ee values from 1 to 98%. These facts indicate that the species of *C. parapsilosis* from various sources could possess different physiological character and have distinctions on the gene encoding the oxidoreductases,[21] so that the efficiency of stereoselective conversion presented different levels by *C. parapsilosis* from various sources. Among the strains screened, *C. parapsilosis* CCTCC M203011 was the best one selected for further research as an efficient catalyst, by which (*S*)-PED with high optical purity of 98% ee was

(15) Xie, S. X.; Ogawa, J.; Shimizu, S. *Biosci. Biotechnol. Biochem.* **1999**, *63*, 1721.
(16) Zheng, C. S.; Phillips, R. S. *J. Chem. Soc., Perkin Trans.* **1992**, 1083.
(17) Bastos, F. M.; Franca, T. K.; Machado, G. D. C.; Pinto, G. F.; Oestreicher, E. G.; Paiva, L. M. C. *J. Mol. Catal. B: Enzym.* **2002**, *19*, 459.
(18) Hidalgo, A. G. D.; Akond, M. A.; Kita, K.; Kataoka, M.; Shimizu, S. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 2785.
(19) Corbier, C.; Clermont, S.; Billard, C. P.; Skarzynski, T.; Branlant, C.; Wonacott, A.; Branlant, G. *Biochemistry* **1990**, *29*, 7101.
(20) Seelbach, K.; Riebel, B.; Hummel, W.; Kula, M.; Tishkov, V. I.; Egorov, A. M.; Wandrey, C.; Kragl, U. *Tetrahedron Lett.* **1996**, *37*, 1377.
(21) Yamamoto, H.; Kawada, N.; Matsuyama, A.; Kobayashi, Y. *Biosci. Biotechnol. Biochem.* **1999**, *63*, 1051.

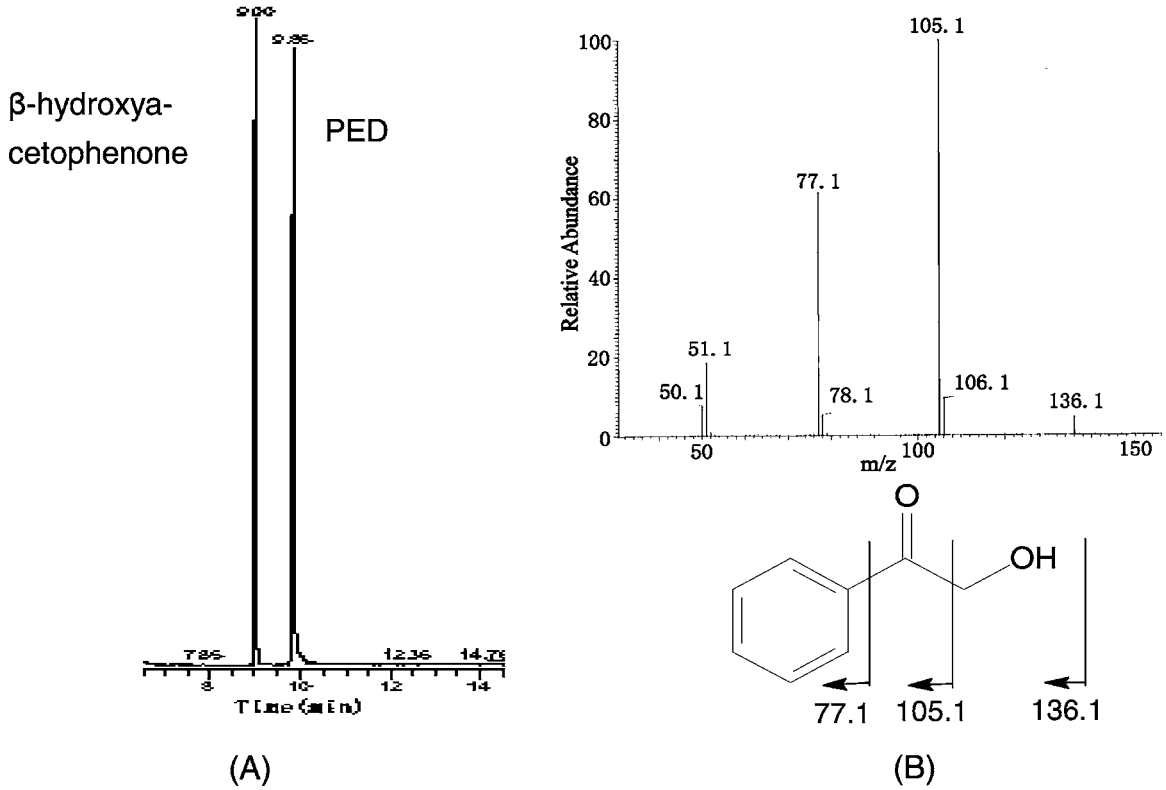


Figure 2. **Analysis of reaction intermediate by GC−MS. (A) Analysis of the intermediate by GC. (B) Mass spectrum of the intermediate and the structure of *β*-hydroxyacetophenone.**

Table 1. **Stereoselective conversion of racemic PED catalyzed by *C. parapsilosis* from different sources**

microorganisms	residual PED (%)(*A*)	ee of (*S*)-PED (%)(*B*)	(*S*)-isomer yield (%)(*C*)[a]
Candida parapsilosis ATCC 10232	80	62	65
C. parapsilosis ATCC 22019	79	62	64
C. parapsilosis ATCC 7330	81	76	71
C. parapsilosis CECT 10211	82	1	41
C. parapsilosis CECT 10304	79	75	69
C. parapsilosis CECT 10434	83	81	75
C. parapsilosis CECT 10437	79	88	74
C. parapsilosis AS 2.491	81	60	65
C. parapsilosis AS 2.590	78	72	67
C. parapsilosis AS 2.1497	82	47	61
C. parapsilosis CICC 1627	90	73	78
C. *parapsilosis* CCTCC M203011	93	98	92
C. parapsilosis IFO 0708	96	8	52

[a] Note: (*S*)-PED yield (*C*) = net yield of (*S*)-PED from (*RS*)-PED = $A \times [1 - (100 - B)/200]$.

obtained in high yield of 92%. In our research, highly enantioselective conversion of racemic PED was carried out with a higher substrate concentration of 0.8% (w/v) and a lower cell concentration of 5% (w/v). Although excessive substrate concentration might inhibit the reaction and decrease the reaction efficiency, from the results of this work, it still has the potentiality of optimizing the reaction conditions to increase the substrate concentration to a high level and enlarging the reaction to large-scale level for industrial application.

Identification of the Intermediate in the Asymmetric Reaction. While analyzing the reaction mixture with GC−MS, it was found that in addition to PED (retention time of 9.85 min), a possible intermediate which appeared at the retention time of 9.00 min on GC was formed during the reaction (Figure 2A). The mass spectrum of this material exhibited a molecular ion (M^+) at *m*/*e* 136.1, and its fragment pattern well explained the structure of *β*-hydroxyacetophenone (Figure 2B).

Reaction Route of the Stereoselective Conversion. During the asymmetric conversion catalyzed by *C. parapsilosis* CCTCC M203011, the amount of (*R*)-PED decreased with time, while the amount of the opposite stereoisomer increased in the same course, and the optical purity of (*S*)-enantiomer reached the highest point after incubation for 48 h (Figure 3).

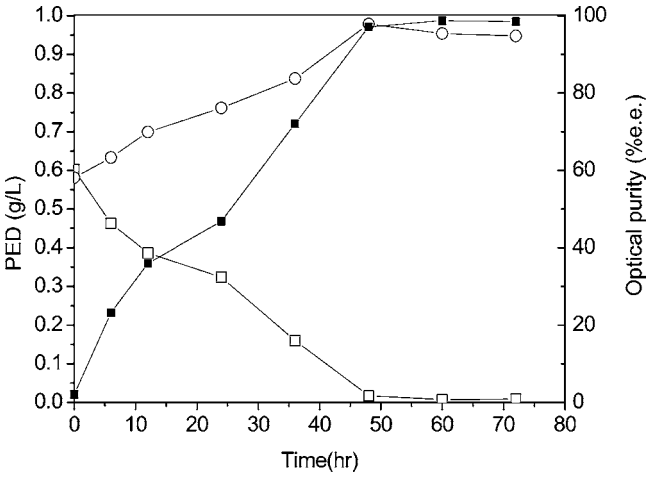


Figure 3. **Asymmetric reaction process catalyzed by *C. parapsilosis* CCTCC M203011. (□) (*R*)-PED; (○) (*S*)-PED; (■) optical purity.**

Scheme 1. **Schematic representation of stereoinversion of (*RS*)-PED to (*S*)-PED catalyzed by *C. parapsilosis* CCTCC M203011**

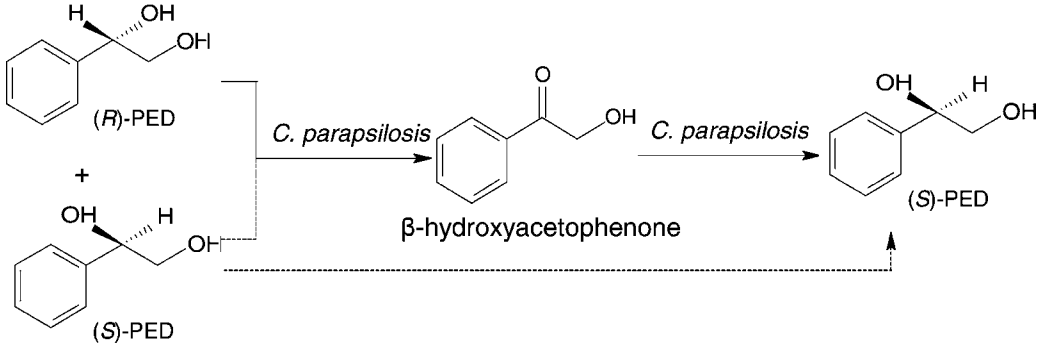


Table 2. **Cofactor requirement and stereospecificity in the oxidation of PED catalyzed by cell-free extract and purified enzyme from *C. parapsilosis* CCTCC M203011, respectively**

		activity (units/mg protein)	
catalyst	cofactor	(*R*)-PED	(*S*)-PED
cell-free extract	NAD^+	0.016	0.005
	$NADP^+$	0.106	0.065
		(*RS*)-PED	
purified enzyme	NAD^+	ND[a]	
	$NADP^+$	2.35	

[a] ND: not detected.

Table 3. **Cofactor requirement in the reduction catalyzed by cell-free extract from *C. parapsilosis* CCTCC M203011**

	activity (units/mg protein)			
cofactor	acetone	2-hexanone	acetophenone	β-hydroxy-acetophenone
NADH	0.212	0.089	0.117	0.039
NADPH	0.012	0.015	0.011	0.009

These results indicated that (*S*)-PED was produced from the intermediate identified as β-hydroxyacetophenone by asymmetric reduction after the oxidation of (*RS*)-PED to β-hydroxyacetophenone (Scheme 1).In addition to the (*S*)-isomer that remained in the substrate of the racemate and that converted from the (*S*)-isomer, the product of (*S*)-PED also included the part converted from the (*R*)-isomer. Therefore, the yield of (*S*)-PED from the racemate was over 50% by *C. parapsilosis* CCTCC M203011. This reaction route of stereoselective conversion from (*RS*)-PED to the (*S*)-enantiomer catalyzed by *C. parapsilosis* CCTCC M203011 coincided with that of asymmetric oxidoreduction catalyzed by *C. parapsilosis* IFO 0708,[5] suggesting that the same species as *C. parapsilosis* showed some similarities in catalyzing asymmetric reactions of the racemates of chiral alcohols to the optical pure enantiomers.

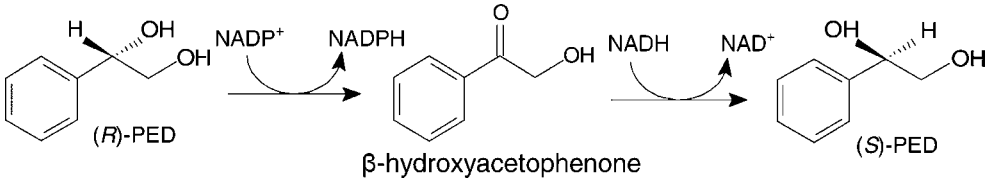
Cofactor Requirement and Stereospecificity. In the reaction of oxidoreduction, oxidoreductases such as alcohol dehydrogenase or carbonyl reductase require $NAD(P)^+$ or NAD(P)H as cofactors to catalyze the oxidoreduction. To obtain the information on the reaction mechanism, the cofactor requirement and stereospecificity in stereoselective oxidation catalyzed by the cell-free extract and the purified enzyme from *C. parapsilosis* CCTCC M203011 were investigated (Table 2). In the presence of $NADP^+$ but not NAD^+, the cell-free extract catalyzed the oxidation, suggesting that the enzyme is an $NADP^+$-dependent dehydrogenase, and in the oxidation of PED, (*R*)-PED was oxidized much faster than (*S*)-PED, which indicated that the cell-free extract contained an (*R*)-specific alcohol dehydrogenase. To confirm this $NADP^+$-dependent dehydrogenase and clarify the mechanism of the asymmetric reaction catalyzed by *C. parapsilosis* CCTCC M203011, the enzyme catalyzing the oxidation of PED was purified from the cell-free extract. The relative molecular mass of the native enzyme was found to be 45 kD by HPLC on a Protein KW-803 Shodex gel filtration column. On the cofactor requirement of this purified alcohol dehydrogenase in catalyzing PED oxidation, the alcohol dehydrogenase catalyzed the oxidation of PED with $NADP^+$ but not NAD^+ as the cofactor, indicating that the enzyme is an $NADP^+$-dependent alcohol dehydrogenase, which coincided with the observation on the cofactor requirement in the oxidation catalyzed by the cell-free extract from *C. parapsilosis* CCTCC M203011. For its dissimilarity with other alcohol dehydrogenases from *C. parapsilosis* reported in cofactor requirement,[5,22] this $NADP^+$-dependent alcohol dehydrogenase was proposed to be an alcohol dehydrogenase with novel cofactor dependence. The result that alcohol dehydrogenases from same species of *C. parapsilosis* showed different cofactor requirements in catalyzing oxidation could be explained by saying that these strains of the same species were different on the gene that encodes dehydrogenases, so that the three-dimensional structures and the active-site binding cofactors of the enzymes presented dissimilarity.

The cofactor requirements in the reduction of various ketones catalyzed by the cell-free extract from *C. parapsilosis* CCTCC M203011 were also investigated. As shown in Table 3, the activities of the cell-free extract with NADH as cofactor were much higher than those with NADPH as the coenzyme, suggesting that an NADH-dependent reductase catalyzing asymmetric reduction was contained in the cell-free extract from *C. parapsilosis* CCTCC M203011.

From these observations, the reaction mechanism of stereoinversion of (*RS*)-PED to (*S*)-PED by *C. parapsilosis* CCTCC M203011 can be explained as follows. (*R*)-PED in the racemate is oxidized to the intermediate of β-hydroxy-

(22) Yamamoto, H.; Matsuyama, A.; Kobayashi, Y.; Kawada, N. *Biosci. Biotechnol. Biochem.* **1995**, *59*, 1769.

Scheme 2. **Stereoinversion of (*R*)-PED to (*S*)-PED catalyzed by *C. parapsilosis* CCTCC M203011**



acetophenone by an $NADP^+$-dependent (*R*)-specific dehydrogenase, and then β-hydroxyacetophenone is reduced to (*S*)-PED by an NADH-dependent (*S*)-specific reductase (Scheme 2). This mechanism was found to be different from a previous report, in which the oxidation of 1,2-diols catalyzed by the cell-free extract of *C. parapsilosis* required NAD^+ as cofactor and the reduction catalyzed by the cell-free extract required NADPH as cofactor.[5] For the cell-free extract from *C. parapsilosis* CCTCC M203011 showing some activity to (*S*)-isomer in a low level, this alcohol dehydrogenase purified from the cell-free extract could also catalyze the oxidation of a small quantity of (*S*)-PED to the intermediate in this asymmetric reaction. It appears that the (*S*)-specific reductase might play a more important role in catalyzing this enantioselective oxidoreduction to achieve such high optical purity of the product.

In this study, *C. parapsilosis* CCTCC M203011 presented some distinctions with other strains of the same species in catalyzing asymmetric conversion, especially on the efficiency of catalyzing stereoselective conversion and the mechanism of asymmetric reaction. In addition, the enzyme catalyzing oxidation was purified and was found to be an alcohol dehydrogenase with novel cofactor specificity. To take a good advantage of the enzyme and make the mechanism more clear, further research on characterization of the purified enzyme and purification of the other enzyme catalyzing reduction is now under investigation.

Experimental Section

Microorganisms and Chemicals. All strains were obtained from American Type Culture Collection (ATCC), China Center for Type Culture Collection (CCTCC), China Center of Industrial Culture Collection (CICC), Spanish Type Culture Collection (CECT), Chinese Academy of Science (AS), and Institute for Fermentation of Osaka (IFO). (*R*)-, (*S*)-, and (*R*,*S*)-PED as standard samples were purchased from the Sigma-Aldrich Chemical Co. All other chemicals used in this work were of analytical grade and commercially available.

Medium and Cultivation. Medium for yeasts contained 4% (w/v) glucose, 0.3% (w/v) yeast extract, and 10% (v/v) mineral solution (pH 7.0). Medium for molds contained 2% (w/v) glucose, 0.3% (w/v) yeast extract, 0.5% (w/v) meat extract, 0.5% (w/v) peptone, and 5% (v/v) mineral solution (pH 7.0). Medium for bacteria contained 0.5% (w/v) glucose, 0.2% (w/v) yeast extract, 1% (w/v) meat extract, 1% (w/v) peptone, and 5% (v/v) mineral solution (pH 7.0). The mineral solution consisted of 13% $(NH_4)_2HPO_4$, 7% KH_2PO_4, 0.8% $MgSO_4 \cdot 7H_2O$, and 0.1% NaCl. Microorganisms were cultured in 50-mL shaking flasks containing 5 mL of medium at the temperature of 30 °C. After incubation for 48 h, the cells were harvested by centrifugation at 4000 rpm for 20 min, washed twice with physiological saline, and then used for reaction.

Asymmetric Reaction Conditions. The reaction mixture in 2 mL comprised 0.1 M potassium phosphate buffer (pH 6.5), 5% (w/v) wet cells, and 0.8% (w/v) (*RS*)-PED. The reactions were carried out at 30 °C for 48 h with shaking. After reaction, the cells were removed by centrifugation at 4000 rpm for 20 min, and then PED was extracted with three volumes of ethyl acetate by vigorous mixing. Finally, the organic layer was analyzed by HPLC.

Analytical Methods. The optical purity of PED was determined by HPLC using a Chiralcel OB-H column (4.6 mm × 25 cm; Daicel Chemical Ind., Ltd., Japan). Enantiomers were eluted with hexane and 2-propanol (9:1) at a flow rate of 0.5 mL/min. The effluent was monitored at 215 nm, and the areas under each peak were integrated. The enantiomeric excess (ee) was calculated from the peak areas of the stereoisomers. The intermediate was identified by gas chromatography−mass spectrometry (GC−MS) with a Finnigan Trace-MS mass spectrometer (Finnigan, U.S.A.) using a column of OV1701 (30 m × 0.25 mm × 0.25 μm). The initial temperature was 60 °C, followed by an increase of temperature at a rate of 10 °C/min. The final temperature was held at 240 °C for 15 min. The carrier gas was helium (0.8 mL/min).

Enzyme Assay. The assay mixture in 220 μl for oxidation comprised 200 mM potassium phosphate buffer (pH 7.5), 3 mM $NAD(P)^+$, 0.1 M (*RS*)-PED, and an appropriate amount of the enzyme. For reduction, $NAD(P)^+$ and (*RS*)-PED were replaced by NAD(P)H (1 mM) and ketones (0.1 M), respectively. The reaction mixture was incubated for 2 min without the enzyme at 30 °C, and then the reaction was started by the addition of the catalyst. The increase or decrease in the amount of the coenzyme was measured spectrophotometrically at 340 nm. One unit of enzyme activity was defined as the amount of enzyme catalyzing the reduction/oxidation of 1 μmol of $NAD(P)^+$/NAD(P)H per min under the assay conditions.

Enzyme Purification. All purification procedures were done at the temperature of 4 °C, and 20 mM potassium phosphate buffer (pH7.5) was used as the buffer unless otherwise specified.

Preparation of the Cell-Free Extract. The collected cells suspended in buffer were disrupted with a French pressure cells at 20000 psi. The cell debris was removed by centrifugation (10000 rpm × 60 min) at 4 °C, and the supernatant was used as the cell-free extract.

Ethanol Precipitation. The cell-free extract was added to 50% (v/v) ethanol and stirred at 4 °C for 10 min. The precipitate was collected by centrifugation and dissolved in

buffer. The undissolved part was removed by centrifugation, and the supernatant was used for the next step.

DEAE-Sephacel Chromatography. The supernatant was put on a HiPrep 16/10 DEAE-Sephacel column and eluted with a linear gradient of NaCl (0.0−0.5 M). The active fractions were combined and dialyzed against the buffer.

Superdex G-75 Chromatography. The dialyzed solution was put on a HiLoad 16/60 Superdex G-75 column, and the enzyme was eluted with the same buffer. Then the active fractions were collected.

Blue Sepharose Chromatography. The enzyme solution was put on a Blue Sepharose column and eluted with the buffer of $NADP^+$ (0.5 mM). The active fractions were collected and concentrated and then used for characterization.

Measurement of the Molecular Mass of the Enzyme. The relative molecular mass of the enzyme was measured by HPLC on a Protein KW-803 Shodex Gel Filtration column (8 mm × 300 mm) at a flow rate of 0.72 mL/min with 25mM phosphate buffer (pH 6.8).[15]

Acknowledgment

Financial support of Ministry of Education, P.R.China, under the Excellent Young Teachers Program (EYTP), and Department of Science and Technology of Jiangsu Province, P. R. China, under Hi-technology Program are gratefully acknowledged. We also thank the National Science Foundation of China (NSFC) (No. 20376031) and the National Key Basic Research and Development Program of China (973 Program) for their valuable support.

Received for review October 17, 2003.

OP0341519