**CHINESE JOURNAL OF CATALYSIS**
Volume 33, Issue 4, 2012
Online English edition of the Chinese language journal

Available online at www.sciencedirect.com
ScienceDirect

**Cite this article as:** Chin. J. Catal., 2012, 33: 681–687.

ARTICLE

# Efficient Synthesis of (*R*)-2-Chloro-1-(3-chlorophenyl)ethanol by Permeabilized Whole Cells of *Candida ontarioensis*

NI Ye*, ZHANG Beihua, SUN Zhihao

The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, Jiangsu, China

**Abstract:** (*R*)-2-Chloro-1-(3-chlorophenyl)ethanol is a key pharmaceutical intermediate in the synthesis of $\beta_3$-adrenoceptor receptor ($\beta_3$-AR) agonists. The asymmetric reduction of 2-chloro-1-(3-chlorophenyl)ethanone to (*R*)-2-chloro-1-(3-chlorophenyl)ethanol catalyzed by resting cells of *Candida ontarioensis* was studied. At a substrate concentration of 10 g/L, the microbial cells showed excellent catalytic activity under the optimum reaction conditions, giving the product in 99.9% ee and 99.0% yield. After cetyltrimethylammonium bromide-pretreatment, the activity of permeabilized *Candida ontarioensis* cells was increased by more than 2-fold and the product could be produced over the significantly shortened reaction period of 24 h in 99.9% ee and 97.5% yield at a substrate concentration of 30 g/L. This work provides a practical approach for the efficient preparation of the important chiral intermediate (*R*)-2-chloro-1-(3-chlorophenyl) ethanol.

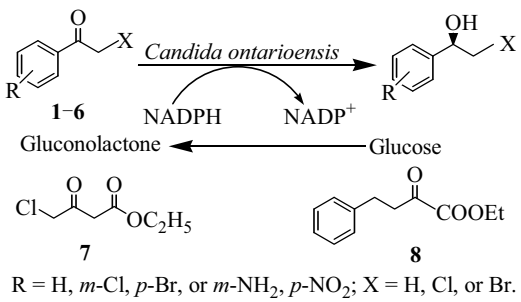
**Key words:** (*R*)-2-chloro-1-(3-chlorophenyl)ethanol; asymmetric reduction; cetyltrimethylammonium bromide; permeability; *Candida ontarioensis*

Optically pure chiral alcohols are valuable building blocks for the synthesis of pharmaceuticals, agrochemicals, and liquid crystals [1]. The α-halohydrins are particularly important as synthons for the preparation of β-adrenergic drugs [2]. Several chemical and biological approaches for the synthesis of optically active α-halohydrins have been developed [3,4] and the asymmetric reduction of the corresponding prochiral aromatic ketones is regarded as the most promising methodology [5]. Owing to its excellent stereoselectivity, mild reaction conditions, and environmental benignancy, biocatalysis has been reported for the production of a variety of optically pure pharmaceutical intermediates [6–8]. In biocatalytic reactions, whole-cell systems are often preferred to isolated enzymes as the tedious enzyme isolation/purification processes required are eliminated and reactions involving multiple enzymes/pathways as well as co-factor regeneration are allowed [9–11].

(*R*)-2-Chloro-1-(3-chlorophenyl)ethanol is a versatile intermediate for the synthesis of $\beta_3$-adrenoceptor receptor ($\beta_3$-AR) agonists, such as the anti-depressant drug SR58611 and the thermogenic anti-obesity drug BRL 37344 [12]. Several groups have reported the biocatalytic synthesis of (*R*)-2-chloro-1-(3-chlorophenyl)ethanol. Sawa et al. [13] reported the asymmetric synthesis of (*R*)-2-chloro-1-(3-chlorophenyl) ethanol using whole cells of *Rhodotorula glutinis* at a substrate concentration of 3.25 g/L. Lin and co-workers [14] reported the bioreduction of 2-chloro-1-(3-chlorophenyl) ethanone by *Saccharomyces cerevisiae*, providing the product after 48 h in 99% ee and 97% yield at a substrate concentration of 28 g/L. Recombinant *E. coli* cells and cell-extracts from *Nocardia globerula* and *Rhodotorula glutinis* have also been reported for the asymmetric production of (*R*)-2-chloro-1-(3-chlorophenyl) ethanol in over 99% ee. However, the inclusion of expensive additives such as $NADP^+/NAD^+$ and glucose dehydrogenase

Received 2 Novebmer 2011. Accepted 6 January 2012.
*Corresponding author. Tel/Fax: +86-510-85329265; E-mail: yni@jiangnan.edu.cn
This work was supported by the National Basic Research and Development Program of China (973 Program, 2011CB710800), New Century Excellent Talents in University (NCET-11-0658), Natural Science Foundation of Jiangsu Province (BK2011150), the Program of Introducing Talents of Discipline to Universities (111-2-06), and a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

DOI: 10.1016/S1872-2067(11)60363-X




**Scheme 1.** Bioreduction of compounds **1**–**8** with *Candida ontarioensis* whole cells.

was required for regeneration of the cofactor [15–18].

In this study, *Candida ontarioensis* was selected from a screening panel of 90 strains in our laboratory collection because of its high carbonyl reductase activity towards various halo-acetophenone substrates and 2-chloro-1-(3-chlorophenyl) ethanone was used as a model substrate to investigate the asymmetric reduction catalyzed by whole-cells of *Candida ontarioensis* (Scheme 1). Using cetyltrimethylammonium bromide (CTAB)-permeabilized cells, the reaction time was significantly reduced from 72 to 24 h and the product was obtained in 99.9% ee and 97.5% yield at a 30 g/L substrate concentration.

## 1 Experimental

### 1.1 Screening and identification of the target strain

Three strains were screened from the 90 strains in our laboratory using 10 g/L 2-chloro-1-(3-chlorophenyl)ethanone (Porton Co., Ltd, China) as the substrate and *Candida* sp. BC 2A showed the highest carbonyl reductase activity. The 18S rRNA gene sequence of *Candida* sp. BC 2A was determined (GenBank number: JN820129), identifying the strain as *Candida ontarioensis*.

### 1.2 Cultivation conditions of *Candida ontarioensis*

The fermentation medium was composed of 1% (w/v) glucose, 2% (w/v) corn steep liquor power, and 0.005% (w/v) $CaCl_2$ and was adjusted to pH 6.5. The microorganism was cultured at 30 °C for 20 h with 180 r/min shaking. The cells were collected by centrifugation at 2350 × *g* for 5 min and washed twice with 0.85% saline.

The cell pellet was treated with 4 g/L CTAB at 4 °C for 20 min to improve permeability. The CTAB-treated and untreated *Candida ontarioensis* cells were used as biocatalysts in the bioconversion of 2-chloro-1-(3-chlorophenyl)ethanone.

### 1.3 Asymmetric bioreduction of 2-chloro-1-(3-chlorophenyl)ethanone in aqueous medium

The reaction system consisted of 1 g of wet cells (collected from 30 ml of culture broth), glucose (5%, w/v), the appropriate amount of 2-chloro-1-(3-chlorophenyl)ethanone, and potassium phosphate buffer (0.2 mol/L, pH 6.5), in a final volume of 10 ml. The reaction was conducted in a 50-ml Erlenmeyer flask with stopper and shaken at 30 °C and 180 r/min for 72 h. The mixture was extracted with an equal volume of ethyl acetate and the organic layer was concentrated under vacuum and diluted with mobile phase for subsequent HPLC analysis.

### 1.4 Activity assay

The carbonyl reductase activity of *Candida ontarioensis* cells was measured according to the reaction conditions mentioned above by analyzing the reaction during the first 30 min to determine the initial velocity. One unit of the enzyme activity (U) is defined as the amount of enzyme required for generation of 1 μmol (*R*)-2-chloro-1-(3-chlorophenyl)ethanol per minute.

The enantiomeric excess (ee) and analytic yield of (*R*)-2-chloro-1-(3-chlorophenyl)ethanol were determined on an Agilent 1100 HPLC (California, USA) equipped with a 5 μm Chiralcel OB-H (0.46 mm × 250 mm, Tokyo, Japan) column using an UV detector at 220 nm. The HPLC was performed using *n*-hexane/isopropanol (9/1, HPLC grade, Xingke Chemical Reagent Co., Ltd, China) as the mobile phase at a flow rate of 1.0 ml/min and the column temperature was 30 °C.

## 2 Results and discussion

### 2.1 Cell growth and carbonyl reductase production by *Candida ontarioensis*

The time course of cell growth and carbonyl reductase production by *Candida ontarioensis* was investigated. As shown in Fig. 1, the cell growth continued throughout the entire fermentation process and the carbonyl reductase activity of whole-cells increased rapidly over the first 20 h. A maximum activity of 25.4 U/L (0.74 U/$g_{WCW}$) and a cell mass of 34.5 $g_{WCW}$/L (WCW = wet cell weight) was reached at the 20 h mark. A decrease in enzymatic activity was evident after 20 h because of the degradation of reductase by protease at the end of the fermentation process. Thus, *Candida ontarioensis* cells were collected after 20 h of the fermentation process for further bioreduction studies.

### 2.2 Optimization of bioreduction conditions

Major parameters in the asymmetric reduction of 2-chloro-1-(3-chlorophenyl)ethanone were investigated, including buffer pH, temperature, cell concentration, and cosubstrate (for

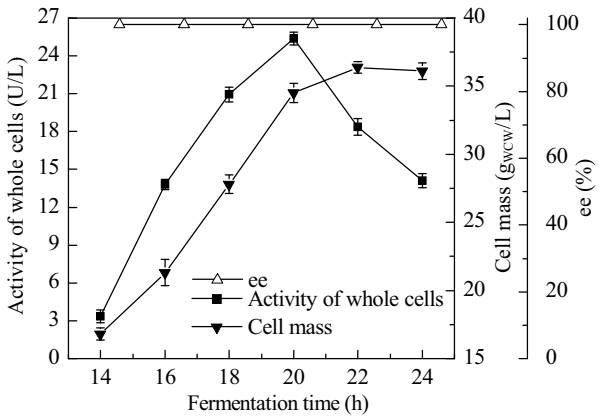



**Fig. 1.** Time courses of carbonyl reductase activity and cell growth of *Candida ontarioensis.* Fermentation conditions: 50 ml fermentation medium in 250 ml shake flask, 2% inoculum, 30 °C, and 180 r/min shaking.

cofactor recycling).

### 2.2.1 Effect of pH and temperature

To evaluate the effect of pH on the carbonyl reductase activity, the reaction was carried out in buffers at various pH values, including sodium acetate buffer (pH 4.5–6) and potassium phosphate buffer (pH 6–8). Figure 2(a) shows that the buffer pH did not affect the ee of the product and that the product yield was greater than 90% in the pH range of 5–8. The asymmetric reduction was sensitive to acidic environments and the product yield was observed to decrease from 94.3% to 72.9% when the pH was reduced from 5.0 to 4.5. Therefore, pH 6.5 was chosen as the optimum pH for this study.

Reaction temperature could also affect the activity and stability of the enzyme. An increase in the temperature could make the activation energy for the reaction easier to reach and the mass transfer of substrate/product could also be improved. The effect of reaction temperature was investigated (Fig. 2(b)) and the results indicated that the product yield was maintained at 98%–99% between a temperature range of 30–45 °C. Therefore, 30 °C was chosen as the optimum temperature for

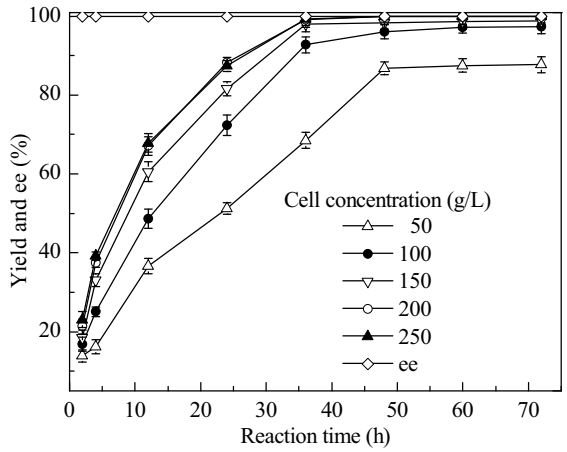



**Fig. 3.** Effects of cell concentration on product ee and yield in the bioreduction. Reaction conditions: 5% (w/v) glucose, 10 g/L substrate, potassium phosphate buffer (0.2 mol/L, pH 6.5) and different cell concentrations in a final volume of 10 ml, 30 °C, 72 h.

the bioreduction reaction.

### 2.2.2 Effect of cell concentration

The amount of cell mass in the biocatalytic reaction system directly affects the product yield of the bioconversion. The reaction time course at different cell concentrations was also investigated (Fig. 3). As shown in Fig. 3, the yield of product increased rapidly over the first 24 h for all of the cell concentrations tested. A yield of 100% was reached at cell concentrations of 200 and 250 g/L after 36 h of reaction. Thus, a cell concentration of 200 g/L was used as the optimum cell concentration.

### 2.2.3 Effect of different cosubstrates

Cofactor regeneration is crucial for the continuous progress of asymmetric reduction, because of the limited amount of NAD(P)H in microbial cells [19,20]. Thus, a cosubstrate should be added to the reaction system for the cofactor to be

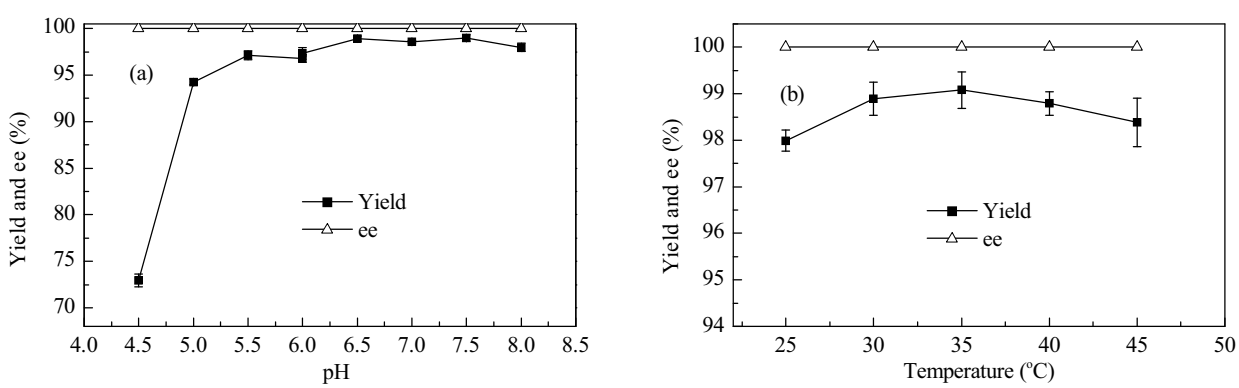



**Fig. 2.** Effects of pH (a) and temperature (b) on product ee and yield in the bioreduction. (a) Reaction conditions: 1 g wet cells, 5% (w/v) glucose, 10 g/L substrate, various buffers (0.2 mol/L, pH 4.5–8.0 sodium acetate buffer and potassium phosphate buffer) in a final volume of 10 ml, 30 °C, 72 h. (b) Reaction conditions: the same as above except different temperatures and potassium phosphate buffer (0.2 mol/L, pH 6.5).

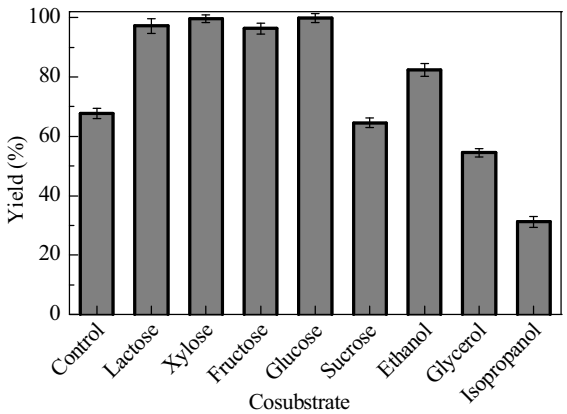



**Fig. 4.** Effect of various cosubstrates on the bioreduction reaction. Reaction conditions: 2 g wet cells, 5% (w/v) cosubstrates, 10 g/L substrate, potassium phosphate buffer (0.2 mol/L, pH 6.5) in a final volume of 10 ml, 30 °C, 72 h.

recycled by glucose dehydrogenase or alcohol dehydrogenase mediated oxidation of the cosubstrate. Sugars and alcohols, including glucose, sucrose, ethanol, and isopropyl alcohol, are commonly used as cosubstrates. In this study, several cosubstrates were investigated (Fig. 4). In comparison with the control (no cosubstrate), the highest yield was observed in groups supplemented with glucose or xylose. In contrast, the use sucrose, glycerol, and isopropanol resulted in no obvious positive effect and negative effects were actually observed. The different cosubstrates did not affect the ee of product and the optical purity achieved in all cases was 99.9%. Glucose was therefore selected as cosubstrate due to its low cost.

### 2.3 Substrate specificity

The asymmetric synthesis of several optically active alcohols by *Candida ontarioensis* was also tested (Table 1). *Candida ontarioensis* whole-cells showed high reaction activity and enantioselectivity towards various halo-acetophenones including 2-chloroacetophenone, 2-bromoacetophenone, 2-chloro-1-(3-chlorophenyl)ethanone, *p*-bromoacetophenone, *m*-aminoacetophenone, and *p*-nitroacetophenone. The corresponding alcohols reached over 99.9% ee and 99.0% yield in all cases. However, *Candida ontarioensis* whole-cells showed no activity for ethyl 4-chloroacetoacetate (COBE) and ethyl 2-oxo-4-phenylbutyrate (OPBE). The results suggest that the carbonyl reductase activity of *Candida ontarioensis* has high substrate specificity and enantioselectivity towards various halo-, amino-, and nitro-substituted acetophenones, but not for keto ester substrates.

**Table 1** Bioreduction of compounds **1**–**8** by *Candida ontarioensis* whole cells

| Substrate | | R | X | ee (%) | Configuration | Yield (%) |
|---|---|---|---|---|---|---|
| 2-Chloroacetophenone | **1** | H | Cl | > 99.9 | *R* | 99.0 |
| 2-Bromoacetophenone | **2** | H | Br | > 99.9 | *R* | 99.0 |
| 2-Chloro-1-(3-chlorophenyl)ethanone | **3** | *m*-Cl | Cl | > 99.9 | *R* | 99.0 |
| *p*-Bromoacetophenone | **4** | *p*-Br | H | > 99.9 | *R* | 99.0 |
| *m*-Aminoacetophenone | **5** | *m*-$NH_2$ | H | > 99.9 | *R* | 99.0 |
| *p*-Nitroacetophenone | **6** | *p*-$NO_2$ | H | > 99.9 | *S* | 99.0 |
| COBE | **7** | — | — | — | — | — |
| OPBE | **8** | — | — | — | — | — |

Reaction conditions: 2 g wet cells, 10 g/L substrate, potassium phosphate buffer (0.2 mol/L, pH 6.5) in a final volume of 10 ml, 30 °C, 72 h.

### 2.4 Reaction acceleration by improved membrane permeability

Whole-cell catalyzed reactions often exhibit much slower reaction rate than that of isolated enzymes due to the mass transfer limitations imposed by the cellular membrane. Our previous studies demonstrated that the reaction rate of whole-cell biocatalysis could be significantly accelerated by modulating membrane permeability [21,22]. Various chemical and physical strategies including detergents, solvents, chelators, and freeze-thaw treatments have been employed to address the permeability issue. CTAB is a cationic surfactant and has long been utilized as an effective permeabilizer to enhance the permeability of microbial cell membranes [23]. Low product yields and prolonged reaction times were often reported in whole-cell asymmetric reductions involving unnatural and hydrophobic substrates. Yu and co-workers [24] reported that the reactions catalyzed by alcohol dehydrogenase and glucose-6-phosphate dehydrogenase from CTAB-permeabilized fresh brewer's yeast were increased by 525- and 7.9-fold, respectively. Cortez and co-workers [25] compared various permeabilization methods including CTAB, Triton X-100 and freezing-thawing and found that CTAB-pretreatment could effectively improve the accessibility of glucose-6-phosphate dehydrogenase in *Candida guilliermondii*. In this study, the effect of CTAB-treatment on the bioreduction reaction was investigated in detail.

#### 2.4.1 Effect of CTAB pretreatment time

Herein, the effect of CTAB-pretreatment time on the initial reaction rate was studied (Fig. 5(a)). The initial reaction rate reached a maximum when *Candida ontarioensis* cells were pretreated with 2% CTAB for 20 min at 4 °C and the activity of whole-cells reached 74.8 U/L, representing a greater than 2-fold increase relative to the cell without CTAB-treatment. Hence the favorable CTAB pretreatment time was determined to be 20 min at 4 °C.

#### 2.4.2 Effect of CTAB pretreatment concentration

The effect of CTAB concentration on the asymmetric reduction was also examined. As shown in Fig. 5(b), the greatest yield was achieved at a CTAB concentration of 4 g/L. Further

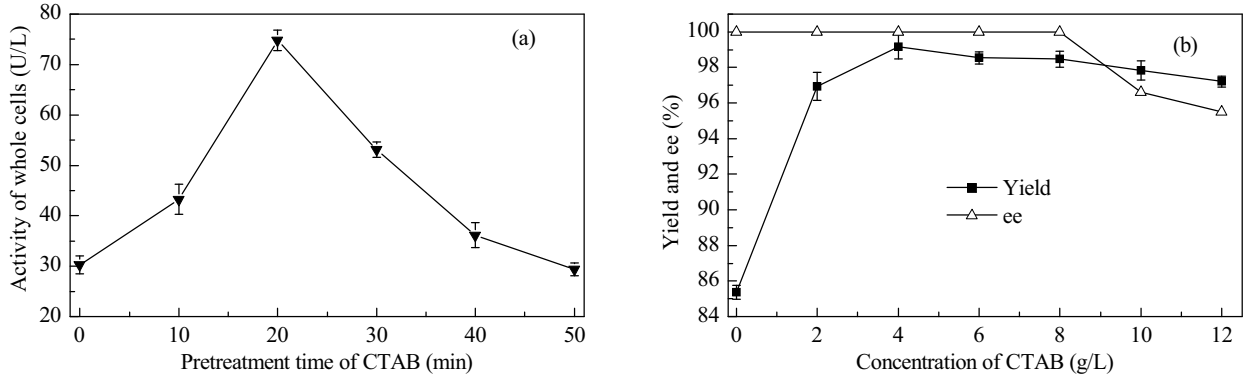



**Fig. 5.** Effects of CTAB pretreatment time (a) and concentration (b) on the activity of *Candida ontarioensis* whole-cells and the product ee and yield. (a) Reaction conditions: 2 g wet cells, 2% CTAB, 30 g/L substrate, and potassium phosphate buffer (0.2 mol/L, pH 6.5) in a final volume of 10 ml, 30 °C, 30 min. (b) Reaction conditions: 2 g wet cells, 30 g/L substrate, and potassium phosphate buffer (0.2 mol/L, pH 6.5) in a final volume of 10 ml, 30 °C, 72 h

increases in the CTAB concentration, however, led to a slight decline in the yield and also compromised the enantioselectivity. An appreciable decrease in product ee at over 10 g/L of CTAB was noted. It was speculated that CTAB might interact with carbonyl reductase and interfere with its enzymatic activity and enantioselectivity. Also, the increased membrane permeability could promote the formation of the alcohol product of the opposite configuration catalyzed by other reductases, leading to a decreased ee value in the product. Therefore, a CTAB concentration of 4 g/L was used for permeabilization treatment.

### 2.5 Time courses of bioreduction catalyzed by CTAB-pretreated and untreated cells

To evaluate the potential of CTAB-pretreated cells as whole-cell biocatalysts in the biosynthesis of (*R*)-2-chloro-1-(3-chlorophenyl)ethanol, an asymmetric reduction catalyzed by CTAB-pretreated and untreated *Candida ontarioensis* cells was conducted (Fig. 6). During the first 10 h, the reaction rate of CTAB-pretreated cells was 2-fold greater than that of the cells without CTAB-treatment. The reaction catalyzed by CTAB-pretreated cells was almost finished after 24 h (97.5% yield), whereas a yield of only 86.6% was attained by untreated cells over a prolonged reaction period of 72 h. It was speculated that the reaction progress was hindered in the later stage of bioconversion by high levels of intracellular product, because of mass transfer resistance across the untreated-cell membrane. The optical purity of product was maintained at 99.9% ee throughout the bioconversion process. Consequently, the CTAB-pretreatment resulted in a greatly reduced reaction time at an elevated substrate concentration of 30 g/L and was therefore feasible for the bioreduction catalyzed by whole- cells of *Candida ontarioensis*. Compared with the bioreduction of 2-chloro-1-(3-chlorophenyl)ethanone by *Saccharomyces cerevisiae* [14] on a 1-L reaction scale, the reaction time was shortened considerably in this study.

### 2.6 Isolation and identification of the bioreduction product

The reaction system consisted of 24 g wet cells, 1200 mg substrate, and 0.2 mol/L potassium phosphate buffer (pH 6.5) in a final volume of 120 ml. The process was carried out at 180 r/min and 30 °C over 24 h. The mixture was extracted with ethyl acetate and the organic layer was concentrated under vacuum. The crude product was separated by silica gel chromatography using ethyl acetate/petroleum ether (1:6) as eluent. The optical purity and yield of (*R*)-2-chloro-1-(3-chlorophenyl)ethanol were determined by HPLC analysis. After purification, 800 mg of the product with > 99.9% ee and 99% purity was obtained. The yields of biotransformation and purification processes were calculated to be 99% and 66.7%, respectively.

The product was identified by $^{1}H$ (400 MHz) and $^{13}C$ (400 MHz) NMR using $CDCl_3$ as solvent, and the $^{1}H$ and $^{13}C$ NMR spectra are as follows. $^{1}H$ NMR ($CDCl_3$, 400 MHz): $\delta$ 2.70 (s, 1H, OH), 3.60 (dd, 1H, $CH_2Cl$), 3.72 (dd, 1H, $CH_2Cl$), 4.87

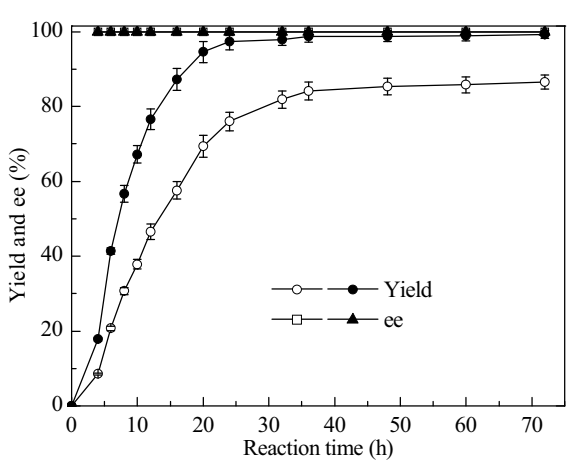



**Fig. 6.** Time courses of bioreduction catalyzed by CTAB-pretreated (solid) and untreated (hollow) *Candida ontarioensis* cells. Reaction conditions: 200 g wet cells, 30 g/L substrate, potassium phosphate buffer (0.2 mol/L, pH 6.5) in a final volume of 1 L, 30 °C, 72 h.

(dd, 4H, aromatic H), 7.26–7.32 (m, 1H, CH); $^{13}$C NMR ($CDCl_3$, 100 MHz): $\delta$ 51, 73, 124, 126, 129, 130, 135, 142.

## 3 Conclusions

The asymmetric reduction of 2-chloro-1-(3-chlorophenyl) ethanone to its corresponding chiral alcohol by *Candida ontarioensis* whole-cells was investigated. In the aqueous system, the microbial cells showed excellent catalytic ability under the optimum reaction conditions (30 °C, pH 6.5, 10 g/L substrate concentration), giving the product in 99.9% ee and 99.0% yield. A substrate specificity study indicated that the carbonyl reductase from *Candida ontarioensis* shows high substrate specificity and enantioselectivity for various halo-, amino, and nitro-substituted acetophenones, but not for keto ester substrates. CTAB-treatment was applied to enhance the cellular membrane permeability and to accelerate the reaction. Using cells pretreated with 4 g/L CTAB for 20 min, the reaction rate was increased by 2-fold over the first 10 h of the biotransformation and a product yield of 97.5% was achieved in 24 h, which was 48 h earlier than that without CTAB-treatment (86.6% yield at 72 h). The results demonstrate the importance of good mass transfer across the cell membrane for the whole-cell biocatalysis and that the efficiency of biocatalytic reactions can be greatly improved by increasing membrane permeability. This study provides a feasible and green approach for the efficient production of (*R*)-2-chloro-1-(3-chlorophenyl)ethanol. To further enhance the enzymatic activity and efficacy of the biocatalytic reaction, asymmetric preparation of (*R*)-2-chloro-1-(3-chlorophenyl) ethanol using recombinant strains overexpressing carbonyl reductase is underway.

## References


1 Kurbanoglu E B, Taskin M, Zilbeyaz K, Hasenekoglu I. *Chin J Catal*, 2009, **30**: 370
2 Bloom J D, Dutia M D, Johnson B D, Wissner A, Burns M G, Largis E E, Dolan J A, Claus T H. *J Med Chem*, 1992, **35**: 3081
3 Ohkuma T, Tsutsumi K, Utsumi N, Arai N, Noyori R, Murata K. *Org Lett*, 2007, **9**: 255
4 Ni Y, Xu J H. *J Mol Catal B*, 2002, **18**: 233
5 Schmid A, Dordick J S, Hauer B, Kiener A, Wubbolts M, Witholt B. *Nature*, 2001, **409**: 258
6 Woodley J M. *Trends Biotechnol*, 2008, **26**: 321
7 Tao J H, Xu J H. *Curr Opin Chem Biol*, 2009, **13**: 43
8 Ma X K, Wang Zh Zh, Chen W L. *Chin J Catal*, 2006, **27**: 20
9 de Carvalho C C. *Biotechnol Adv*, 2011, **29**: 75
10 Wohlgemuth R. *Curr Opin Microbiol*, 2010, **13**: 283
11 Ma X K, Wang Zh Zh, Chen W L. *Chin J Catal*, 2006, **27**: 314
12 Harada H, Hirokawa Y, Suzuki K, Hiyama Y, Oue M, Kawashima H, Yoshida N, Furutani Y, Kato S. *Bioorg Med Chem Lett*, 2003, **13**: 1301
13 Sawa I, Konishi Y, Maemoto S, Hasegawa J. EP 0493617A1. 1992
14 Lin H, Chen Y Z, Xu X Y, Xia S W, Wang L X. *J Mol Catal B*, 2009, **57**: 1
15 Itoh N, Matsuda M, Mabuchi M, Dairi T, Wang J C. *Eur J Biochem*, 2002, **269**: 2394
16 Kizaki N, Yano M, Funaki M, Takesue T, Yasohara Y, Morikawa S, Nakai T, Kataoka M, Shimizu S. US 7915022B2. 2011
17 Kizaki N, Sawa I, Yano M, Yasohara Y, Haseawa J. *Biosci Biotechnol Biochem*, 2005, **69**: 79
18 Shimizu S, Kataoka M, Kizaki N, Yasohara Y. EP 1400594A1. 2002
19 Zhang B L, Pionnier S. *Chem Eur J*, 2003, **9**: 3604
20 Zhang W, Ni Y, Sun Z H, Zheng P, Lin W Q, Zhu P, Ju N F. *Process Biochem*, 2009, **44**: 1270
21 Ni Y, Chen R R. *Biotechnol Bioeng*, 2004, **87**: 804
22 Ni Y, Mao Z C, Chen R R. *Appl Microbiol Biotechnol*, 2006, **73**: 384
23 He Y C, Zhang Z J, Xu J H, Liu Y Y. *J Ind Microbiol Biotechnol*, 2010, **37**: 741
24 Yu M A, Wei Y M, Zhao L, Jiang L, Zhu X B, Qi W. *J Ind Microbiol Biotechnol*, 2007, **34**: 151
25 Cortez D V, Roberto I C. *New Biotechnol*, 2012, **29**: 192