Biotechnology and Bioprocess Engineering **2006**, 11: 48-53

Efficient Bioreduction of Ethyl 4-chloro-3-oxobutanoate to (*S*)-4-chloro-3-hydrobutanoate by Whole Cells of *Candida magnoliae* in Water/*n*-Butyl Acetate Two-phase System

Zhinan Xu[1]*, Limei Fang[2], Jianping Lin[1], Xiaoxia Jiang[1], Ying Liu[1], and Peilin Cen[1]

[1] Institute of Bioengineering, Department of Chemical and Biochemical Engineering, Zhejiang University, Hangzhou 310027, China
[2] Zhejiang University City College, Huzhou Road 51, Hangzhou 310015, China

Abstract The asymmetric biosynthesis of ethyl (*S*)-4-chloro-3-hydrobutanoate from ethyl 4-chloro-3-oxobutanoate was investigated by using whole cells of *Candida magnoliae* JX120-3 without the addition of glucose dehydrogenase or $NADP^+$/NADPH. In a one-phase system, the bioconversion yield was seriously affected on the addition of 12.1 g/L ethyl 4-chloro-3-oxobutanoate. In order to reduce this substrate inhibition, a water/*n*-butyl acetate two-phase system was developed, and the bioreduction conditions optimized with regard to the yield and product enantiometric excess value. The optimal conditions were as following: water to *n*-butyl acetate volume ratio of 1:1, 4.0 g DCW/L active cells, 50 g/L glucose and 35°C. By adopting a dropwise substrate feeding strategy, high concentration of ethyl 4-chloro-3-oxobutanoate (60 g/L) could be asymmetrically reduced to ethyl (*S*)-4-chloro-3-hydrobutanoate with high yield (93.8%) and high enantiometric excess value (92.7%).

Keywords: asymmetric reduction, ethyl (*S*)-4-chloro-3-hydrobutanoate, ethyl 4-chloro-3-oxobutanoate, *Candida magnoliae*, two-phase system

INTRODUCTION

Chiral alcohols are useful starting materials in the synthesis of various pharmaceuticals. The needs for optically active drugs have increased in the pharmaceutical and agrochemical fields over recent years. Chiral alcohols are important intermediates for the synthesis of these optically active drugs. Optically active D-(*S*)-4-chloro-3-hydroxybutanoate ethyl ester ((*S*)-CHBE) is a key chiral intermediate in the enantioselective synthesis of slagenins B and C, as well as the total synthesis of HMG-CoA reductase inhibitors, which can also be converted into the 1,4-dihydropyridine-type blocker [1-3]. Several synthetic routes have been developed to obtain this enantiomer, of which the biocatalytic asymmetric reduction of ethyl 4-chloro-3-oxobutanoate (COBE) is the most economical for obtaining optically active (*S*)-CHBE, since COBE can be easily synthesized from cheap available materials [4].

The asymmetric bioconversion from COBE to (*S*)-CHBE can be catalyzed by various microorganisms such as *Saccharomyces cerevisiae*, *Sporobolomyces salmonicolor*, and *Candida magnoliae*, *etc.* [4]. Further studies have shown that the bioreduction of COBE is catalyzed by NADPH-dependent aldehyde reductase or carbonyl reductase [5,6]. And about 5 and 13 g/L (*S*)-CHBE were produced by *Geotrichum candidum* and *Zygosaccharomyces rouxii* respectively [7,8]. Since both the substrate (COBE) and product ((*S*)-CHBE) exhibit poor solubility and stability in water, and they are also harmful to cells or the function of the enzymes used, some new strategies have been suggested to carry out the bioconversion in a two-phase system to alleviate the above problem and improve the productivity of (*S*)-CHBE [4]. With this biphasic system strategy, a high concentration of COBE (90 g/L) can be transformed to (*S*)-CHBE, in a high yield and with a high product enantiometric excess (e.e.) value using the heated or acetone pretreated cells. However, such pretreatment of bioactive cells led to relatively high enzyme activity for (*S*)-CHBE biosynthesis and an improved product e.e. value, but the ability of the cell to recycle coenzyme would be harmed. Therefore, glucose dehydrogenase and $NADP^+$ must be supplemented to this bioreduction reaction when the pretreated cells are employed, which definitely increased the cost of this process.

For this asymmetric reduction, NADPH will act as an electron donor; therefore, whole cells are preferred for their higher ability of regenerating this kind of coenzyme. In active yeast cells, glucose metabolism can provides key sources of NADPH: the two oxidative steps of the pentose phosphate pathway and isocitrate dehydrogenase, which is part of the citric acid cycle. The coupling of COBE reduction and glucose oxidation in the yeast cells

***Corresponding author**
Tel: +86-571-87951220 Fax: +86-571-87951220
e-mail: znxu@zju.edu.cn

will maintain the bioreduction of COBE by recycling $NADP^+$/NADPH, so the addition of extra glucose dehydrogenase and NADPH may be not necessary. However, the low substrate tolerance of active cells always affects the performance of the bioreduction system. The water/organic solvent two-phase system will be beneficial to lower the substrate concentration in the aqueous phase and alleviate substrate inhibition. Many factors, such as solvent selection and operation conditions, are important factors for a successful bioconversion in a two-phase system. Therefore, the economics of this process were decided by many factors, such as product yield, product e.e. value, substrate concentration, and supplements of glucose dehydrogenase and $NADP^+$, *etc.*

In our laboratory, a new strain, *C. magnoliae* JX120-3, has been screened out for its high activity to convert COBE to (*S*)-CHBE without the addition of any glucose dehydrogenase and $NADP^+$/NADPH [9]. In this work, a two-phase bioconversion system from COBE to (*S*)-CHBE was developed for the production of (*S*)-CHBE using active cells of *C. magnoliae* JX120-3, and the reaction conditions were further optimized to complete the bioconversion efficiently.

MATERIALS AND METHODS

Chemicals

The COBE was purchased from Acros Organics (NJ, USA) and the (*S*)-CHBE was a product from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). The glucose-6-phosphate dehydrogenase (GDH, from *Leuconostoc masenteroides*) was supplied by Worthington Biochemical Corporation (NJ, USA), and the β-NADPH-Na_4 and $NADP^+$ was respectively obtained from Roche (Shanghai, China) and Amresco (Ohio, USA), respectively. All the other chemicals were of analytical grade and commercially available.

Strains, Medium, and Cultivation

C. magnoliae JX120-3 was obtained with high carbonyl reductase activity using several runs of the strain naturally isolated from soil, which was preserved in our laboratory [9]. The strain was cultivated at 35°C and 200 rpm in 30 mL of fermentation medium, containing glucose 40 g/L, yeast extract 5 g/L, KH_2PO_4 5 g/L, $(NH_4)_2HPO_4$ 10 g/L, $CuSO_4 \cdot 5H_2O$ 0.005 g/L, $MnSO_4 \cdot 4H_2O$ 0.01 g/L, and NaCl 0.1 g/L.

The culture broth was harvested by centrifugation (5,000 g, 10 min, 4°C), the cells were collected and washed with 100 mM potassium phosphate buffer (pH 6.0), and then used directly for the biotransformation without pretreatment.

Bioconversion of COBE to CHBE

Bioconversion of COBE to CHBE in Aqueous Phase

A reaction mixture comprising 200 mM potassium phosphate buffer (pH 6.0), different concentration of COBE, 30 g/L glucose, 10 mg (dry cell weight, DCW) active cells, in a final volume of 10 mL, was incubated at 35°C on a reciprocal shaker (100 rpm) for 6 h.

Bioconversion of COBE to CHBE in Two-phase System

A reaction mixture comprising 200 mM potassium phosphate buffer (pH 6.0), different concentration of COBE, 30 g/L glucose, 40 mg (DCW) active cells, in a final volume of 10 mL, was mixed with an equal volume of *n*-butyl acetate in a 50 mL reaction vessel, and the mixture was then incubated at 30°C on a reciprocal shaker (100 rpm) for 30 h. COBE was initially added, or with different feeding modes, according to the experiment design.

Analysis

The concentrations of COBE and CHBE were determined by gas chromatography [10]. The enantiomeric excess of CHBE was determined by high-performance liquid chromatography on a Chiralcel OB packed column (4.6 × 250 mm; Daicel Chemical Industries, Japan) and detected at 217 nm after elution with *n*-hexane/2-propanol (9:1, v/v) at the flow rate of 1 mL/min. Under these conditions, the (*R*)- and (*S*)-CHBE were eluted at 10.5 and 11.6 min, respectively.

RESULTS AND DISCUSSION

Bioreduction of COBE in Aqueous System

In many bioconversion reactions, the inhibitions cau-sed by substrates and products are always thought to be the limiting factor to obtaining high yields and product e.e. values. The effects of the initial COBE concentration on the performance of the bioreduction with *C. mag-noliae* JX120-3 were examined, with the results shown in Fig. 1. The time course study showed that a reaction time of 5 h was enough to obtain the peak data of the bioconversion under various batch conditions (data not shown), thus the subsequent batch bioreactions were termined after 5 h unless otherwise indicated. With the increase in the initial COBE concentration in the aqueous phase system, it was observed that both (*S*)-CHBE yield and e.e. value decreased. Especially, when the initial COBE concentration was higher than the critical value of about 12.1 g/L, a sharp decrease was observed, which was explained by the existence of substrate inhibition. The substrate inhibition can be reduced by monitoring the substrate concentration, such as, adding 24.2 g/L COBE evenly at 0 and 3 h (Fig. 2). The results showed that the inhibition effect was reduced with this new operation mode, but the yield and e.e. value were still lower than when only 12.1 g/L COBE was initially added. This suggested that the product inhibition was not eradicated just using this simple addition mode.

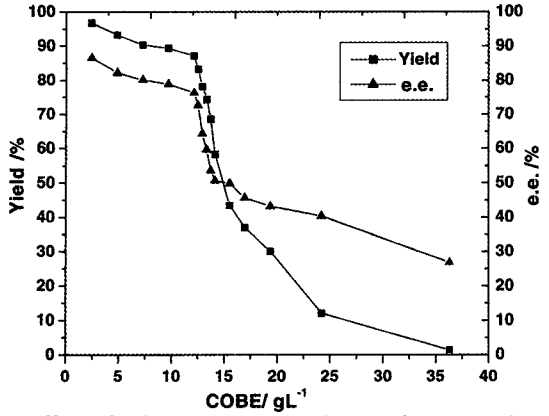

Fig. 1. Effect of substrate concentration on the conversion yield and product e.e. value in the mono-phase system. All the tests were operated at 30°C and ended after 6 h of reaction.

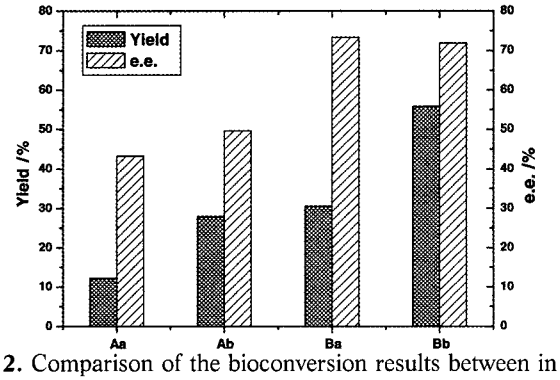

Fig. 2. Comparison of the bioconversion results between in the one- and two-phase systems. Aa, 24.2 g/L COBE was initially added to the one-phase system; Ab, 24.2 g/L COBE was evenly added, at 0 and 15 h, to the one-phase system; Ba, 24.2 g/L COBE was initially added to the two-phase system; Bb, 24.2 g/L COBE was evenly added, at 0 and 15 h, to the two-phase system. Also, all the tests were operated under the same conditions (1 g/L (DCW) active cells and 20 g/L glucose, at pH 6.0 and 30°C) and ended after 30 h of reaction.

Solvent Selection

Much research has reported that a two-phase system was a good choice for reducing substrate inhibition. The use of organic solvent is a key factor for a successful bioconversion in a two-phase system [11,12]. For a specific bioconversion, there is still no general theory that guides the solvent selection, but the hydrophobicity (log*P*) of the organic solvent is perhaps a reference indicator [13]. As shown in Table 1, five organic solvents were used for our two-phase system for the asymmetric biosynthesis of (*S*)-CHBE. When the log*P* was changed from 0.68 to 1.7, the product yield and product e.e. were improved; however, further increase on the log*P* value will not guarantee good results. This was partly explained by the higher toxicity of solvents with higher log*P* values. The highest yield and e.e. value were obtained in a water/*n*-butyl acetate two-phase system, which was explained by the high partition coefficients of COBE and (*S*)-CHBE and the stability of carbonyl reductase (CAR) and glucose dehydrogenase (GDH) in *n*-butyl acetate [13,14]. Therefore, *n*-butyl acetate was selected to construct the two-phase system in the subsequent experiments.

Table 1. Effects of organic solvents on the product yield and product e.e.

Organic solvents	Log*P*	Yield/%	e.e./%
Ethyl acetate	0.68	35.6	64.8
n-Butyl acetate	1.70	86.7	90.8
Hexane	3.50	85.3	86.5
Dibutyl *o*-phthalate	5.40	82.6	82.1

Ten mL of different organic solvents was added into a 10 mL reaction system, consisting of 24.2 g/L COBE, 4 g/L (DCW) active cells, and 5 g/L glucose, pH 6.0. All the tests were conducted at 30°C and ended after 30 h incubation.

Further study (Fig. 2) showed that, compared with a one-phase system, the (*S*)-CHBE yield was improved significantly by using our two-phase system, even without the addition of any glucose dehydrogenase and $NADP^+$/NADPH. When 24.2 g/L COBE was added, the (*S*)-CHBE yield could be improved from 27.9 to 55.8% changing from an aqueous system to our two-phase system; similarly, the e.e. value could be improved from *ca.* 45 to *ca.* 72%. This reduced inhibition effect was contributed to by lower concentration of COBE and (*S*)-CHBE in the water phase due to the high partition coefficients of COBE (10.6) and (*S*)-CHBE (15.8) in the water/*n*-butyl acetate system. However, when the same concentration of substrate (24.2 g/L COBE) was added into different two-phase systems, a big difference in the yield (shown in Fig. 2 and Table 1) was observed. It was explained by the addition of different quantities of active cells to the systems, *i.e.*, 1 g/L (DCW) active cells in Fig. 2, 4 g/L (DCW) active cells, as shown in Table 1.

Effect of Water/*n*-Butyl Acetate Volume Ratio

The product yield and e.e. value were examined with different volume ratios of water phase to organic phase (V_w/V_o). As shown in Table 2, the volume ratio of the water to that of the organic phase significantly influenced the product yield and e.e. value. Generally speaking, enzymes and active cells may be inactivated once directly brought into contacting with organic solvents due to the toxicity of the solvents. Therefore, the product yield and e.e. value were expected to increase with increasing the V_w/V_o ratio, because there would be less chance for enzymes and active cells to come into contact with organic solvent. The results (Table 2) were however, conflicted with this expectation. The possible reason was that the existing carbonyl reductase and other related coenzyme regenerating enzymes were relatively stable when *n*-butyl acetate was employed. Conversely, the lower concentration of the substrate and product in the water phase, due to the decreased V_w/V_o value, could improve the product yield and e.e. value by alleviating the inhibition effect ob-

Table 2. Effects of the water/*n*-butyl acetate volume ratio on the product yield and product e.e. value

V_w/V_o	Yield/%	e.e./%
1/0.2	67.6	75.6
1/0.3	73.8	78.6
1/0.5	81.3	88.7
1/1.0	86.7	90.8
1/2.0	85.6	89.8
1/3.0	70.2	83.5

According to different water to oil phase ratios, *n*-butyl acetate was added into a 10 mL reaction system, consisting of 24.2 g/L COBE, 4 g/L (DCW) active cells, and 5 g/L glucose, at pH 6.0. All the tests conducted at 30°C and ended after 30 h incubation.

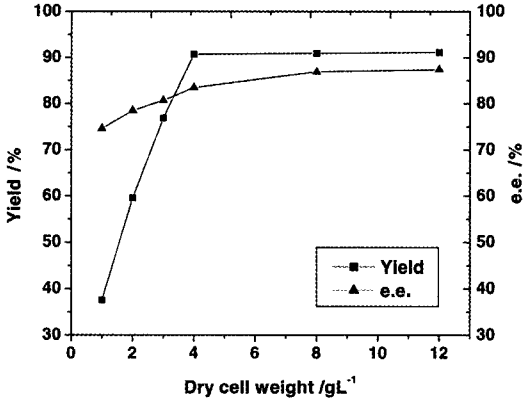

Fig. 3. Effects of cell mass on the product yield and e.e. value in the two-phase system. Different quantities of active wet cells were added to a water/*n*-butyl acetate two-phase (1:1) system. 24.2 g/L COBE and 20 g/L glucose were used for the bioconversion, which was carried out at 30°C and ended after 30 h of reaction.

served in the one phase system. In the meantime, the partition coefficient of (*S*)-CHBE was higher than that of COBE; therefore, a much lower (*S*)-CHBE concentration in the aqueous phase was favorable for the production of (*S*)-CHBE. With a decrease of the volume ratio (V_w/V_o), the yield and e.e. value improved (Table 2). Further decreases in the ratio, less than 1:1, did not obviously improve the biotransformation. The preferred ratio was set at 1:1 in the subsequent experiments. The high product e.e. value contributed to the quick transfer of (*S*)-CHBE, after its formation, from the aqueous phase to the organic phase, as no racemic enzymes were found in the organic phase.

Effect of Biomass

Fig. 3 showed the effect the amount of biomass has on the product yield and e.e. value. These results indicated that both the product yield and e.e. value increased with increasing biomass. When active wet cells were less than 4.0 g DCW/L, a linear increase in the product yield was observed. When the amount of biomass was higher than

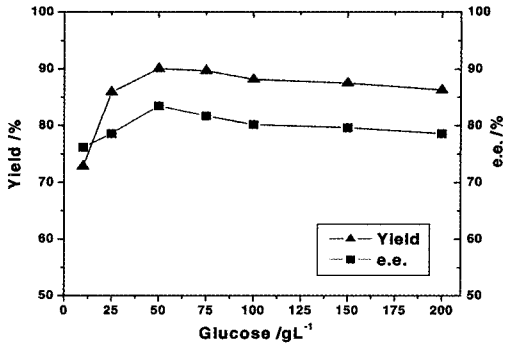

Fig. 4. Effects of glucose concentration on product yield and e.e. value in two-phase system. Different concentrations of glucose were added to a water/*n*-butyl acetate two-phase (1:1) system. 24.2 g/L COBE and 4 g/L (DCW) active cells were used for the bioconversion which was carried out at 30°C and ended after 30 h of reaction.

4.0 g DCW/L, the product yield remained stable. The increase in the product e.e. value was smoother with a greater amount of biomass. A high product yield (90.1%) and e.e. value (83.5%) could be obtained in this two-phase system when 4.0 DCW g/L cells were initially added.

The larger amount of active *C. magnoliae* cells the higher will be initial activity of enzymes involved in the bioreduction of COBE to (*S*)-CHBE and the NADPH regeneration, which are favorable for increasing the reaction rate. The reason for the higher conversion ratio was that the higher NADPH concentration and higher initial enzyme activity might counteract the effect of the enzyme deactivation in the two-phase system.

Effect of Glucose Concentration

The bioreduction of COBE to (*S*)-CHBE is catalyzed by the carbonyl reductase in *C. magnoliae*, which is a NADPH-dependent oxido-reductase. In this yeast, glucose metabolism can provides key sources of NADPH due to three key enzymatic reactions involved in HMP and TCA cycles. In order to eliminate the heterogenous addition of expensive GDH and NADPH from the system, glucose was supplemented instead for the NADPH regeneration in our two-phase system. The effect of the glucose concentration on the bioreduction was examined, with the results shown in Fig. 4. It was obvious that without glucose addition, the yield of (*S*)-CHBE yield was very low and would be improved by increasing the glucose concentration. The highest (*S*)-CHBE yield was achieved when glucose concentration was about 50 g/L. The effect of the glucose concentration on the product e.e. value was not significant. More than 50 g/L glucose would lead to decrease in the product yield and product e.e. This implies that 50 g/L glucose was enough for NADPH regeneration in our two-phase system, with some glucose metabolism byproducts having a harmful effect on the bioreaction.

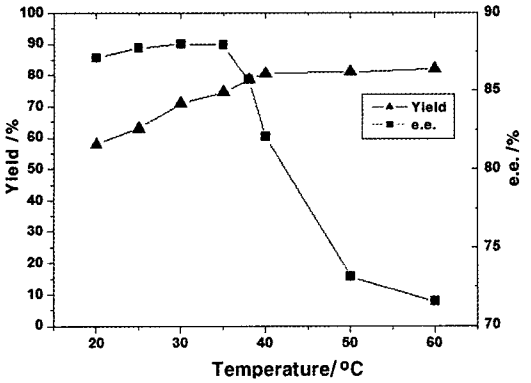

Fig. 5. Effect of reaction temperature on the product yield and e.e. value in the two-phase system. The bioconversion tests were carried out at different temperatures and ended after 30 h of reaction. 24.2 g/L COBE and 4 g/L (DCW) active cells were added to a water/*n*-butyl acetate two-phase (1:1) system.

Effect of Reaction Temperature

The effects of temperature on the product yield and e.e. value are shown in Fig. 5. When the temperature was adjusted from 20 to 35°C, the product yield increased slightly, but the high yield was maintained. Further increases in temperature led to a drastic drop in the product yield, due to the increased deactivation rate of the enzymes. The optimal temperature should be 35°C. The effect of temperature on the product e.e. value was relatively small, but a higher temperature slightly improved the product e.e. value, which was probably due to the higher reaction rate and mass transfer rate between two phases [15]; thus, the synthesized (*S*)-CHBE would be transferred to the organic phase more quickly.

Effects of COBE Addition Modes

From the above experimental data, the bioreduction of COBE to (*S*)-CHBE was greatly improved in our water/*n*-butyl acetate two-phase system. However, the inhibition due to a high concentration of substrate still existed. As shown in Table 3, when the initial COBE concentration was increased to 60 g/L, the (*S*)-CHBE yield and e.e. value dropped to only 12.4 and 73.6%, respectively. In order to reduce the substrate inhibition, the substrate was divided into equal parts, which were then added one at a time into the two-phase system at given time intervals; these results are also listed in Table 3. It was obvious that the more equal parts the substrate were divided into, the higher the product yield and e.e. value. Then, a dropwise feeding strategy of COBE was adopted. Using this new strategy, the (*S*)-CHBE yield and e.e. value reached 93.8 and 92.7%, respectively. The supply of extra GDH or $NADP^+$/NADPH did not further improve the performance of this asymmetrical biotransformation (data not shown), suggesting that the recycling of NADP by this yeast could maintain the bioreduction efficiently, even when the bioreaction time was extended From 6 to 42 h from one-phase system to our biophase system. The strategy of substrate dropwise addition has previously been used in a one-phase reaction system (Shimizu, S. *et al.*, 1990). This present work further showed that this substrate feeding mode was also very efficient at eliminating the substrate inhibition in our two-phase system, and the two-phase system developed was very attractive as a high concentration of substrate was efficiently reduced without the addition of extra GDH and NADPH.

Table 3. Effects of the substrate feeding modes on product yield and product e.e. value in the two phase system

Substrate addition manner	Total time/h	Yield/%	e.e./%
Once	30	12.36	73.6
Twice	40	38.70	79.8
Thrice	50	53.60	84.6
Dropwise	60	93.80	92.7

In the water/*n*-butyl acetate two-phase (1:1) system, 6 g/L (DCW) active cells and 50 g/L glucose were added, with an operation temperature of 35°C. A total of 60 g/L COBE was fed according to several different modes: once, 0 h (60 g/L); twice, 0 h (30 g/L), 20 h (30 g/L); thrice, 0 h (20 g/L), 13 h (20 g/L), 26 h (20 g/L); dropwise, 0~40 h. The pH value of the system was carefully adjusted to 6.0 ± 0.2 during the substrate feeding process, and all the bioconversions were ended after 42 h.

CONCLUSION

The obstacle associated with the oxidoreductase catalyzed bioreduction from COBE to (*S*)-CHBE is the requirement for the expensive coenzyme NADPH. With the use of active cells of *C. magnoliae* JX120-3 as catalyst, the NADPH is successfully regenerated due to intracellular glucose metabolism with cheap glucose as the substrate, thus avoiding the supplement of additional GDH and NADPH. The inhibitory effects of the substrate on the participated enzymes will limit the increase in the substrate concentration, and cause a low product yield and e.e. value. A water/*n*-butyl acetate two-phase system was developed and proven to be effective at reducing this inhibition as a result of the high partition coefficients of COBE and (*S*)-CHBE in the organic phase. The bioreduction conditions were also systematically optimized. When the water to *n*-butyl acetate volume ratio was 1:1, the addition of 4 g/L active cells and 50 g/L glucose into the system, and monitoring the reaction at a temperature of 35°C, the product yield and e.e. value were able to reach their maxima. Under these optimal conditions, and with the dropwise feeding strategy, a high concentration of COBE (60 g/L) was efficiently transformed to (*S*)-CHBE with both a high yield and e.e. value.

REFERENCES

[1] Jiang, B., J. F. Liu, and S. Y. Zhao (2001) Enantioselective synthesis for the antipodes of slagenins B and C: es-

tablishment of absolute stereochemistry. *Org. Lett.* 3: 4011-4013.

[2] Karanewsky, D. S., M. C. Badia, C. P. Ciosek, Jr., J. A. Robl, M. J. Sofia, L. M. Simpkins, B. DeLange, T. W. Harrity, S. A. Biller, and E. M. Gordon (1990) Phosphorus-containing inhibitors of HMG-CoA reductase. 1. 4-[(2-arylethyl) hydroxyphosphinyl]-3-hydroxy-butanoic acids: a new class of cell-selective inhibitors of cholesterol biosynthesis. *J. Med. Chem.* 33: 2952-2956.

[3] Kita, K., M. Kataoka, and S. Shimizu (1999) Diversity of 4-chloroacetoacetate ethyl ester-reducing enzymes in yeasts and their application to chiral alcohol synthesis. *J. Biosci. Bioeng.* 88: 591-598.

[4] Yasohara, Y., N. Kizaki, J. Hasegawa, S. Takahashi, M. Wada, M. Kataoka, and S. Shimizu (1999) Synthesis of optically active ethyl 4-chloro-3-hydroxybutanoate by microbial reduction. *Appl. Microbiol. Biotechnol.* 51: 847-851.

[5] Wada, M., M., Kataoka, H. Kawabata, Y. Yasohara, N. Kizaki, J. Hasegawa, and S. Shimizu (1998) Purification and characterization of NADPH-dependent carbonyl reductase, involved in stere-oselective reduction of ethyl 4-chloro-3-oxobuta-noate, from *Candida magnoliae*. *Biosci. Biotechnol. Biochem.* 62: 280-285.

[6] Shimizu, S., M. Kataoka, and K. Kita (1998) Chiral alcohol synthesis with yeast carbonyl reductases. *J. Mol. Catal., B Enzym.* 5: 321-325.

[7] Hallinan, K. O., D. H. G. Crout, J. R. Hunt, A. S. Carter, H. Dalton, R. A. Holt, and J. Crosby (1995) Yeast catalysed reduction of β-keto esters. II. Optimization of the stereospecific reduction by *Zygosaccharomyces rouxii*. *Biocatal. Biotransformation* 12: 179-191.

[8] Patel, R. N., C. G. McNamee, A. Banerjee, J. M. Howell, R. S. Robinson, and L. Szarka (1992) Stereoselective reduction of β-keto esters by *Geotrichum candidum*. *Enzyme Microb. Technol.* 14: 731-738.

[9] Liu, Y., Z. Xu, K. Jing, *et al.* (2004) A novel NADPH-regenerating system for production of chiral alcohols. *The 8th China-Japan-Korea Joint Symposium on Enzyme Engineering*. pp. 105. Hangzhou, China.

[10] Liu, Y., Z. Xu, K. Jing, X. Jiang, J. Lin, F. Wang, and P. Cen (2005) Asymmetric reduction of COBE to CHBE with two co-existing, recombinant *Escherichia coli* strains. *Biotechnol. Lett.* 27: 119-125.

[11] Lou, W., M. Zong, Y. Zhang, and H. Wu (2004) Efficient synthesis of optically active organosilyl alcohol via asymmetric reduction of acyl silane with immobilized yeast. *Enzyme Microb. Technol.* 35: 190-196.

[12] Bar, R. (1988) Effect of interphase mixing on a water-organic solvent two-liquid phase microbial system: ethanol fermentation. *J. Chem. Technol. Biotechnol.* 43: 49-62.

[13] Shimizu, S., M. Kataoka, M. Katoh, T. Morikawa, T. Miyoshi, and H. Yamada (1990) Stereoselective reduction of ethyl 4-chloro-3-oxobutanoate by a microbial aldehyde reduction in an organic solvent-water diphasic system. *Appl. Environ. Microbiol.* 56: 2374-2377.

[14] Hocknull, M. D. and M. D. Lilly (1988) Stability of the steroid delta-dehydrogenation system of *Artehrobacter simplex* in organic solvent-water two liquid phase environments. *Enzyme Microb. Technol.* 10: 669-674.

[15] Darrugo, P., G. P. Fantoni, S. Servi, *et al.* (1997) The effect of absorbing on substrate concentration and enantiomeric excess in yeast reduction. *Tetrahedron Asymmetry* 14: 375-379.

[Received July 11, 2005; accepted October 4, 2005]