Available online at www.sciencedirect.com
ScienceDirect
Tetrahedron: Asymmetry
Tetrahedron: *Asymmetry* 17 (2006) 3219–3225

Immobilisation of alcohol dehydrogenase from *Lactobacillus brevis* and its application in a plug-flow reactor

Falk Hildebrand and Stephan Lütz*

Institute of Biotechnology 2, Research Centre Jülich, D-52425 Jülich, Germany

Received 3 October 2006; accepted 9 November 2006

Abstract—The immobilisation of alcohol dehydrogenase from *Lactobacillus brevis* (E.C. 1.1.1.2) on an amino-epoxy support (amino-epoxy Sepabeads®) has been investigated with regards to increasing stability under storage and process conditions. After the standard immobilisation procedure resulted in no significant stabilisation, we found a fourfold increase in stability by blocking the remaining functional groups on the enzyme-support preparations with glycine or mercaptoethanol. However, stabilising the multi-point covalent attachment could only be achieved by additionally cross-linking the adsorbed proteins with glutardialdehyde. By this means, we achieved a high stabilisation effect, resulting in a half-life time of over 1200 h when stored at 30 °C. This means a 60-fold increase in stability compared to the soluble enzyme.

To determine the operational stability, the enzyme-support preparation was applied to the production of (*R*)-phenylethanol from acetophenone in a plug-flow reactor, which could be operated for over 10 weeks with an excellent enzyme utilisation.

1. Introduction

The industrial application of enzymes as highly selective biocatalysts for the production of fine chemicals is a fast-growing field.[1,2] When working on an industrial scale, continuously operated reactor setups should be favoured, since they offer the advantages of high volumetric productivities and low expenditure of human labour.

However, less than a quarter of all biotechnological processes conducted on an industrial scale are continuous processes.[3] To become economically feasible, attention must be paid to efficient utilisation of the biocatalyst[4,5] in industrial processes. In continuous processes, this can be achieved either by retaining the catalyst within the reactor by a membrane[6] or by immobilising it. An immobilised biocatalyst can easily be removed from the product solution, yielding protein-free product of a high purity. Over the last few decades, different methods for the immobilisation of biocatalysts have been developed, about which extensive reviews are available.[7–10] Among them covalent coupling to carrier supports,[11] cross-linking between enzyme molecules [cross-linked enzyme aggregates (CLEAs) or crystals (CLECs)],[12,13] adsorption on carriers,[14] ionic interactions[15] and encapsulation in polymeric gels or membranes[16,17] can be distinguished.

In addition to the facile handling of the biocatalyst in many cases the stability of the enzyme is increased by immobilisation.[18,19] However, these modifications often come with changes in the enzymatic structure, altering activity or selectivity of the enzyme. The possible loss in activity therefore has to be balanced against the benefits gained by the immobilisation.

Herein, we report the first immobilisation of the synthetically and industrially[20] important alcohol dehydrogenase from *Lactobacillus brevis* (*Lb*-ADH; E.C. 1.1.1.2) by covalent coupling to a carrier support. *Lb*-ADH catalyses the enantioselective reduction of a broad spectrum of carbonyl compounds to enantioenriched (*R*)-alcohols (Fig. 1),[21,22] which play an important role as precursors to a great number of pharmaceutical agents and natural products.[23] We will also show that by immobilising the biocatalyst, not only are we able to benefit from the advantages of a continuous reactor setup, but significant stabilisation effects are achieved as well.

Surprisingly, attempts to immobilise alcohol dehydrogenases are mostly confined to adsorption methods,[24] while

* Corresponding author. Tel.: +49 2461 61 4388; fax: +49 2461 61 3870; e-mail: s.luetz@fz-juelich.de

0957-4166/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetasy.2006.11.013

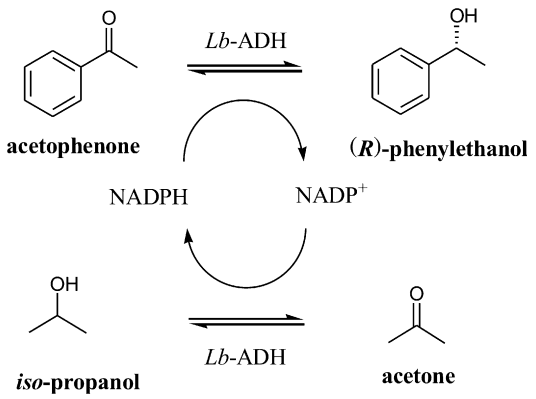

Figure 1. Enantioselective reduction of acetophenone by *Lactobacillus brevis*; substrate-coupled cofactor regeneration.

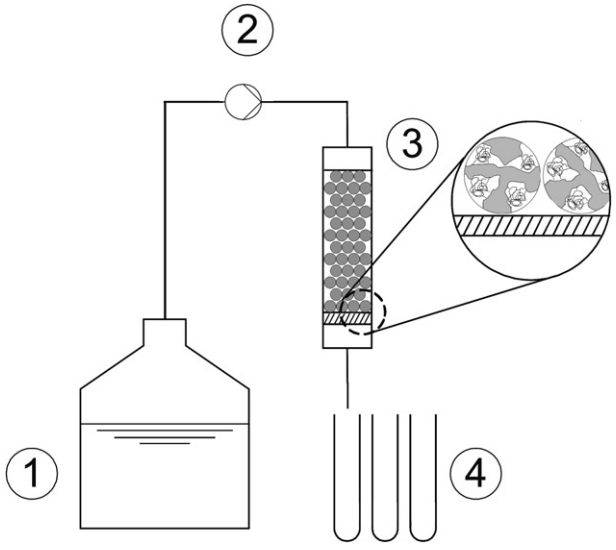

Figure 2. Process chart of the flow-tube reactor: (1) substrate solution; (2) pump; (3) immobilised biocatalyst, retained by a filter; (4) product solution.

only very few examples of covalent binding are known.[25,26] Furthermore, these immobilisation techniques are often designed for use in sensor equipment and not for synthetical applications. Concerning the alcohol dehydrogenase investigated from *L. brevis*, to the best of our knowledge, only one example of immobilisation is yet known, a physical adsorption on glass beads for use in gas-phase reactions.[27]

Thus, our aim was to find an immobilisation procedure for *Lb*-ADH, which gives the best combination of increase in stability and remaining activity. Sepabeads® were chosen as the carrier material because of their superior mechanical stability under reactor conditions when compared to other carrier materials. Among the different functionalities available, preliminary investigations showed the amino-epoxy species to be the most suitable carriers for the given enzyme. Furthermore, additional modifications of the enzyme-support preparations were also investigated. These preparations will be compared to each other regarding their remaining activities and stabilities in storage. The most promising ones will then be utilised in a continuously operated plug-flow reactor to determine their operational stability.

2. Results and discussion

2.1. Immobilisation of *Lb*-ADH on amino-epoxy Sepabeads®

According to the manufacturer, the immobilisation of proteins on amino-epoxy carrier supports proceeds via a two-step mechanism. Prior to covalent binding to the epoxy groups of the support, the protein must physically adsorb to the surface. Through additional amino groups on the support, this adsorption process is facilitated and proceeds at low buffer concentrations.[28,29] After the adsorption process, bonds are formed between the epoxy groups and the nucleophilic groups of the enzyme, resulting in a covalent multi-point attachment in which the enzyme conformation is more rigid and therefore more stable against inactivation.

At the beginning of our work, we examined the influence of the buffer type and concentration on the immobilisation process. Of the buffer systems investigated, a phosphate buffer with a pH of 7 proved to be the most suitable one. As was expected, the immobilisation rate depending on the buffer concentration showed the predicted tendency; it became more rapid when taking place in buffers of a low ionic strength (see Fig. 3). It is noteworthy that even at high buffer concentrations, more than 90% of the enzyme were adsorbed on the support, while other enzymes were reported to stay almost completely in the supernatant when mixed with the carrier material in buffers of a high ionic strength.[29]

When working in buffers of a low ionic strength, we obtained a remaining activity of the enzyme-support preparation of 15%. To investigate whether the immobilisation procedure had a stabilising effect on the enzyme, the thermal stabilities of the different enzyme preparations at

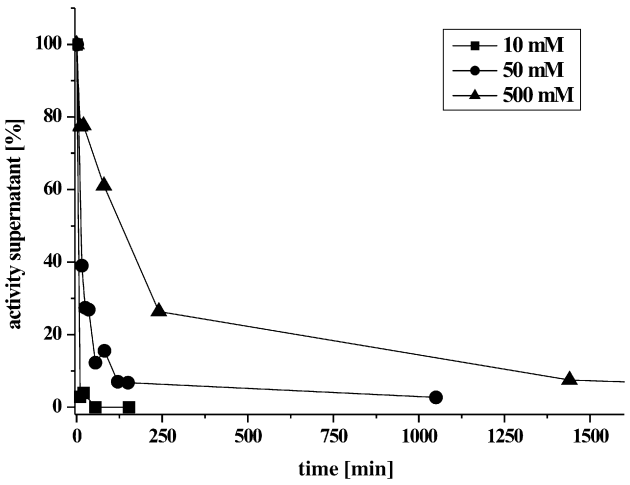

Figure 3. Adsorption rate of *Lb*-ADH on amino-epoxy Sepabeads® in different buffer concentrations.

30 °C were studied and compared to the half-life time of 20 ± 1 h determined for the soluble enzyme under equal conditions. The enzyme-loaded carriers prepared at low to moderate buffer concentrations were of equal stability, showing a half-life time of 18 ± 4 h (10 mM) and 20 ± 2 h (50 mM), respectively. For the enzymes immobilised at high buffer concentrations, the initial remaining activity was very low, though most of the protein had adsorbed on the carriers. After 24 h, no activity could be measured at all, indicating that the enzyme conformation was severely distorted during the immobilisation process.

The unaltered stability indicates that no stabilising multipoint attachment has occurred so far, and the enzymes seem to be bound to the surface in a way, which still allows the usual mechanism of thermal inactivation.

In terms of activity no difference could be detected between the enzyme-support preparations prepared in 10 or 50 mM buffer. Furthermore, it should be noted that all enzyme-support preparations kept their full activity for over four weeks, when stored at 4 °C.

2.2. Blocking of the remaining functional groups

After the immobilisation of enzymes on the support, many functional groups of the support still remain unchanged and may interact with enzyme residues, influencing the catalytic properties of the enzyme.

In order to diminish these effects and to achieve a stabilising effect on the immobilised enzymes, the enzyme-support preparations were treated with 1 M glycine or 5% mercaptoethanol solution, respectively, for different incubation times. The amino and thiol groups act as nucleophiles in this reaction, attacking the remaining epoxy groups and forming covalent bonds.

Blocking of the remaining functional groups shows a significant increase in enzyme stability. For the treatment with glycine, an incubation time of 40 min yielded an enzyme-support preparation with an increased half-life time of 27 ± 3 h, for incubation times of 60 min or more, a half-life time of even 83 ± 6 h was achieved. The same tendency was observed when comparing the stabilities of the preparations treated with mercaptoethanol. For an incubation time of 40 min, the half-life time was increased to 48 ± 8 h; longer incubation ($\geqslant$60 min) resulted in even 73 ± 24 h (see Fig. 4).

Concluding these findings it becomes evident that an incubation time of 60 min gives the greatest benefits. By both blocking methods a fourfold increase in stability can be achieved.

2.3. Cross-linking of immobilised enzymes

Another approach to stabilise immobilised enzymes is the additional cross-linking with glutardialdehyde after their immobilisation on the support. Since glutardialdehyde is a bifunctional molecule, the protein molecules are linked with each other or with functional groups of the support, if still available. This leads to a rigidification of the enzymatic structure, lessening the influence of thermal deactivation.[30]

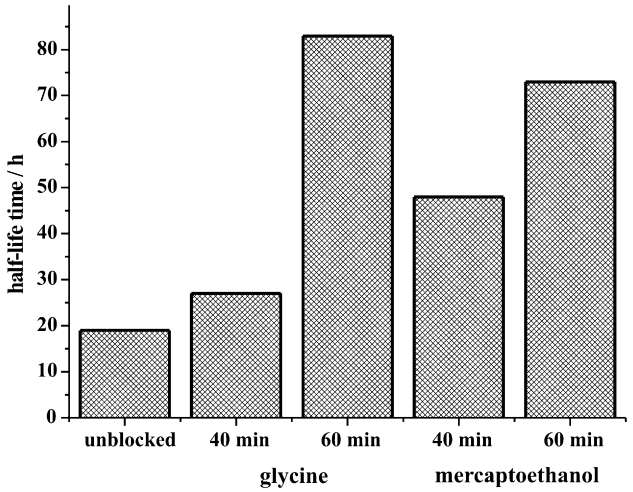

Figure 4. Enzyme stability after blocking procedure.

Preliminary experiments showed that incubation times for more than 1 h, as indicated in literature, resulted in almost complete loss of enzymatic activity, possibly by reaction between glutardialdehyde and residues of the active centre of the enzyme. This is why all further cross-linking experiments were carried out with an incubation time of 30 min.

The enzyme-support preparations were treated with glutardialdehyde solutions of different concentrations ranging between 0.2% and 2.0%. As shown in Figure 5, it became obvious that any treatment with glutardialdehyde resulted in a major loss of activity, which became more severe when increasing the concentration of glutardialdehyde, probably for the reason described.

Concerning the stability, the stabilising effect of the cross-linking procedure gives the greatest benefits for a concentration of only 0.2% glutardialdehyde. Higher concentrations gave only smaller stabilisations, if any, while

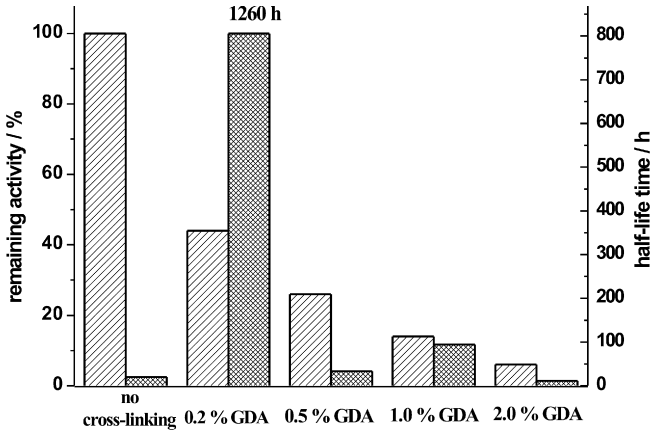

Figure 5. Enzyme properties after cross-linking.

the corresponding remaining activities were greatly diminished. In contrast to that, the stability of the enzyme-support preparations incubated with 0.2% glutardialdehyde was increased dramatically, resulting in a half-life time of 1260 ± 390 h (see Fig. 3). Compared to the soluble enzyme, this means a stabilising effect by a factor of 60. This huge increase in stability indicates that the protein molecules are now bound to the surface and to each other by additional bonds via glutardialdehyde molecules, resulting in intense rigidification.

2.4. Combination of blocking and cross-linking

After investigating the two methods of stabilisation described, we further investigated whether the combination of both blocking and cross-linking enables us to reap the beneficial effects of both methods. Since the cross-linking with 0.2% glutardialdehyde yields the best combination by far of stabilisation and remaining activity, we treated both glycine-blocked and mercaptoethanol-blocked derivatives with this concentration of glutardialdehyde.

However, in the case of glycine-blocked preparations, it is noteworthy that glycine, as a secondary amino compound, acts as a weak nucleophile and thus may reduce the part of glutardialdehyde actually used for the cross-linking of the protein. Nevertheless, as Figure 4 indicates, we found a stabilising effect after treatment with glutardialdehyde, resulting in an improved half-life time of 42 h, while keeping 33% of the original activity.

For the enzyme-support preparations blocked with mercaptoethanol, we found a remaining activity of 20%. However, the stabilising effect of the cross-linking was definitely more pronounced, yielding a half-life time of 560 ± 67 h (see Fig. 6).

Despite the smaller remaining activity, the combination of blocking with mercaptoethanol and glutardialdehyde cross-linking is definitely the more promising combination, since it yields an enzyme-support preparation, which is stabilised by a factor of 28.

2.5. Loading capacity

To determine the maximum loading capacity of the amino-epoxy carriers, different amounts of protein were offered to the same amount of support. As can be seen in Figure 7, up to an enzyme loading of 1 mg protein per gram support, a complete adsorption occurs. Afterwards, only a decreasing fraction of the offered protein amount is actually immobilised.

2.6. Process stability

In order to evaluate the enzyme stability under process conditions, we chose to utilise the immobilised biocatalyst in a plug-flow reactor.[31] In contrast to more complex reactor setups employing membranes, which could be blocked by protein content, the plug-flow reactor setup described herein can be operated, even with flow rates of 100 mL h^{-1} without suffering a significant drop in pressure in the reactor. For these reasons, this reactor setup represents a very convenient and facile way of operating a continuously operated biocatalytical reactor.

We used the substrate-coupled approach for the regeneration of cofactor (Fig. 1),[32] adding 100 mM *iso*-propanol to the substrate solution, which is oxidised to acetone in a second reaction catalysed by *Lb*-ADH. The first reactor was operated with enzyme-support preparation, which was blocked with glycine.

As Figure 8 shows, for a residence time of 15 min, almost a complete conversion of 98% could be achieved. Unfortunately, the conversion starts to decrease after 20 residence times. Since the enzyme stability was expected to be of a much higher value, we incubated the enzyme-support preparation with 1 M sodium chloride solution for 1 h to check whether the covalent bonds between the protein molecules and the surface had been formed. After the incubation, the enzyme-support preparation showed no remaining activity, indicating that the protein molecules had been completely desorbed from the surface. It became clear that despite

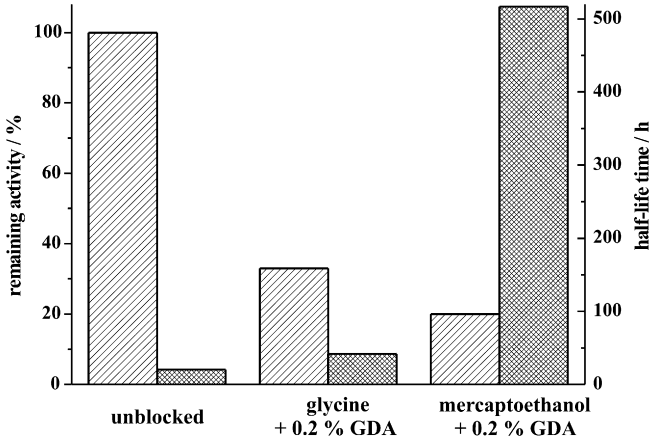

Figure 6. Enzyme properties after combined blocking and cross-linking.

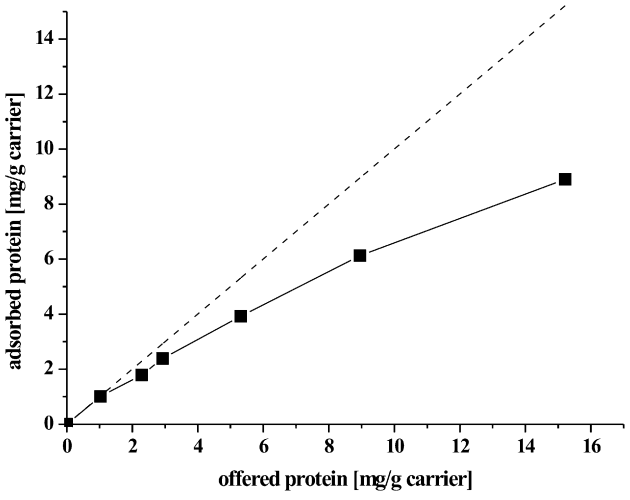

Figure 7. Loading capacity of *Lb*-ADH on amino-epoxy Sepabeads®.

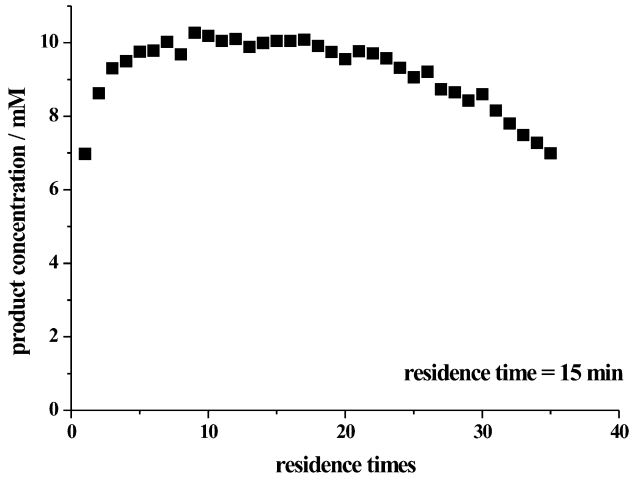

Figure 8. Plug-flow reactor with glycine-blocked enzyme preparation.

the fact that we followed the normal immobilisation protocol described by the manufacturer, no covalent bonds were formed.

Nevertheless, a moderate catalyst utilisation, described by a total turnover number of about total turnover number = 25,000, which is defined as the quotient of mole product produced by protein deployed, could be achieved. Furthermore, a volumetric productivity of 144 g L^{-1} d^{-1} was determined during *steady state* conditions.

Until now, it has not been investigated in detail to what extent the reduction reaction of acetone to *iso*-propanol competes with the reduction of acetophenone. However, the almost complete conversion (98%) obtained in this plug-flow reactor clearly indicates that the excess of *iso*-propanol used is large enough to neglect this reaction.

The enzyme-support preparations, which had been cross-linked with glutardialdehyde, kept their activity after incubating them in 1 M sodium chloride solution. This is why we chose to set up a second reactor utilising enzyme-support preparation, which had been blocked with mercaptoethanol and was afterwards cross-linked by 0.2% glutardialdehyde solution. As Figure 9 clearly shows, the

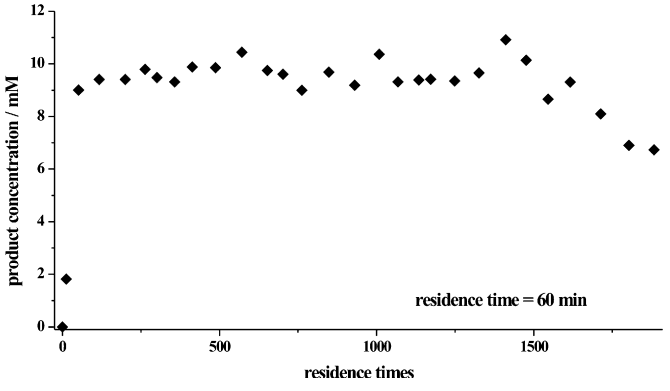

Figure 9. Plug-flow reactor with mercaptoethanol-blocked and cross-linked enzyme preparation.

operational stability could be dramatically increased. It was possible to operate the reactor for more than 70 days without loss of activity. Due to instrumental limitations, the longest residence time that could be applied was 60 min, leading to a constant conversion of 60%.

This conversion was not limited by thermodynamic reasons, as an increase in *iso*-propanol up to 200 mM showed no effect on the conversion. Additionally, the cofactor concentration was reduced during the experiment to a value of 0.5 mM, without any effect on the conversion. In comparison to the first reactor the drop in volumetric productivity to 30 g L^{-1} d^{-1} is more than compensated by the enzyme utilisation, reaching total turnover number = 2,500,000.

It is not easy to evaluate this degree of enzyme utilisation since there are very few publications, which actually give definite numbers for the ratio of product formed and enzyme used. Furthermore, it is only partially feasible to compare total turnover numbers gained for different substrates. To at least roughly estimate the quality of catalyst utilisation in our system, we compared it to the numbers achieved in the application of *Lb*-ADH in non-continuous reactor setups in our group.[33] The reaction investigated here was the reduction of methyl acetoacetate to (*R*)-methyl-3-hydroxybutanoate. For the use in a batch reactor, a total turnover number of 168,000 was reported. When operated in a repetitive batch mode, the total turnover number could be increased to 560,000.

Even though this comparison gives only a rough estimate of the quality of enzyme utilisation, we found it justified to claim that the combination of an enzyme immobilisation and a continuous reactor setup we herein report shows a very high enzyme utilisation and might therefore prove more economical than non-continuous reactor setups reported so far.

Furthermore, it is noteworthy that during the first 1700 residence times, no (*S*)-phenylethanol was produced, that is, the stereoselectivity of the enzyme had been fully retained during the immobilisation, leading to an enantiomeric excess of over 99.5%. After 10 weeks of operation, traces of (*S*)-phenylethanol were detected in the product solution. The presence of the second enantiomer indicates that the stereoselectivity of the enzyme starts to decrease. This is why the reactor run was aborted after 78 days of operation.

3. Conclusion

To our knowledge, we have reported the first immobilisation of alcohol dehydrogenase from *L. brevis* by covalent coupling to a carrier support. Up to an enzyme loading of 1 mg protein per gram support, the protein can be completely adsorbed to the support within an hour, when working with 10 mM phosphate buffer solution. The enzyme-support preparation shows a remaining activity of 15% with excellent, undiminished stereoselectivity (over 99.5%), and is stable for over a month when stored at 4 °C. To achieve a significant stabilisation effect, further modifications of the enzyme-support preparation proved

to be necessary. Blocking of the remaining functional groups with amino compounds or mercaptoethanol resulted in a stability increase up to a factor of four. However, when following the standard immobilisation procedure, no covalent bonds were formed between the enzyme and the support. Covalent multi-point attachment could only be realised by cross-linking the adsorbed protein molecules by treatment with 0.2% glutardialdehyde. We achieved a supreme stabilisation effect, yielding storage stability with a half-life time of over 1200 h at 30 °C. This stands for a 60-fold stability increase compared to the soluble enzyme.

This stability was also demonstrated under process conditions: enzyme-support preparation, which had both been blocked and cross-linked was utilised in a plug-flow reactor. This reactor could be operated for 11 weeks without loss of activity and superior biocatalyst utilisation (total turnover number = 2,500,000). The use of this plug-flow reactor combines the advantages of continuous operation over a long period of time with facile handling of the biocatalyst and easy product separation and is therefore an excellent way to produce enantioenriched alcohols.

In the future we will investigate whether these findings can be transferred to other alcohol dehydrogenases with a low thermal stability or contrary stereoselectivity.

4. Experimental

4.1. Materials

Amino-epoxy Sepabeads® (EC-HFA) were kindly donated by Resindion S.R.L., Milan, Italy, from the Mitsubishi Chemical Corporation. Alcohol dehydrogenase from *L. brevis* (MW = 105 kDa) was purchased from Julich Chiral Solutions, Jülich, Germany. All other reagents were purchased from Sigma, Germany and were of analytical grade or better. The standard buffer solution used is 50 mM phosphate buffer with 1 mM magnesium chloride and a pH of 7.

4.2. Enzyme activity assay

Enzymatic activity was determined spectrophotometrically by using acetophenone as a substrate and measuring the difference in the concentration of reduced cofactor at a wavelength of 340 nm after 1 min. Photometrical measurements were performed on a Shimadzu UV 1601 from Shimadzu, Duisburg, Germany. The reaction mixture consisted of 970 μL of 11 mM acetophenone in standard buffer solution, 20 μL of 9.5 mM NADPH solution and 10 μL of the analysed enzyme solution. The reaction was performed at 20 °C.

One unit is defined as the amount of enzyme reducing one micromole of acetophenone per minute under the described conditions.

The activity of the immobilisate was determined by adding a definite amount of enzyme-support preparation to a 1 mL batch consisting of 980 μL of 11 mM acetophenone and 20 μL of 9.5 mM NADPH and stirring it with a magnetic mixer at 400 rounds per minute. After distinct time intervals, samples are taken from the reaction mixture and are analysed by GC. The starting reaction velocity is calculated from the collected data by linear regression.

4.3. Protein determination

The protein content was determined according to Bradford.[34] For calibration, bovine serum albumin was used.

4.4. Gas chromatography (GC)

Analytical measurements were carried out on an Agilent 6890N system (Agilent Technologies, CA, USA) with a Cyclodex β-1/P column of 25 m × 250 μm and a hydrogen gas flow of 0.2 mL min^{-1}. Sample solution (0.2 μL) were injected at 250 °C, initial column temperature was 100 °C, kept for 11.5 min; afterwards the temperature was increased to 180 °C within 3 min. Acetophenone was detected after 5.7 min, (*R*)-phenylethanol after 9.8 min and (*S*)-phenylethanol after 10.5 min.

4.5. Immobilisation of the protein on amino-epoxy Sepabeads®

The support was washed with water and an amount of 100–1500 mg wet support was suspended in a phosphate buffer of different concentrations. Enzyme lyophilisate was also dissolved in buffer solution and the activity and protein content were measured as described previously. The enzyme solution was given to the carrier suspension, shaken for different time intervals and stored for 24 h at 4 °C.

4.6. Blocking of the remaining functional groups

To block all the remaining epoxy-groups on the carrier surface, the immobilisate was treated with 1 M glycine or 5% mercaptoethanol solution for different time intervals. Afterwards the enzyme-support preparation was rinsed 10 times with 5 mL of water each and was stored in buffer solution at 4 °C.

4.7. Cross-linking with glutardialdehyde

For further stabilisation, the enzyme-loaded carriers were treated with glutardialdehyde in concentrations between 0.2 and 2.0% for different time intervals. Subsequently, the enzyme-support preparation was rinsed 10 times with 5 mL of water each, and stored in buffer solution at 4 °C.

4.8. Thermal stability

The thermal stabilities of the soluble (1 U/mL) and immobilised enzymes were determined by incubating the samples at 30 °C in a standard buffer solution. Periodically withdrawn samples were assayed for activity as described earlier. The thermal stability is given as half-life time, the time after which half the original activity remains, assuming exponential decay.

4.9. Loading capacity

Different amounts of protein (0–3 mg) were offered to 100 mg of wet support. The samples were shaken for 24 h; afterwards, the protein contents in the supernatant were determined. The protein amount bound to the carriers is defined as the difference between the original protein content and the protein remaining in the supernatant.

4.10. Plug-flow reactor

In the operation of the plug-flow reactor the following substrate concentrations were used: 11 mM and 16 mM acetophenone, respectively, 100/200 mM *iso*-propanol, 0.5–1.5 mM NADPH or NADP and 1 mM Mg^{2+} in 50 mM phosphate buffer. As indicated in Figure 9, a Pharmacia 500 pump (Freiberg, Germany) was used to pump the cooled substrate solution into the 0.8 mL flow-tube, filled with a 5 cm packed bed of enzyme-support preparation, which was retained by a filter (Minisart RC4, Sartorius, Germany). A fraction collector was used to collect the samples, which are then analysed by GC (see Fig. 2).

The first reactor was employed with 2.3 mg *Lb*-ADH immobilised on a 1200 mg support; the second one was loaded with 0.7 mg protein on a 980 mg support.

Acknowledgements

The authors would like to thank Professor C. Wandrey for support and helpful discussions, Resindion for generously providing the Sepabeads®, Dr. Caimi and Dr. Giesecke for helpful discussions. Additionally, the authors would like to thank Professor Dr. M. Ansorge-Schumacher, Tina Kubitzki and Sven Kühl for the exchange of ideas about immobilisation procedures. Expert technical assistance by Heike Offermann is gratefully acknowledged. This work was funded by the Deutsche Forschungsgemeinschaft (graduate college 1166 'Biocatalysis in non-conventional media' (BioNoCo)).

References

1. Koeller, K. M.; Wong, C. H. *Nature* **2001**, *409*, 232–240.
2. Panke, S.; Held, M.; Wubbolts, M. *Curr. Opin. Biotechnol.* **2004**, *15*, 272–279.
3. Straathof, A. J. J.; Panke, S.; Schmid, A. *Curr. Opin. Biotechnol.* **2002**, *13*, 548–556.
4. Kragl, U.; Kruse, W.; Hummel, W.; Wandrey, C. *Biotechnol. Bioeng.* **1996**, *52*, 309–319.
5. Wandrey, C. *Chem. Rec.* **2004**, *4*, 254–265.
6. Lütz, S.; Rao, N.; Wandrey, C. *Chem. Ing. Techn.* **2005**, *77*, 1669–1681.
7. Sheldon, R. A.; Schoevaart, R.; Van Langen, L. M. *Biocatal. Biotransform.* **2005**, *23*, 141–147.
8. Bornscheuer, U. T. *Angew. Chem., Int. Ed.* **2003**, *42*, 3336–3337.
9. Cao, L. Q. *Curr. Opin. Chem. Biol.* **2005**, *9*, 217–226.
10. Katchalski-Katzir, E. *Trends Biotechnol.* **1993**, *11*, 471–478.
11. Mateo, C.; Fernandez-Lorente, G.; Abian, O.; Fernandez-Lafuente, R.; Guisan, J. M. *Biomacromolecules* **2000**, *1*, 739–745.
12. Schoevaart, R.; Wolbers, M. W.; Golubovic, M.; Ottens, M.; Kieboom, A. P. G.; van Rantwijk, F.; van der Wielen, L. A. M.; Sheldon, R. A. *Biotechnol. Bioeng.* **2004**, *87*, 754–762.
13. Margolin, A. *Trends Biotechnol.* **1996**, *14*, 223–230.
14. Bastida, A. *Biotechnol. Bioeng.* **1998**, *58*, 486–493.
15. Kurlemann, N.; Liese, A. *Tetrahedron: Asymmetry* **2004**, *15*, 2955–2958.
16. Pierre, A. C. *Biocatal. Biotransform.* **2004**, *22*, 145–170.
17. De Temino, D. M.; Hartmeier, W.; Ansorge-Schumacher, M. B. *Enzym. Microb. Technol.* **2005**, *36*, 3–9.
18. Mozhaev, V. V. *Trends Biotechnol.* **1993**, *11*, 88–95.
19. Bolivar, J. M.; Wilson, L.; Ferrarotti, S. A.; Fernandez-Lafuente, R.; Guisan, J. M.; Mateo, C. *Biomacromolecules* **2006**, *7*, 669–673.
20. Daussmann, T.; Hennemann, H.-G.; Rosen, T. C.; Dünkelmann, P. *Chem.-Ing.-Techn.* **2006**, *78*, 249–255.
21. Müller, M. *Adv. Biochem. Eng./Biotechnol.* **2005**, *92*, 261–287.
22. Niefind, K.; Riebel, B.; Müller, J.; Hummel, W.; Schomburg, D. *Acta Crystallogr. Sect. D-Biol. Crystallogr.* **2000**, *56*, 1696–1698.
23. Ager, D. *Handbook of Chiral Chemicals*; Dekker: New York, USA, 1999.
24. Pithawala, K. A.; Bahadur, A. *Cellulose Chem. Technol.* **2002**, *36*, 265–273.
25. Soni, S. *J. Appl. Polym. Sci.* **2001**, *82*, 1299–1305.
26. Bolivar, J. M.; Wilson, L.; Ferrarotti, S. A.; Guisan, J. M.; Fernandez-Lafuente, R.; Mateo, C. *J. Biotechnol.* **2006**, *125*, 85–94.
27. Trivedi, A.; Heinemann, M.; Spiess, A. C.; Daussmann, T.; Büchs, J. *J. Biosci. Bioeng.* **2005**, *99*, 340–347.
28. Mateo, C.; Abian, O.; Fernandez-Lorente, G.; Pedroche, J.; Fernandez-Lafuente, R.; Guisan, J. M. *Biotechnol. Prog.* **2002**, *18*, 629–634.
29. Torres, R.; Mateo, C.; Fernandez-Lorente, G.; Ortiz, C.; Fuentes, M.; Palomo, J. M.; Guisan, J. M.; Fernandez-Lafuente, R. *Biotechnol. Prog.* **2003**, *19*, 1056–1060.
30. Hernandez, C. E. L.; Lütz, S.; Liese, A.; Bon, E. P. S. *Enzym. Microb. Technol.* **2005**, *37*, 582–588.
31. Rao, N.; Lütz, S.; Seelbach, K.; Liese, A. Basics of Bioreaction Engineering. In *Industrial Biotransformations*; Wiley-VCH: Weinheim, 2006.
32. Findrik, Z.; Vasic-Racki, D.; Lütz, S.; Daussmann, T.; Wandrey, C. *Biotechnol. Lett.* **2005**, *27*, 1087–1095.
33. Tan, I. *Applications of Whole Cell Biotransformations for the Production of Chiral Alcohols*; Rheinische Friedrich-Wilhelms Universität, 2006.
34. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248–254.