Available online at www.sciencedirect.com

SCIENCE DIRECT®

Tetrahedron: Asymmetry

Tetrahedron: *Asymmetry* 16 (2005) 899–901

Asymmetric synthesis of the chiral synthon ethyl (*S*)-4-chloro-3-hydroxybutanoate using *Lactobacillus kefir*

Maya Amidjojo and Dirk Weuster-Botz*

Lehrstuhl für Bioverfahrenstechnik, Technische Universität München, Boltzmannstr. 15, D-85748 Garching, Germany

Received 30 November 2004; accepted 10 January 2005

Abstract—*Lactobacillus kefir* was used as the whole cell biocatalyst for the asymmetric reduction of ethyl 4-chloro acetoacetate **1** to the chiral synthon ethyl (*S*)-4-chloro-3-hydroxybutanoate **2**. Ketoester **1** was obtained as micro-droplets, without the use of an organic solvent as substrate reservoir. **2** (1.2 M) was produced using 2-propanol as co-substrate with a final yield of 97% within 14 h. A high space-time yield and a high specific product capacity of 85.7 mmol/L h and of 24 mmol/g_{DCW} were measured. The enantiomeric excess of the (*S*)-alcohol **2** was 99.5%.
© 2005 Elsevier Ltd. All rights reserved.

1. Introduction

Chiral C3- and C4- building blocks are important intermediates for the synthesis of pharmaceuticals and fine chemicals.[1] Ethyl (*S*)-4-chloro-3-hydroxybutanoate **2** ((*S*)-CHBE), for instance, is a key intermediate for HMG-CoA reductase inhibitors, a compound class which lowers the cholesterol level in human blood.[2]

Microbial transformations using whole cells as biocatalysts for these bioreductions have been well investigated. For the stereoselective reduction of the prochiral ketoester ethyl 4-chloro acetoacetate (CAAE) **1** to the corresponding (*S*)-CHBE **2** various processes using yeasts have been published.[1,3,4] Nevertheless, a main problem with yeast bioreductions is the low enantiomeric excess of the alcohols, due to many reductases with competitive stereoselectivities present in the yeasts.[5] To overcome this problem various approaches have been suggested, such as heat treatment of the cells or their preparation with acetone,[1,3,6] the use of a two-phase organic/aqueous-system,[5] a slow release of the ketoester substrates,[5] the selection of a suitable co-substrate,[5] the addition of enzyme inhibitors[7] and the use of recombinant microorganisms.[4,8]

The wildtype bacterium *Lactobacillus kefir* was proven to perform the asymmetric synthesis of the (*S*)-alcohol **2** with an enantiomeric excess >99%.[9] Herein 15 mM of CAAE **1** were converted with a yield of 100% into the corresponding (*S*)-CHBE **2**. This (*S*)-CHBE **2** concentration is rather low and thus not relevant for further industrial applications.

We herein report the stereoselective reduction of **1** to **2** using wildtype *L. kefir* aiming at (*S*)-CHBE **2** concentrations comparable to existing yeast processes (see Fig. 1).

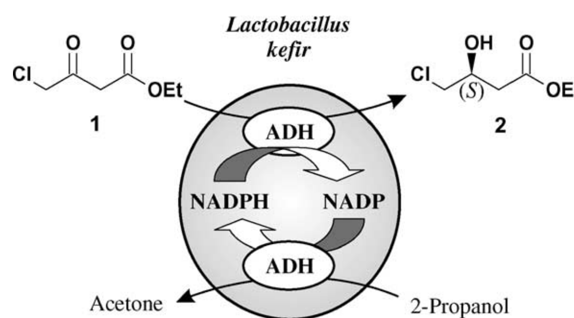

Figure 1. Scheme of the asymmetric reduction of ethyl 4-chloro acetoacetate **1** to ethyl (*S*)-4-chloro-3-hydroxybutanoate **2** by *Lactobacillus kefir* with regeneration of the cofactor NADPH using 2-propanol as co-substrate.

2. Results and discussion

For the conversion of CAAE **1** (300 mM) the specific product capacity and the enantiomeric excess were

* Corresponding author. Tel.: +49 89 2891 5712; fax: +49 89 2891 5714; e-mail: d.weuster-botz@lrz.tum.de

0957-4166/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetasy.2005.01.013

compared at a co-substrate concentration of 450 mM glucose and 20% v/v 2-propanol, respectively. The conversion stopped with both co-substrates after 5 h. A yield of 100% was measured using 2-propanol as co-substrate. The yield with glucose as co-substrate was much lower (22%). The same difference was observed with respect to the specific product capacity (see Fig. 2). With 2-propanol as co-substrate (*S*)-CHBE **2** (5.7 mmol) was formed by 1 g of the biocatalyst (dry cell weight, DCW). The specific product capacity was much lower (1.3 $mmol/g_{DCW}$) for the conversion with glucose as co-substrate. Furthermore the enantiomeric excess was increased from 87% with glucose as co-substrate to >99% if 2-propanol was used.

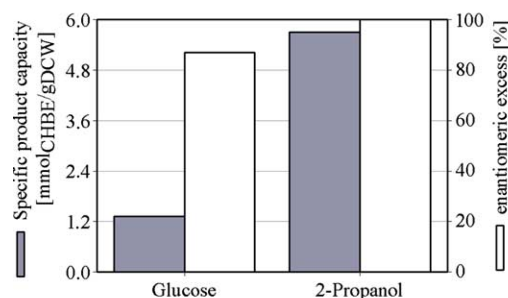

Figure 2. Comparison of the specific product capacity and the enantiomeric excess of **2** for the cofactor regeneration with glucose and 2-propanol, respectively.

Investigations with different 2-propanol concentrations between 5% and 20% v/v and 450 mM CAAE **1** showed that a 2-propanol concentration of 5% v/v was sufficient to achieve a yield of 100% within 3 h.

Based on these results the asymmetric reduction of CAAE **1** to the corresponding (*S*)-alcohol **2** was carried out in a stirred-tank reactor on a 200 mL-scale with 5% v/v 2-propanol (see Fig. 3a). CAAE **1** was added in two steps with a concentration of 620 mM at a time, due to its instability in aqueous media and an assumed decrease of stereoselectivity at too high ketoester **1** concentrations.[5] A final (*S*)-CHBE **2** concentration of 1.2 M and an enantiomeric excess of 99.5% was achieved within 14 h. The final yield was 97%. The space-time yield and specific product capacity were 85.7 mmol/(L*h) and 24 $mmol/g_{DCW}$, respectively.

Figure 3b shows the conversion rates of the bioreduction. The drawn lines represent first order decay of the biocatalyst. At the end of the first section, a CAAE **1** limitation could be observed. This is indicated by an increase of the conversion rate after the second CAAE **1** dosage at 3.5 h. A half-life of 2.9 h was estimated for *L. kefir* based on the measured (*S*)-CHBE **2** concentrations.

Compared to the reference conversion with *L. kefir* reported by Arragozini et al. the final concentration of **2** was improved 80-fold.[9] Despite the often observed decreases in stereoselectivity for high ketoester concentrations in the aqueous phase,[5,10] no losses in stereoselectivity were observed with *L. kefir*.

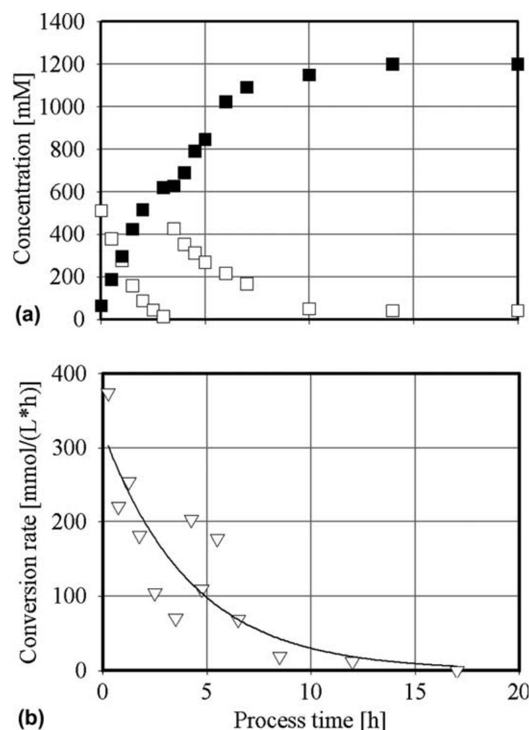

Figure 3. (a) Concentrations of CAAE **1** (□) and (*S*)-CHBE **2** (■) in a stirred-tank reactor (potassium phosphate buffer, 0.2 M, pH 6.5, 5 mM $MgCl_2$, 30 °C). CAAE **1** was added at $t = 0$ h and $t = 3.5$ h, respectively. (b) Conversion rates (▽) and estimation of the conversion rates based on a first order decay of the *Lactobacillus kefir* cells (drawn line).

Our process with *L. kefir* resulted in an improvement to the final (*S*)-CHBE **2** concentration, as well as in stereoselectivity, if compared to yeast bioreductions performed in organic/aqueous two-phase systems with NADP addition. For the synthesis of **2** with the yeast *Candida magnoliae* a final product concentration of 680 mM was reported at complete conversion. The enantiomeric excess was 96%.[3] Using a recombinant *Pichia pastoris* strain, 350 mM of **1** were converted with a yield of 91% at an enantiomeric excess of 95%.[4]

High stereoselectivity comparable to our *L. kefir* conversion was reported using a recombinant *E. coli* with glucose as co-substrate.[8] Herein the final (*S*)-CHBE **2** concentration was 1.25 M in aqueous media and 2.58 M in an organic/aqueous two-phase system. Nevertheless the addition of NADP was necessary in both cases, because the *E. coli* cells needed to be permeabilized by a pretreatment with Triton X-100.

3. Conclusion

L. kefir was proven to be an effective wildtype biocatalyst for the asymmetric reduction of the ketoester **1** to its corresponding (*S*)-alcohol **2** without making use of an organic solvent. Compared to all the other reported processes, no addition of cofactor was necessary, due to the successful use of the intracellular cofactor regeneration system with 2-propanol as co-substrate. Compared to existing (*S*)-CHBE **2** syntheses the

bioreduction process by whole cells of *L. kefir* is thus a competitive alternative considering the process parameters stereoselectivity, yield and final product concentration.

4. Experimental

4.1. Chemicals

All chemicals were purchased from VWR/Merck except ethyl 4-chloro 3-hydroxybutanoate [((*R*)- and (*S*)- enantiomers)] used for analytical purposes, which were obtained from Sigma-Aldrich.

4.2. Microorganism

The biocatalyst (*L. kefir* DSMZ 20587) was purchased from Juelich Fine Chemicals, Juelich, Germany and stored at −20 °C.

4.3. Bioreductions

The bioreductions were performed with resting cells of *L. kefir*. Prior to the conversions, the cells were washed once in 0.2 M potassium phosphate buffer, pH 6.5. After centrifugation at 4500 rpm for 10 min (Hettich GmbH & Co.AG, Tuttlingen, Germany) the supernatant was removed.

For the comparison of co-substrates potassium phosphate buffer with 0.2 M, pH 6.5, 5 mM $MgCl_2$, 450 mM glucose and 20% v/v 2-propanol, respectively, were used. The cell pellet was suspended in the buffer (25 mL) in a way that 50 g_{DCW}/L was achieved. The reaction was started by adding **1** (1 mL). The bioreductions were performed at 30 °C in stirred flasks (multipoint magnetic stirrer, H + P Labortechnik AG, Oberschleissheim, Germany). The conversion of **1** and the production of **2** was monitored by GC analysis throughout the whole process.

The investigations on initial 2-propanol concentrations followed the same procedure. The same buffer (25 mL) with 5% v/v to 20% v/v 2-propanol was applied. The reduction was started by addition of 1.5 mL of **1**.

The cells for the bioreduction with dual CAAE **1** addition were dissolved in 183.5 mL buffer as described before with 5% v/v 2-propanol. The reaction was started by adding 16.5 mL of **1**. The initial *L. kefir* concentration was adjusted to 50 g_{DCW}/L. After 3.5 h, 16.5 mL of the ketoester **1** was added again. The bioreductions were performed at 30 °C in a stirred tank reactor (Infors AG, Bottmingen, Switzerland). Ketoester CAAE **1** was provided as micro-droplets. The power input for the bioreduction was 0.54 kW/m^3. The estimated mean droplet diameter for **1** was <43 μm. The conversion of **1** and the production of **2** was monitored by GC analysis throughout the whole process. for all bioreductions performed. The conversion rate was estimated based on the measured (*S*)-CHBE **2** concentrations using the central difference approximation.

4.4. Analysis

The concentrations of CAAE **1** and (*S*)-CHBE **2** and (*R*)-CHBE were determined by chiral gas chromatography on a CP-3800 system (Varian, Darmstadt, Germany) equipped with a Chiraldex-GTA column (20 m × 0.25 mm ID, Astec Inc., Whippany, USA) and an FID-detector (Varian, Darmstadt, Germany). Ethyl acetate was used as the organic solvent for the extraction of the components from the aqueous phase. For this 800 μL of ethyl acetate and 500 μL of glass beads (0.2 mm diameter) were added to 400 μL of reaction suspension. For the extraction, the samples were mixed using a Thermomixer (Eppendorf AG, Hamburg. Germany) for 15 min at 1400 min^{-1}. After centrifugation at 13,000 rpm for 5 min the ethyl acetate supernatant was diluted in anhydrous ethyl acetate (1:5 v/v). The diluted ethyl acetate phase could then be used for GC analytics.

References

1. Shimizu, S.; Kataoka, M. *Adv. Biochem. Eng./Biotechnol.* **1999**, *63*, 109–123.
2. Thottathil, J. K.; Pendri, Y.; Li, W.-S.; Kronenthal, D. United States Patent 1994; US5278313.
3. Yasohara, Y.; Kizaki, N.; Hasegawa, J.; Takahashi, S.; Wada, M.; Kataoka, M.; Shimizu, S. *Appl. Microbiol. Biot.* **1999**, *51*, 847–851.
4. Engelking, H.; Pfaller, R.; Wich, G.; Weuster-Botz, D. *Tetrahedron: Asymmetry* **2004**, *15*, 3591–3593.
5. Sybesma, W. F. H.; Straathof, A. J. J.; Jongejan, J. A.; Pronk, J. T.; Heijnen, J. J. *Biocatal. Biotransform.* **1998**, *16*, 95–134.
6. Patel, R. N. *Enzyme Microb. Technol.* **2002**, *31*, 804–826.
7. Nakamura, K.; Inoue, K.; Ushio, K.; Oka, K.; Ohno, A. *Chem. Lett.* **1987**, *9*, 679–682.
8. Kizaki, N.; Yasohara, Y.; Hasegawa, J.; Wada, M.; Kataoka, M.; Shimizu, S. *Appl. Microbiol. Biotechnol.* **2001**, *55*, 590–595.
9. Arragozini, F.; Valenti, M.; Santaniello, E.; Ferraboschi, P.; Grisenti, P. *Biocatalysis* **1992**, *5*, 332–352.
10. Kometani, T.; Yoshii, H.; Kitatsuji, E.; Nishimura, H.; Matsuno, R. *J. Ferment. Technol.* **1993**, *76*, 33–37.