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The effect of absorbing resins on substrate concentration and enantiomeric excess in yeast reduction

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Abstract: The correlation between the enantiomeric excess (e.e.) of the (*S*)-(+)-ethyl-3-hydroxybutanoate **2**, obtained in the baker's yeast reduction of ethyl acetoacetate **1**, and the concentration of the substrates in the fermentation mixture has been studied by the use of two different techniques (absorbing resins and organic solvents) The presence of resins undoubtedly influences the enantiomeric excess of the product. © 1997 Elsevier Science Ltd

Introduction

The use of whole cell biocatalysts is increasingly applied for the synthesis of small molecules especially for the preparation of enantiomerically pure compounds[1]. Purified hydrolytic enzymes, which do not require added cofactors, are among the most popular biocatalysts for this purpose along with reducing enzymes (oxidoreductases) found in micro-organisms. However, purified oxidoreductases require expensive cofactors for effecting the catalytic step. Therefore, whole cells are preferentially used, thus circumventing the difficult problem of efficient recycling of the cofactor[2]. Whole cells usually express a multitude of enzymatic activities. Therefore, when using micro-organisms as biocatalysts, the crucial problem encountered is selectivity. It is well known that in the reduction of a carbonyl group by yeast cells, unsatisfactory enantioselectivity is often the result of the simultaneous action of several enzymes, which display both different kinetics and different selectivity on the same substrate[3]. In these cases the selectivity of the reductive step can be strongly influenced by substrate concentration[4]. In order to achieve a more selective system in bakers yeast (b.y.) reductions, it is of great importance to be able to control the reaction conditions so that the desired enzyme activity is favoured and/or the undesired ones are suppressed[5]. Ideally, the properties of all the enzymes that are able to interfere with the desired enzyme activity should be well known. However, due to the complexity of the whole cell systems, the many techniques used for obtaining an improved selectivity are usually only empirical variation based on trial and error.

Enantiomerically pure 3-hydroxybutanoates are chiral building blocks of wide use and importance in organic synthesis. They can be obtained using biocatalysis either by reduction of the corresponding ketoesters or by alternative biocatalytic procedures[6]. The reduction of 3-oxo-esters with baker's yeast is well established. Thus, (*S*)-ethyl-3-hydroxybutanoate **2** of 85% e.e. is obtained under normal fermenting conditions[7]. Hydroxyester **2** of considerably higher enantiomeric purity is obtained if the concentration of the substrate **1** is kept low by slow addition to the fermenting brew[8]. The product **2** is obtained in very high e.e. (>97%) albeit after a long time[9]. Another report describes the large scale preparation of the same compound by using high dilution techniques as well but employing ethanol as the energy source during fermentation[10]. It is evident that new methods for adjusting the substrate concentrations to optimum, should be of value in many biocatalytical processes especially those involving whole cell systems. Here we report a study on two techniques which can be used to reduce the substrate concentration in order to optimise the e.e. of the product. Thus, we have investigated

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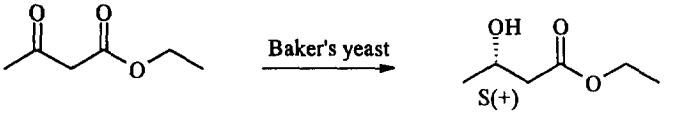
the use of absorbing resins and of organic solvents as means of reducing the substrate concentration in the water phase. Absorbing resins are widely used for the recovery of organic compounds from water. Their use in suspensions containing yeast biomass is possible since the significant difference in particle size allows the recovery of the resin by filtration using a sintered glass funnel with suitable porosity[11]. The b.y. reduction of ethyl 3-oxobutanoate **1** was selected, as a model reaction (Scheme 1) for our study, because this reaction is well known in many details and because the enantiomeric excess of the alcohol **2** produced can now easily be monitored by GLC on a chiral stationary phase (see Experimental).



1 **2**

Scheme 1.

Results and discussion

Of the resins used, four showed an effect on the e.e. of the product (Figure 1) which was raised from 78% in the blank experiment to 89 and 91% when Dow24D or Amberlite XAD1 were employed and to 93 and 94% when Amberlite XAD7 and XAD1180 were used respectively.

Since the resins do not interfere in other ways with the fermentation medium, and the yield of recovered product was not effected by the resin itself, the observed increased enantioselectivity can be attributed to reduced concentration of the substrate in the water phase. The higher the partition coefficient resin/water, the higher was the e.e. observed. The enantiomeric excess was also dependent on the amount of resin used (Figure 2). However, if higher e.e. values than previously reported are desired the amount of resin required becomes too large for practical applications.

In a series of experiments the substrate concentration in water was controlled and measured with different amount of resin or without absorbing resin. The presence of a resin clearly influenced the product e.e. For instance, when a concentration of substrate of 3 g/l was used, the e.e. of the product was 82% (dried yeast) and dropped to 70%, when the concentration was raised to 6 g/l. After adding resin to a mixture with the initial concentration at 6 g/l, the measured concentration in the water phase became 3.8 g/l. The product **2** was obtained in 84% e.e. This shows that absorbent resins can be used profitably in controlling the substrate concentration and hence the enantioselectivity of reductions in whole cell biotransformation. The use of ethanol instead of glucose as energy source consistently gave slightly higher e.e.. When dried yeast YSC-2 from Sigma was used the product e.e.s were lower both with and without resin present.

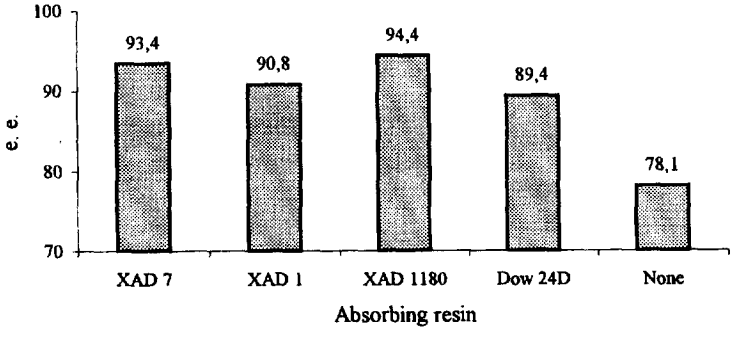


Figure 1. Enantiomeric excess of **2** using different absorbing resins.

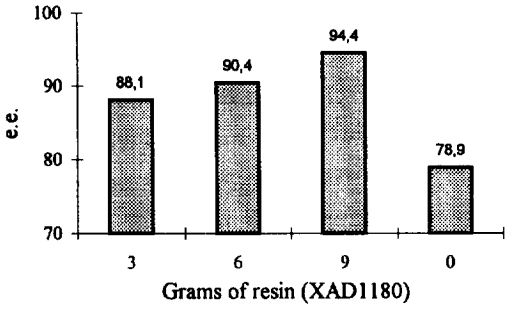


Figure 2. Enantiomeric excess of **2** and amount of resin.

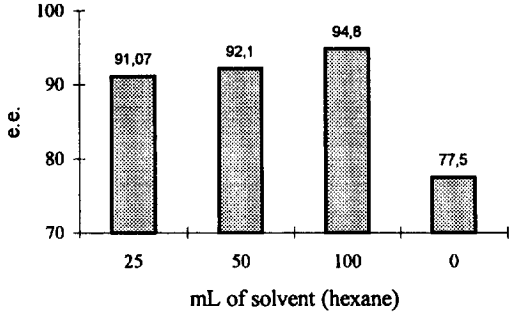


Figure 3. Enantiomeric excess of **2** and water/organic solvent ratio (water vol. 50 mL).

Organic solvents have been used with whole cells systems[12] in order to increase the substrate availability. This can be due either to increased mass-transfer following cell adhesion to the organic phase or simply due to an increased solvent induced cell wall permeability. In several cases increased e.e.s of the reduction products have been observed. Sometimes the solvent has been considered as being responsible either for an activation of enzymes or for selective inactivation of others. However, no conclusive experimental results have been presented.

We have now used biphasic organic solvent/water systems in b.y. reductions of compound **1**. The hypothesis was that these systems would allow efficient control of the substrate concentration in the water phase. Some solvent/water systems (EtOAc, MTBE, CH_2Cl_2) were not suitable for the biotransformation (conversion less than 3% in 24 h). In toluene the enantiomeric excess of **2** was 95.8% but the conversion was low (16%) due to a very slow rate. Since toluene is known to cause autolysis of yeast cells, it is not surprising that the conversion obtained in this reduction is limited. However, in the *n*-hexane/water system the conversion was complete in 24 h and the e.e. was 92.1%. Most of these reactions were carried out in a biphasic system containing equal amount of the two solvents. However, the best results (94.8% e.e.) were obtained when *n*-hexane was used as the solvent in a *n*-hexane/water ratio of 2:1 (Figure 3). Similar solvent like *n*-pentane or *n*-eptane gave comparable results with e.e. values ranging from 89.6 to 89.9% in a 1:1 mixture organic solvent/water.

The effect of the increased enantiomeric purity of the product in the presence of the organic solvent could be also attributed to diminished water concentration of the substrate. However, experiments of partition of the substrate showed that the water concentration of the ketoester using *n*-hexane as a solvent was not sensibly reduced. On the other hand experiments in complete anaerobiosys also showed an increased enantiomeric excess of the product (93.1% when *n*-hexane is present in a 1:1 ratio with water and 89.8 without organic solvent). In conclusion it seems that absorbing resins can alter the enantiomeric purity of the product in yeast reduction and that this is in connection with reduced

substrate concentration. The effect observed in the presence of a non polar solvent is probably due to the combination of several factors.

Experimental

General methods

Pressed yeast and dried yeast type II were obtained from Distillerie Italiane and from SIGMA, respectively. Absorbing resins were obtained from Fluka or directly from the producer.

Analytical procedures

GLC analysis were run on a DANI 8610 gas chromatograph equipped with PTV injector and FID detector. The conversion was followed by GLC on a glass capillary column, MEGAWAX (MEGA, Italy), 25 mt × 0.25 mm i.d., film thickness 0.25 μm, carrier gas H_2 0.7 bar. Temperature program: 40°C for 1 min, 10°C min^{-1}, 110°C, 2 min, 2°C min^{-1}, 130°C, detector 250°C. Rt: ethyl 3-oxobutanoate 8.54, ethyl 3-hydroxybutanoate 9.92. Diethyl malonate was used as an internal standard. The enantiomeric composition of the product **1** was monitored by direct GLC analysis on a chiral phase capillary column diAc–*t*BuSi–βCdx (MEGA, Italy), 25 mt × 0.25 mm i.d., film thickness 0.25 μm, carrier gas H_2 0.7 bar. Temperature program 40°C for 1 min, 20°C min^{-1}, 75°C 2 min, 1°C min^{-1}, 90°C, detector 250°C. Rt *S*-enantiomer 16.40, *R*-enantiomer 17.34. This method for the e.e. determination should be more accurate than the methods previously reported requiring derivatisation or relying on rotation values.

General procedures

The partition coefficient of the keto ester between the resin and water was determined in the following way: the substrate (0.4 g) was dissolved in 20 mL of ethyl acetate and the resin (2 g) was added to this solution and mixed for five min. The organic solvent was evaporated under reduced pressure and the residual resin poured into 20 mL of water and shaken for 10 min. The residual **1** in the resin was determined as follows: the resin was filtered off and washed with 2×10 mL of water. These combined water washings were extracted twice with 20 mL of ethyl acetate, which gave after evaporation of the organic solvent, 0.17 g of **1**. From the resin, extracted with 2×20 mL of ethyl acetate, additional 0.11 g of **1** was recovered. Thus the resin (in this case XAD1180) retained 0.28 g of **1** while releasing 0.12 g to the water phase in the first equilibration with water.

Microbial transformation

In a typical experiment the substrate **1** (1.3 g) was dissolved in ethyl acetate (20 ml) and the solvent removed in the presence of the added resin (6 or 9 g). The resin was then added to a prefermented (1 h) suspension of 13 g of pressed yeast in 100 ml of water containing 20 g of glucose at 28°C. The mixture was stirred on a linear shaker (120 movements min^{-1}) until all the substrate was consumed (12–56 h). The resin was filtered off and the suspension and the beads extracted with ethyl acetate separately. In experiments with dried yeast, 2.6 g were used with the same amount of substrate. Variations were: the type of resin and its relative amount, the use of ethanol instead of glucose, the use of non fermenting conditions. When organic solvents were used the typical procedure was similar to the one described, but various ratio of water/organic solvent were employed. A blank experiment without resin or solvent was run in parallel.

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References

1. Servi, S. *Chim. Ind.*, **1996**, *78*, 959–963.
2. Kula, M.R. Proceedings *Chiral Europe*, **1994**, 27–33.
3. Shieh, W.R.; Gopalan, A.S.; Sih, C.J. *J. Am. Chem. Soc.*, **1985**, *107*, 2993.

4. Chen, C.S.; Zhou, B.N.; Girdaukas, G.; Shieh, W.R.; VanMiddlesworth, F.; Gopalan, A.S.; Sih, C.J. *Bioorg. Chem.*, **1984**, *12*, 98–117.
5. Nakamura, K.; Inoue, K.; Ushio, K.; Oka, S.; Ohno, A. *Chemistry Letters*, **1987**, 679. Ushio, K.; Hada, J.; Tanaka, Y.; Ebara, K. *Enzyme Microb. Technol.*, **1993**,
6. Sheldon, R. A. *Chirotechnology*, **1993**, Marcel Dekker Inc. N.Y., pp 159 and 374.
7. Seebach, D.; Sutter, M.A.; Weber, R.H.; Züger, M.F. *Org. Synth.*, **1984**, *63*, 1–9.
8. Wipf, R.; Kupfer, E.; Bertazzi, R.; Leuenberger, H.G.W. *Helv. Chim. Acta*, **1983**, *66*, 485.
9. Liese, A.; Karutz, M.; Kamphuis, J.; Wandrey, C.; Kragl, U. *Biotechnol. Bioeng.*, **1996**, *51*, 544–550
10. Kometani, T.; Yoshii, H.; Kitatsuji, E.; Nishimura, H.; Matsuno, R. *J. Ferment. Bioeng.*, **1993**, *76*, 33–37.
11. Anderson, B.H.; Hansen, M.M.; Harkness, A.R.; Henry, C.L.; Vicenzi, J.T.; Zmijewski, M.J. *J. Am. Chem. Soc.*, **1995**, *117*, 12358–12359.
12. Nakamura, K.; Inoue, K.; Ushio, K.; Oka, S.; Ohno, A. *J. Org. Chem.*, **1988**, *53*, 2589–2593. Nakamura, K.; Inoue, K.; Ohno, A. *Tetrahedron Lett.*, **1995**, *36*, 265–266. Nakamura, K.; Kondo, S.I.; Kawai, Y.; Ohno, A. *Tetrahedron Lett.*, **1991**, *32*, 7075–7078.

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