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Stereoselective reduction of ethyl 4-chloro acetoacetate with recombinant *Pichia pastoris*

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Abstract—A recombinant strain of *Pichia pastoris* coexpressing the carbonyl reductase of *Candida magnoliae* and the glucose dehydrogenase of *Bacillus subtilis* was used for the stereoselective reduction of ethyl 4-chloro acetoacetate **1** to (*S*)-4-chloro-3-hydroxybutanoate **2**. The bioreduction was performed in a two-phase reaction system with *n*-butyl acetate as organic solvent. Complete conversion of 350 mM **1** with a final yield of 91% and an enantiomeric excess of 95% was achieved by continuous feeding of cell suspension and ketoester **1**.

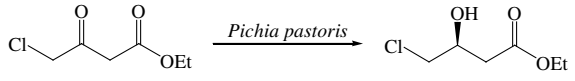
1. Introduction

Optically active alcohols can easily be obtained by microbial reduction of prochiral ketones. The stereoselective reduction of ethyl 4-chloro acetoacetate (CAAE) **1** to (*S*)-4-chloro-3-hydroxybutanoate [(*S*)-CHBE] **2**, a chiral building block in the pharmaceutical industry, can be catalyzed by various yeasts.[1,2] The enantiomeric excess (ee) is often low due to many different stereoselective reductases present in yeasts.[3,4] The use of recombinant microorganisms holds great potential to overcome these limitations.[3,5]

Ketoester **1** is unstable in aqueous media. A two-phase organic-solvent reaction systems can be used to prevent the decomposition of **1** by minimizing its concentration in the aqueous phase.[5–7] At the same time, the inhibitory effect of the ketoester on the biocatalyst is reduced.

The yeast *Candida magnoliae* reduces ketoester **1** to the corresponding alcohol **2** with an enantiomeric excess of 96%.[8] This NADPH-depending carbonyl reductase with (*S*)-directed stereoselectivity has been isolated, characterized and expressed in *E. coli*.[9] By coexpressing a glucose dehydrogenase of *Bacillus megaterium* for cofactor regeneration, the (*S*)-alcohol **2** was obtained with an ee of 100% and a yield of 85%.[9]

Herein we report the stereoselective reduction of **1** to the corresponding (*S*)-alcohol **2** using a recombinant strain of *Pichia pastoris* (Fig. 1). The carbonyl reductase of *C. magnoliae* and a glucose dehydrogenase derived from *Bacillus subtilis* for improved cofactor regeneration were coexpressed under control of the methanol induced AOX promotor.



Ketoester **1** (*S*)-Alcohol **2**

Figure 1. Reduction of ethyl 4-chloro acetoacetate **1** to (*S*)-4-chloro-3-hydroxybutanoate **2** by recombinant *Pichia pastoris*.

2. Results and discussion

The bioreduction of **1** was carried out in aqueous media as well as in organic-solvent/water two-phase systems. The addition of small amounts of NADP (0.1 mM) was necessary to achieve sufficient reduction rates. In the aqueous reaction system, a final concentration of **2** of 21 mM and an enantiomeric excess of 97% was achieved within 24 h. The reaction was limited by the inhibitory effects on the biocatalyst and the decomposition of ketoester **1**. To overcome these limitations, four biphasic reaction systems were studied by adding water immiscible organic solvents to the reaction medium.

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With *n*-butyl acetate as the organic solvent, product concentration was increased by 50% when compared to the reduction in an aqueous reaction system with the enantiomeric excess reaching >98% (Fig. 2). (*S*)-alcohol **2** (4.1 mmol) was formed by 1 g of biocatalyst (dry cell weight).

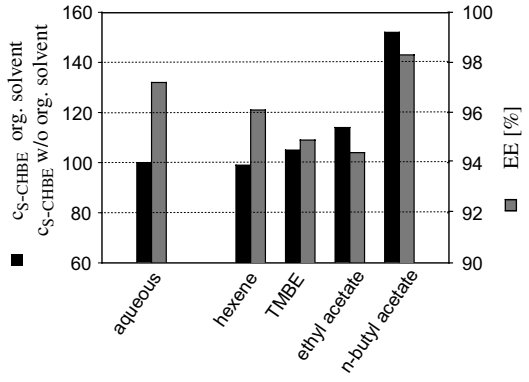

Figure 2. Effect of the organic solvents on the concentration of **2** and the enantiomeric excess. The relative concentration of **2** is calculated relative to the bioreduction in a monophasic aqueous reaction system.

Previously published results have demonstrated that the carbonylreductase is most stable in the presence of *n*-butyl acetate when compared to other organic solvents.[10] Furthermore, *n*-butyl acetate shows good extraction properties towards **1** and **2**.

The reduction efficiency was further improved by adding 6.5% v/v Triton X100 thus increasing the yield by 60% compared to the aqueous reaction system. Triton X100 leads to a permeabilization of the cells and therefore to an increased mass transfer.[11] Furthermore, the surfactant reduces the equilibrium droplet size of the *n*-butyl acetate in water and therefore increases the interfacial area.

Lysis was observed in the presence of *n*-butyl acetate. In order to maintain the stability and activity of the biocatalyst cell suspension and substrate solved *n*-butyl acetate were fed into the reactor.

The feeding rate of the substrate led to an accumulation of **1** during the first 15 h of the process (Fig. 3). The cell

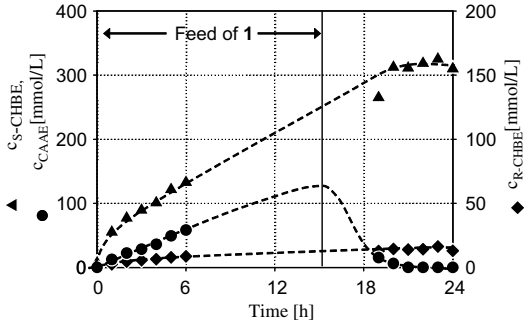

Figure 3. Concentration of CAAE **1**, (*R*)-CHBE and (*S*)-CHBE **2** in a fedbatch reduction. CAAE was fed for 15 h and cell suspension for 20 h; dotted line: expected course of concentrations.

suspension was fed for 20 h. Complete conversion of **1** (350 mM) was achieved within 24 h. The yield of the reaction and the enantiomeric excess was 91% and 95%, respectively. Compound **1** (5.8 mmol) was formed by 1 g of biocatalyst (dry cell weight). This is an improvement of 41% when compared to the batch reaction. The yield was higher than reported for bioreductions with recombinant *E. coli* being 85%, while the enantiomeric excess did not reach 100%.[5]

3. Conclusion

The recombinant yeast *P. pastoris* is a useful tool for the stereoselective reduction of β-keto ester **1** to the corresponding (*S*)-alcohol **2**. The decomposition of the substrate and the inhibitory effect on the biocatalyst were significantly reduced in a two-phase reaction system containing *n*-butyl acetate as the organic phase and by continuously feeding substrate **1** and the biocatalyst.

4. Experimental

4.1. Chemicals

All chemicals were purchased from VWR/Merck except yeast nitrogen base (YNB), which was obtained from Difco.

4.2. Microorganism

The methylotrophic yeast strain *Pichia pastoris* GC909 over-expressing the glucose dehydrogenase gene of *Bacillus subtilis* and the carbonyl reductase gene of *Candida magnoliae*, both under control of the AOX promoter, was provided by the Consortium für Elektrochemische Industrie GmbH, München, Germany.

4.3. Growth conditions

Cells were grown in 1 L shaking flasks with 200 mL complex media (10 g/L yeast extract, 20 g/L peptone, 13.4 g/L yeast nitrogen base, 10 g/L glycerol, 0.4 mg/L biotin, 0.1 mol/L potassium phosphate puffer, pH 6.0) at 28 °C for three days on a rotary shaker at 250 rpm. The expression of recombinant proteins was induced by adding methanol (2 mL) every 24 h for three days. The biomass was collected by centrifugation.

4.4. Bioreductions

For batch reductions, cells were dissolved in 20 mL reaction buffer (1 M glucose, 0.1 M sodium chloride, 0.1 M potassium phosphate buffer, pH 7.0 or 0.1 mM NADP). The reaction was started by adding **1** (0.5 mL) and 1.5 mL of ethyl acetate, *n*-butyl acetate, *tert*-butyl methyl ether (TBME) or hexane, respectively. Flasks were incubated on a magnetic stirrer at 30 °C for 24 h.

For fedbatch bioreductions, cells were dissolved in reaction buffer as described above to a final volume of

180 mL and 60 g/L dry cell weight. Sixty millilitres of the suspension was used as starting volume and 120 mL of the suspension continuously fed for 22 h at 5.5 mL/h using a cassette pump (Watson-Marlow, England). Ketoester **1** (10 mL) was added to *n*-butyl acetate (30 mL) and continuously fed for 15 h (2.6 mL/h) using a parallel titration system (FedBatch Pro, Dasgip, Germany). The pH was controlled at pH 7.0 by adding 5 M NaOH. The reaction vessel was magnetically stirred and incubated at 30 °C for 24 h. NADP was added to a final concentration of 0.1 mmol/L after 6 h.

4.5. Analytics

The reaction mixture (0.5 mL) was extracted by adding ethyl acetate (1 mL), phosphoric acid (0.1 mL) (45% v/v) and 0.2 mL glass beads (0.5 mm in diameter). The samples were mixed in a ball mill (Mixer Mill MM200, Retsch GmbH, Haan, Germany) for 10 min at $30\,s^{-1}$. The samples were centrifuged at 12,000*g* for 5 min for separating the phases. The supernatant was diluted in anhydrous ethyl acetate (1:5 v/v). The concentrations of ketoester **1**, (*S*)-alcohol **2** and (*R*)-alcohol were measured using chiral gas chromatography (Lipodex E column, 25 m × 0.32 mm i.d., Macherey Nagel, Düren, Germany). Acetophenone was used as the internal standard.

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