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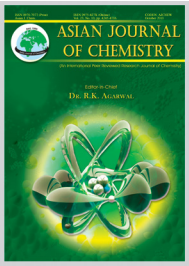

Asymmetric Reduction of Ethyl (S)-4-chloro-3-hydroxy butanoate in an Aqueous-Organic Solvent Biphasic System Using Dried Baker's Yeast

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A process of highly stereoselective reduction of ethyl 4-chloro-3-oxobutanoate (ECOB) to ethyl (S)-4-chloro-3-hydroxy butanoate [(S)-ECHB] with Baker's yeast was established. The influence of volumetric phase ratio, substrate concentration, the amount of dried yeast cells and glucose on the yield of ethyl (S)-4-chloro-3-hydroxybutanoate and the enantiomeric excess (e.e.) (S) value were also investigated. A high yield and e.e. of ethyl (S)-4-chloro-3-hydroxybutanoate were observed in a biphasic system composed of phosphate buffer (0.05 mol/L, pH 7.0) and isooctane. Under the optimum conditions (in the water/isooctane biphasic system, Vaq/Vorg 3/1, dried yeast cells 50 g/L, glucose 0.6 mol/L, 30 ºC, pH 7.0, substrate concentration 0.08 mol/L), the yield of ethyl (S)-4-chloro-3-hydroxybutanoate and the e.e. value of (S)-ECHB reached 93.9 % and 91.4 %, respectively, after 24 h reaction.

Key Words: Asymmetric reduction, Ethyl (S)-4-chloro-3-hydroxybutanoate, Aqueous-organic solvent biphasic system, Baker's yeast.

INTRODUCTION

(S)-Ethyl-4-chloro-3-hydroxybutanoate [(S)-ECHB] is a versatile key intermediate for the synthesis of a variety of hypercholesterolemia drugs and can be produced by the asymmetric reduction of ethyl-4-chloro-3-oxobutanoate (ECOB). Biological catalyst method is one of the main ways of preparing chiral compounds and the catalysts consist of isolated enzyme[1-3] and whole cell system[3-5]. Using whole cells as biocatalysts is low cost because there is no need for enzyme isolation[6] and the addition of NAD(P)H as a cofactor. The cofactor can be regenerated in whole cells. Also, in some cases, enzymes within the cells are more stable than that being isolated. Several microbes have been reported to be used for the reduction of ECHB, such as Aureobasidium pullulans CGMCC 1244[7], Baker's yeast[8], *Pseudoraonas* sp. OS-K-29[9]. The reduction of ECOB with dried Baker's yeast in the presence of the additives allyl bromide and allyl alcohol was performed by Forni *et al.*[8]. Under the optimum conditions a complete conversion of the substrate within 1-2 h and a high enantios-electivity, 90-97 % e.e. were obtained. However, the reduction had a yield of 75 %.

It is well known that water is necessary for whole-cell biocatalysis. However, there have been several disadvantages in the reduction of α,β-unsaturated carbonyl compounds with whole cells in an aqueous system, including low solubility of organic substrates and products, undesired side reactions such as hydrolysis and difficult separation of products[10]. When ECOB or ECHB was at a high concentration, product inhibition or toxicity seemed to cause a drastic decrease in the reaction rate and eventually the total cessation of the biocatalyst productivity. Thus it is necessary to keep a high viability of a cell biocatalyst as well as its high metabolic activity. The water/organic solvent biphasic system may be a well-known alternative to ease the above problem.

In the present work, Baker's yeast was applied to catalyze ECOB to (S)-ECHB in the aqueous-organic solvent biphasic system. The selection of the organic solvent was in the first place. The reaction conditions in the biphasic system, such as V_{aq}/V_{org}, amount of yeast cells, amount of glucose, substrate concentration were optimized to improve the yield of products and the e.e. value of (S)-ECHB.

EXPERIMENTAL

Microorgansim and chemicals: Baker's yeast was kindly provided by meishan-mali yeast Co. Ltd. (Guangdong, China). ECOB (> 98 %, analytical grade) were purchased from Leqi Chemical Co. Ltd. (Shangdong, China). Enantiopure standards, (R)-(96 %) and (S)-ECHB (97 %) were obtained from Sigma-Aldrich, inc., (Steinheim, Germany). All other chemicals were

of analytical grade and were used without further purification unless otherwise stated.

Activation: The activating solution contained: glucose, 50 g/L; $(NH_4)_2SO_4$, 2 g/L; K_2HPO_4, 1 g/L; $CaSO_4$, 1 g/L and citric acid, 1 g/L and its pH was adjusted to 7.0. The activation was carried out in 1000 mL shaking flask containing 250 mL activating solution and 7.5 g Baker's yeast at 30 ºC and 175 rpm with rotary shaking. After activation for 1 h, the strains were harvested by centrifugation (5000 rpm, 10 min) at 4 ºC and washed twice with phosphate buffer (0.01 mol/L, pH 7.0). The gained strains were preserved at 4 ºC.

Bioreduction assay: The reduction of ECOB in an organic-aqueous system was conducted in a 50 mL shaking flask. A certain amount of wet cells or dried cells and the organic solvent were suspended in 30 mL phosphate buffer (0.05 mol/L, pH 7.0) with a certain amount of glucose. After pre-incubated with rotary shaking at 30 ºC and 180 rpm for 15 min, 0.1 mol/L ECOB was added to the medium and the incubation was continued. At time intervals, a fixed amount of medium was sampled and centrifuged (8000 rpm, 10 min) at 4 ºC to remove the cells. The supernatant was extracted by ethyl acetate and mixed with octane as the internal standard for GC determination. The concentration of ECOB and the ratio of V_{aq}/V_{org} were regulated according to the experimental design in the experiments.

Analytical methods: The concentrations of ECOB and ECHB and enantiomeric excess(e.e.) of (S)-ECHB were determined by GC (Aglient GC_6890, USA) equipped with a chiral column (CP-Chirasil Dex CB, 0.32 mm diameter, 25 m length, Varian USA). For the determination of e.e., the ECHB should be derivated. The ethyl acetate layer was dried *via* the rotating evaporation and the remainder reacted with acetic anhydride containing excessive amount of pyridine catalyzed by DMAP at 100 ºC for 20 min.

Analysis method was similar to literature[11]: Sample size 1.0 µL; injector and detector temperature, 250 ºC; column temperature, 100 ºC for 1min and 100-130 ºC at a rate of 3 ºC/min for 1 min, then 130-180 ºC at a rate of 10 ºC/min for 1 min; carrier gas, nitrogen; split ratio, 50:1; detector, FID.

RESULTS AND DISCUSSION

Comparation of the asymmetric reduction with wet cells and dried cells as catalysts: The amount of protein in 1 g dried Baker's yeast was 0.707 g protein, which is equivalent to that of 2.08 g wet yeast[12]. The asymmetric reduction was catalyzed by dried cells and wet cells with a constant protein content and initial water content of Baker's yeast based on the above data (Table-1). Table-1 showed that the dried cells or wet cells could catalyze the reduction of ECOB in the aqueous system and (S)-ECHB was the main product. Wet yeast showed a higher stereoselectivity compared to dried yeast which may be due to a higher activity of alcohol dehydrogenase catalyzing ECOB to (S)-ECOB. However, the reduction using wet yeast had a worse yield than using dried yeast. Wet yeast was more sensitive to confront a bad environment. When glucose (as co-facter) was not added in this reduction, fresh wet yeast could not produce enough NAD(P)H to remain a high activity continued in the metabolism. The conversion of ECOB and the yield of ECHB with different substrate concentrations catalyzed by wet yeast were much lower than that by dried yeasts. Increasing substrate concentration could inhibit the activity of alcohol dehydrogenase and generate hydrolysis side effects of ECOB or ECHB. Detailed investigations to optimize various reaction parameters were conducted using dried yeast as catalyst since it was a simple, convenient and great repeatable operation.

TABLE-1
EFFECT OF DRIED YEAST AND WET YEAST ON THE REDUCTION OF ECOB[a]

Entry	Type of baker's yeast	Substrate concentration (mmol/L)	e.e. (%)	Yield (%)	Conversion of ECOB (%)	Conf.
1	Dry	50	53.9	41.3	88.9	S
2	Fresh wet	50	76.3	15.2	63.9	S
3	Fresh wet	25	76.1	14.8	61.5	S

[a]Reaction condition: 1 g dry yeast (2.08 g wet yeast), 1.55 mL H_2O, 50 (25) mmol/L ECOB, 30 mL phosphate buffer (0.05 mol/L, pH 7.0), 30 ºC, 24 h. conf. means configuration

Selection of organic solvents for the asymmetric reduction of ECOB to (S)-ECHB: The selection of the organic solvent is important in an aqueous-organic solvent biphasic system. The presence of the organic solvent was helpful to generate a high product yield and enantioselectivity of the cells present in the aqueous phase, if the solvent had a low toxicity to the microbe. The influence of organic solvent in the biphasic systems on the biocatalysis synthesis of (S)-ECHB was studied. The results were listed in Table-2, which showed that the yield of ECHB increased with the increase of the log*P* value of the organic solvent ($2.1 < \log P < 5.4$) used. The maximal yield of ECHB and the moderate value of e.e. (S) were achieved in the water/dibutyl phthalate system. The water/isooctane system gave a high yield of ECHB and best value of e.e. (S). In presence of the organic solvent with low log*P* value (0-1) such as acetonitrile or *n*-butyl alcohol, ECHB was not found because toxicity of the solvent resulted in poor viability of the yeast cell. The yield and e.e. (S) of glycerol ($\log P = -1.8$) and glycol ($\log P = -1.4$) were also low and the (R)-ECHB was the main product. The reason may be that water-solubility of organic solvents destroyed cell membrane which reduced catalytic activity. Based on the consideration of the yield and the e.e. (S) influenced by the organic solvent, the water/isooctane biphasic system was chosen for further experiments.

Effect of phase ratio on the reduction: As biotransformation in an aqueous-organic solvent biphasic system, the volumetric phase ratio influences the phase interfacial area and biotransformation rate[13]. So we investigated the effect of the volume ratio of the aqueous phase to the organic phase (V_{aq}/V_{org}) on the asymmetric reduction of ECOB with dried Baker's yeast in water/isooctane biphasic system. The total volume was 30 mL with varying V_{aq}/V_{org} values (Table-3). As shown in Table-3, the reaction rate, e.e. (S) and the yield of the reduction increased with the increase of V_{aq}/V_{org}. The optimum V_{aq}/V_{org} was 3:1. The increase of the amount of water could decrease the opportunity of cells to contact with the organic solvent. The cells could keep high catalytic acitivity. More water in the biphasic system was not necessary and

caused the decrease of the capacity of the reactor. The volumetric phase ratio of 3:1 was considered to be the optimum condition under which a higher yield (89.3 %) with an excellent optical purity of the product (86.8 % ee) was obtained.

TABLE-2
PARAMETERS EVALUATED FOR THE SCREENING OF REACTION SOLVENTS[a]

Organic solvent	log *P*	Yield (%)	e.e. (S) (%)
Glycerol	-1.8	66.1	22.1
Glycol	-1.4	46.1	47.6
Acetonitrile	0	nil	nil
n-butyl alcohol	0.9	2.0	nil
Benzene	2.1	41.0	12.9
Toluene	2.7	49.6	11.5
Cyclohexane	3.4	56.7	40.5
n-Hexane	3.9	58.1	42.6
Petroleum ether	4.0	67.1	57.7
Isooctane	4.5	90.0	67.2
Dibutyl phthalate	5.4	98.1	36.6

[a]Reduction conditions: 1.2 g dried cells, 3.78 g glucose, 7.5 mL phosphate buffer (0.05 mol/L, pH 7.0), 22.5 mL organic solvent containing 0.1 mol/L ECOB, aerobic, 30 ºC, 180 rpm, 24 h

TABLE-3
EFFECT OF THE V_{aq}/V_{org} ON THE REDUCTION OF ECOB IN WATER/*n*-DODECANE BIPHASIC SYSTEM[a]

V_{aq}/V_{org} (mL/mL)	Yield (%)	e.e.(S) (%)	Initial specific reduction rate (mM/h)
1:4	10.0	-19.9	94.3
1:3	18.5	-16.8	102.6
1:2	28.9	-6.0	112.7
1:1	57.3	59.6	137.0
2:1	86.2	80.1	135.5
3:1	89.3	86.8	145.6
4:1	83.2	76.7	138.6

[a]Reaction conditions are similar to Table-2.

Effect of amount of dried yeast cells on the reduction: The influence of amount of dried yeast on the reduction of ECOB was shown in Fig. 1. As the amount of dried yeast increased, the yield of ECHB increased. When 55 g/L of dried yeast was used, 87 % of ECHB was obtained. There were enough oxidoreductases for the reaction when the amount of dried yeast was 55 g/L. However, when the amount of catalyst exceeded 45 g/L, the e.e.(S) of ECHB decreased sharply. The stereoselectivity of the yeast was influenced by the growth period. It may be possible that too much yeast resulted in growth competition and glucose may consumed much faster. Later yeasts had not enough energy and kept in the condition of growth end. The experiment was repeated three times. The reason is further investigated in detail. Considering the yield and the e.e. (S) of ECHB, the optimum amount of dried yeast should be around 50 g/L.

Effect of the amount of glucose as co-substrate on the reduction: In the cell metabolic processes, glucose as co-substrate was applied to produce NAD(P)H continuously, which performed the catalytic reaction successfully. In contrast to isolated enzymes, this approach thus constitutes a good solution to avoid the addition of very costly cofactors like NAD(P)H during the process[14]. As shown in Fig. 2, the amount of glucose (0.6 mol/L) was considered to be the optimum condition under which a higher yield (90.68 %) with an excellent optical purity of the product (88.82 % e.e.) was obtained. In addition, both the yield and the purity of product decreased with a high concentration of glucose, which may result from the inhibition of activity of (S)-alcohol dehydrogenase.

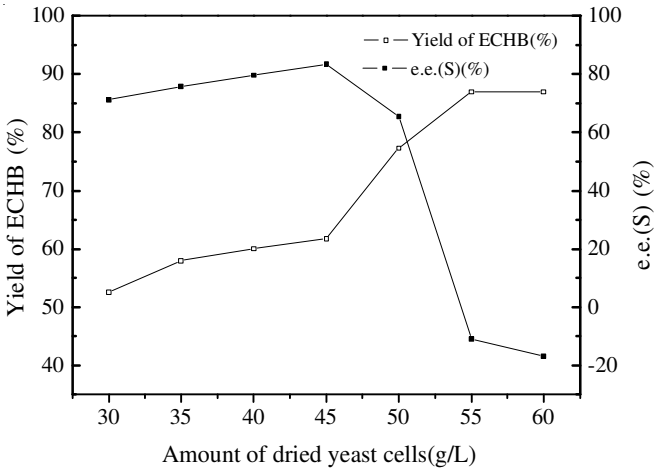

Fig. 1. Effect of the amount of dried yeast cells in water/isooctane biphasic system at 30 ºC, pH 7.0, Vaq/Vorg =3/1

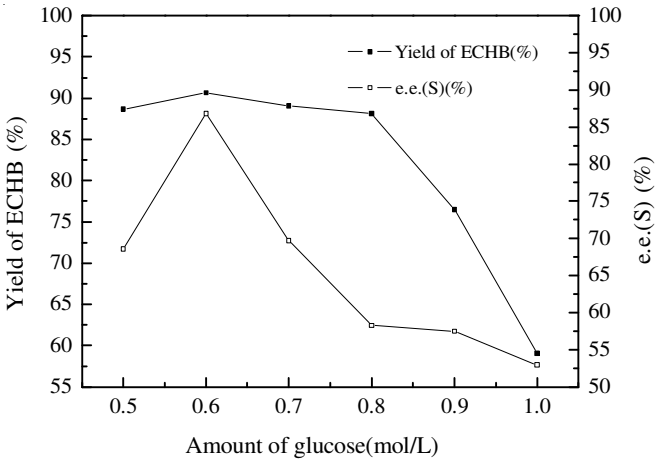

Fig. 2. Effect of the amount of glucose in water/isooctane biphasic system. 30 ºC, pH 7.0, V_{aq}/V_{org} =3/1, 35 g/L dried Baker's yeast

Effect of the substrate concentration on the reduction: Experiments with different original substrate concentrations from 0.02 mol/L to 0.3 mol/L were arranged to study the effect of the substrate concentration on the reduction of ECOB in the water/isooctane biphasic system. The results shown in Fig. 3. From Fig. 3, we found that the yield and the e.e.(S) of ECHB increased with increasing substrate concentration. When substrate concentration was 0.08 mol/L, the best yield and e.e.(S) of ECHB was 92.0 % and 89.1 %, respectively. However, when the substrate concentration exceeded 0.08 mol/L, both the yield and e.e. (S) of ECHB decreased sharply. High concentration of organic solvent had the effect of the toxin on the yeast and inhibited the activity of alcohol dehydrogenase catalyzing ECOB to (S)-ECHB.

Conclusion

Baker's yeast possessed a good catalytic activity and enantioselectivity for the reduction of ECOB to produce (S)-

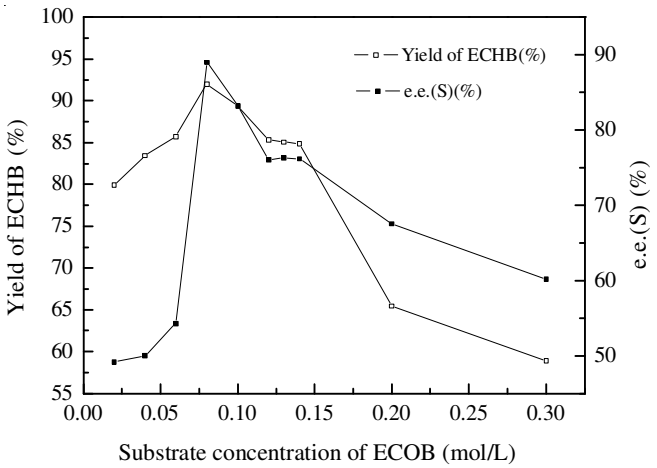

Fig. 3. Effect of the substrate concentration in water/isooctane biphasic system. 30 ºC, pH 7.0, V_{aq}/V_{org} = 3/1, 35 g/L dried Baker's yeast, 0.6 mol/L glucose

ECHB. The water/isooctane biphasic system was recommended for the reduction of ECHB. Among eleven biphasic systems assayed, isooctane has the lowest toxicity to the yeast cells. In the water/isooctane biphasic system, Baker's yeast could keep high viability and metabolicactivity and possessed a satisfactory catalytic activity and enantioselectivity. The optimum volumetric phase ratio (V_{aq}/V_{org}), substrate concentration, the amount of dried yeast cells and glucose were 3/1, 0.08 mol/L, 50 g/L and 0.6 mol/L, respectively, under which 93.9 % of molar conversion and 91.4 % e.e. of the optical purity of the product were obtained.

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REFERENCES

1. S. Shimizu, M. Kataoka and K. Kita, *J. Mol. Catal. B: Enzym.*, **5**, 321 (1998).
2. J. Jung, H.J. Park, K.N. Uhmb, D. Kimc and H.K. Kim, *Biochimica et Biophysica Acta*, **1804**, 1841 (2010).
3. K. Buchholz, V. Kasche and U.T. Bornscheuer, Biocatalysts and Enzyme Technology, Wiley-VCH, Weinheim, pp. 111-170 (2005).
4. M. Amidjojo and D.W. Botz, *Tetrahedron: Asymm.*, **16**, 899 (2005).
5. E.M.T. Buque, A.J. Straathof, J.J. Heijnen and L.A. van der Wielen, *Biotechnol. Bioeng.*, **86**, 795 (2004).
6. D.J. Pollard and J.M. Woodley, *Trends Biotechnol*., **25**, 66 (2006).
7. J.Y. He, Z.H. Sun, W.Q. Ruan and Y. Xu, *Proc. Biochem.*, **41**, 244 (2006).
8. A. Forni, E. Caselli, F. Prati, M. Bucciarelli and G. Torre, *ARKIVOC*, 45 (2002).
9. T. Suzuki, H. Idogaki and N. Kasai, *Bioorg. Med. Chem. Lett.*, **6**, 2581 (1996).
10. A. Wolfson, C. Dlugy, D. Tavor, J. Blumenfeld and Y. Shotland, *Tetrahedron: Asymm.*, **17**, 2043 (2006).
11. Y. He, Z.H. Sun, P. Zhen and L. Han, *J. Chem. Eng. Chin. Univ.,* **2**, 310 (2007) (in Chinese).
12. Y.G. Shi, Ph Dissertation, Asymmetric Reduction of Ethyl 2-oxo-4-phenyl Butyrate Catalyzed by Baker's Yeast, p. 38 (2009) (in Chinese).
13. P. Fernandes, P. Vidinha, T. Ferreira, H. Silvestre, J.M.S. Cabral and D.M.F. Prazeres, *J. Mol. Catal. B: Enzym.*, **19-20**, 353 (2002).
14. D. Dennewald, W.R. Pitner and D.W. Botz, *Process Biochem.*, **46**, 1132 (2011).