

Practical application of different enzymes immobilized on sepabeads

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Abstract The immobilization of an endoglucanase, benzoylformate decarboxylase (BFD) from *Pseudomonas putida*, as well as of lipase B from *Candida antarctica* (CALB) onto the carrier supports Sepabeads EC-EP, Sepabeads EC-EA, and Sepabeads EC-BU was accomplished. It is shown that via these immobilized biocatalysts the synthesis of both fine and bulk chemicals is possible. This is illustrated by the syntheses of polyglycerol esters and (*S*)-hydroxy phenyl propanone. The benefit of immobilization is illustrated by repetitive use in a bubble column reactor as well as in a stirred tank reactor. High stability of two biocatalysts was achieved and reusability up to eight times was demonstrated. The comparison of CALB immobilized on Sepabeads EC-EP to Novozym 435 shows similar activity.

Keywords Immobilized enzyme · Ester oil · Solvent free · Carboligation · Esterification

Introduction

A requirement of a successful industrial bioprocess is rational design and development, taking into considerations both technical and economic aspects. Fundamental aspects of bioreaction engineering, which need particular considerations include enzyme kinetics, basic reactor types, immobilization of enzymes [1, 2], and scale up. To facilitate downstream processing or increase the turnover number of biocatalysts, it is often beneficial to remove the biocatalyst from the reaction solution and to recycle it. One approach for recycling and recovery of the biocatalyst is immobilization on a heterogeneous support [3].

Industrial applications using enzymes as biocatalysts are very divers. The major market is the use of enzymes within detergents. Other applications are in the food and feed sector as well as in the paper and textile production [4]. Hydrolases like lipases and cellulases are used as washing agents and in the production of bulk chemicals like surfactants [5] and of fine chemicals [6], whereas lyases like decarboxylases can be used for enantioselective carbon–carbon couplings. These enantioselective reactions are especially important in the synthesis of high-price products like pharmaceuticals [7]. When aligning all enzymatic reactions leading to a product, which will come into contact with the human body like pharmaceuticals, personal care products, or detergents, it must be taken into consideration that allergy to enzymes can be a problem. The increasing prevalence of occupational allergy [8] emphasizes this need to immobilize and retain enzymes.

Dedicated to Prof. Dr. Christian Wandrey on the occasion of his 65th birthday.

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In this work, we report the immobilization of, a thermoactive endoglucanase optimally active at 70 °C and pH 6.0, the synthetically important benzoylformate decarboxylase (BFD) from *Pseudomonas putida* as well as the industrially important lipase B from *Candida antarctica* (CALB) (Table 1) onto the carrier supports Sepabeads EC-EP, EC-EA, and EC-BU. The gene encoding for the thermoactive endoglucanase was derived from a metagenomic gene library based on a mixed culture grown anaerobically on cellulose at 70 °C and pH 7. The mixed culture, a first enrichment culture, has its origin in a hot spring from the Azores, Portugal [9–11]. Sepabeads EC have a particle size distribution of 150–300 µm and a specific gravity of 1.13 g/mL. They have a highly porous methacrylic polymer matrix and show low swelling tendency. They are available with a wide variety of functional groups for immobilization (EP, epoxy; EA, amine; BU, butyl). Different types of Sepabeads have been successfully applied for the immobilization of many enzymes (Fig. 1). Extensive work in this area has been carried out by Guisan et al. [12, 13], Fernandez-Lafuente et al. [14–17], and Lopez-Gallego et al. [18–20].

Examples concerning the application of Sepabeads on an industrial scale are the production of 6-amino penicillanic acid [21] or the conversion of cephalosporin C into alpha-keto-adipoyl-7-amino-cephalosporanic acid [22]. One recent application on a preparative scale is the application of an alcohol dehydrogenase in a plug-flow-reactor for the synthesis of acetophenone with a volumetric productivity of 144 g/L/day [23]. Next to this continuous process Palomo et al. [24] have shown the application of CALB on octadecyl Sepabeads in a repetitive batch mode and its stability in the hydrolysis of 6-(5-chloropyridin-2-yl)-5-(*O*-butyryl)-7-oxo-5,6-dihydropirrol[3,4b]pyrazine. Hereby 50% of conversion was obtained after 45 min and maintained during ten cycles. Furthermore, detailed analytical work has been done on a µL-scale determining activities by spectrophotometric assays.

Regarding the technical applications as mentioned above we show in this work that by the immobilized biocatalysts both the syntheses of fine and bulk chemicals are possible. Fine chemicals from petrochemicals and products based on renewable resources can be obtained by lipases (triacylglycerol hydrolases). They can synthesize aliphatic

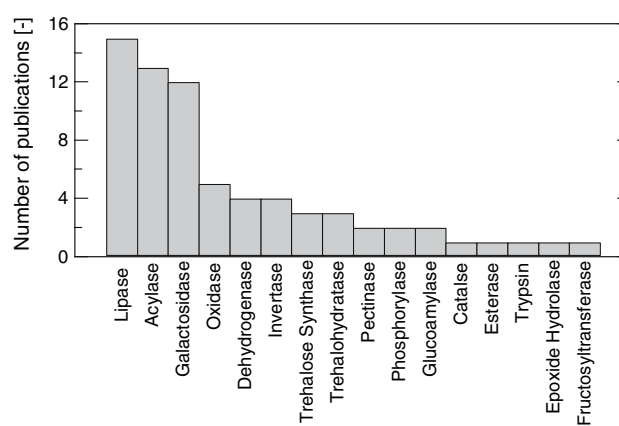


Fig. 1 Number of publications regarding the immobilization of different enzymes on Sepabeads in the time of 1994–2007. Multiple mentions are possible (ISI Web of Knowledge 22.10.2007)

and aromatic esters in non-aqueous, biphasic and solvent-free systems. The most industrially important lipases do not require cofactors for activity. BFD catalyses the enantioselective carbonylase reaction leading to a broad range of carbonyl 2-hydroxy ketones, useful as synthons for organic chemistry [25–28]. These are important intermediates in the production of antifungals [29] as well as of pharmaceuticals like Wellbutrin® (treatment of depression) and Zyban® (smoking cessation) (both from Glaxo Wellcome) [30]. The use of cellulases is found mainly in textile sector for denim washing as an eco-friendly technique to achieve desirable appearance for cotton fabrics. Therefore, CALB, BFD and endoglucanase (cellulase) are good starting points for the demonstration of their preparative use as new immobilized biocatalyst.

Experimental

Materials

The recombinant thermophilic endoglucanase was actively expressed in *E. coli* TunerTM(DE3)pLacI by induction with 1 mM IPTG. The strain was cultivated in 2 L fed-batch fermentations with a feeding strategy based on oxygen balance probing [31]. Benzoylformate decarboxylase was produced as hexahistidin fusion protein [27] by

Table 1 Enzymes used for immobilization

| Enzyme class | Enzyme | MW (kDa) | EC | Source | Host |
|--------------|------------------------------|----------|---------|---------------------------|----------------|
| Hydrolase | Lipase B | 33 | 3.1.1.3 | <i>Candida antarctica</i> | – ^a |
| | Endoglucanase | 85 | 3.2.1.4 | Metagenome | <i>E. coli</i> |
| Lyase | Benzoylformate decarboxylase | 240 | 4.1.1.7 | <i>Pseudomonas putida</i> | <i>E. coli</i> |

^a Commercially available product

fermentation of *E. coli* cells as host strain. The fermentation was carried out in a glucose-limited fed batch mode as described by Buchmann et al. [31]. The cultivation was monitored by oxygen balance probing to control the feeding. After achievement of OD 70 the enzyme production was induced by adding ITPG (1.5 mM), and the fermentation was aborted after 6 h. The enzymes were purified by Ni-NTA column purification. The endoglucanase was pretreated by heat denaturation to remove accompanying proteins [32]. Lipase B from *Candida antarctica* (CALB) was generously donated by Evonik Goldschmidt GmbH. (Essen, Germany).

Immobilization of different enzymes on different supports

Three different enzymes have been immobilized on Sepabeads EC-EP, EC-EA, and EC-BU. Preparation was done using different immobilization strategies. Samples of supernatant were withdrawn. Protein concentration was determined by the Bradford method [33]. Maximum enzyme loading of the carriers was determined and afterwards the immobilized biocatalysts were applied within different reactions.

Adsorption of endoglucanase on Sepabeads EC-BU support

Sepabeads and lyophilized enzyme were mixed in defined amounts in desalting/immobilization buffer. Experimental conditions for adsorption were 24 h at 20 °C on an orbital shaker at 300 rpm; the protein concentrations before and after the binding procedure were carried out in microtiter plates according to the Bradford method.

Activity assay for endoglucanase

Fresh enzyme stock solution was prepared by mixing desalting buffer and lyophilized enzyme. After the immobilization procedure the carriers were washed five times and used for activity tests. Therefore, carboxymethylcellulose (CMC) was used as substrate. The reaction mixture contained 1 mL 0.1% (w/v) CMC per 0.1 g carrier solvated in 0.05 M citrate acid buffer; pH 7. The enzyme reaction was carried out for 30 min at 70 °C. Afterwards, 30 µL of this solution were added to 1 mL bicinchoninic acid (BCA) reagent. Then the absorbance at 562 nm was recorded. The activity A (U/mL) was calculated as follows, whereby V_F , dilution factor; m , slope; t_{inc} , incubation time:

$$A = \frac{\Delta E \cdot V_F \cdot m}{t_{inc}}$$

Adsorption of BFD on Sepabeads EC-EA support

Lyophilized enzyme was mixed with defined amount of adsorption buffer (pH 8; 50 mM potassium phosphate; 1 mM magnesium sulfate; 0.5 mM thiamine diphosphate) and Sepabeads were added. Afterwards, the mixture was shaken for 24 h (orbital shaker 300 rpm). The addition of the cofactor thiamine diphosphate is necessary to maintain the activity of the enzyme after drying. Thereupon, the Sepabeads/BFD were washed five times using 30 mL adsorption buffer and used as suspension for activity tests. The not-bound amount of enzymes was determined by the Bradford method and the fraction of bound enzyme was counted back.

C–C-coupling by BFD/Sepabeads EC-EA

To a thermostatted amount of 4.5 mL reaction buffer (30 °C; pH 8; 50 mM potassium phosphate; 1 mM magnesium sulfate; 0.5 mM thiamine diphosphate; 50 mM benzaldehyde; 500 mM acetaldehyde) 0.5 mL Sepabeads/BFD suspension were added. Sampling was done after 0.5, 1, 2, and 5 min by stopping the stirring, allowing sedimentation for 5 s, and taking a volume of 300 µL. The samples were quenched by adding 300 µL of acetonitrile and analyzed by HPLC. Subsequent to the reaction, Sepabeads/BFD were dried in vacuum to determine the dry weight.

Stability of the BFD preparations

In a stirred tank reactor, 49.5 mL reaction buffer was thermostatted at 30 °C. Afterwards, 0.5 mL Sepabeads/BFD suspension was added. The samples were withdrawn after 0.5, 1, 2, 5, 15, 30, 45, and 60 min following the protocol as outlined above. Then, the Sepabeads/BFD were removed from the reactor and washed five times by immobilization buffer. New reaction buffer was added to the cleaned reactor and thermostatted. The washed carriers were added again. This repetitive batch could be carried out six times, and then the dry weight of the carriers was determined as mentioned above.

Immobilization of CALB on Sepabeads EC-EP support

The commercial enzyme solution was mixed with Sepabeads EC-EP for 24 h at 20 °C. Afterwards the carrier was filtrated and washed five times with distilled water. After

drying under vacuum the immobilized enzyme was applied in esterification reactions. The bound amount of enzyme was determined by analyzing the supernatant by the Bradford-method.

Enzymatic esterification by CALB

The activities of different immobilized preparations of CALB were analyzed in the solvent-free esterification reaction of different substrates. Equimass amounts of lauric acid and polyglycerol were used, 5% (w/w) immobilized enzyme was added. The concentration of the fatty acid was determined by withdrawing samples periodically, dissolving them in ethanol and titrating them.

Stability of the lipase preparations

The immobilized lipase was used in a bubble column within the solvent-free reaction of polyglycerol and lauric acid at 75 °C for 3 h. The conversion of lauric acid was determined at different time points by titration. Remaining substrates and products were removed by filtration and new substrates were added. This repetitive batch was repeated until ~20% of the enzyme activity concerning the first batch was reached.

Results and discussion

The choice of the right immobilization procedure is one of the necessities to get a highly active and long-term stable biocatalyst. Therefore, the same type of carrier, Sepabeads EC, has been used, but different functionalities on its surface were tested in combination with the three enzymes mentioned above. Starting with the determination of the amount of bound enzyme while fixing the amount of enzyme per mass carrier we concluded from a pre-choice which support should be to be used (Fig. 2). Hereby the criterion was to reach the highest ratio of enzyme adsorbed at the carrier to enzyme applied. The percentage reached was in case of CALB 100, 84, and 65% regarding the carriers EC-EP, EC-EA, and EC-BU, in case of BFD was

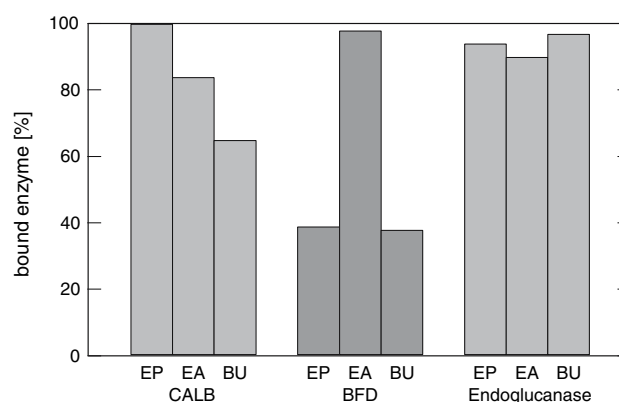


Fig. 2 Binding of the different enzymes to the supported Sepabeads EC-EP, EC-EA, EC-BU. Experiments were carried out at a free enzyme concentration of ~20 mg/g carrier

39, 98, and 38%, respectively, and in case of endoglucanase 94, 90, and 97%. In the later preparative application it is necessary to have a high percentage of enzymes bound while having a low amount of enzymes staying within the stock solution used for immobilization. Preparing the immobilized biocatalyst for examining the preparative applicability we therefore chose the combinations Sepabeads EC-BU/endoglucanase, Sepabeads EC-EA/BFD and Sepabeads EC-EP/CALB for further investigations regarding the preparative applicability.

Following these investigations the maximum load of the chosen carrier with the different enzymes was determined. These experiments were done to reduce the amount of carrier, which is necessary to carry out the synthesis shown later. The amount of enzyme applied within the stock solution was increased and the amount of enzyme bound to the carrier was afterwards determined. These experiments lead to a linear slope of the bound enzyme. The maximum mass of enzyme being immobilized on the chosen supports is illustrated in Table 2.

The endoglucanase, the BFD, and the lipase showed a different binding behavior regarding the different supports Sepabeads EC-BU, EC-EA, and EC-EP, but all of them were bound to the carriers. The enzymes differ in polarity and the functional groups on the surface of the proteins are of different hydrophobicity and state of ionization. Furthermore, the carriers have different functionalities on top of their surface. These complex combinations of functional

Table 2 Concentration and maximal loading of enzyme on supports reached

| | Concentration of enzyme solution (mg/mL) | Applied amount of enzyme per carrier (mg/g) | Immobilized amount of enzyme per carrier (mg/g) | Bound enzyme (%) |
|----------------------------|------------------------------------------|---------------------------------------------|-------------------------------------------------|------------------|
| Sepabeads BU/endoglucanase | 2.4 | 23.8 | 8.8 | 37 |
| Sepabeads EA/BFD | 44.6 | 356.3 | 153.2 | 43 |
| Sepabeads EP/CALB | 69.0 | 138.7 | 117.9 | 85 |

groups on the proteins and carriers have led to the differences in the percentage of bound enzyme. Regarding the Sepabeads EC-BU, hydrophobic interactions are the driving force for the adsorption of the enzyme to the carrier [34]. The amino-functionalized carriers Sepabeads EC-EA will interact in a first step via hydrogen-bridges; therefore, they bind to regions of the enzyme surface with complementary ionic state. The Sepabeads EC-EP has epoxy-functions on their surface, which can build up covalent bindings to the enzyme. The results regarding the percentage of bound enzyme per applied enzyme have led to the combinations Sepabeads BU/endoglucanase, Sepabeads EA/BFD, and Sepabeads EP/CALB, which have been used on a preparative scale for further characterization. In comparison to the data obtained for a fructosyltransferase on Sepabeads EC-EP5 (54.7 mg/g) and Sepabeads EC-EP3 (31.4 mg/g) [35], the amount of enzyme (CALB) which was bound on Sepabeads EC-EP (117.9 mg/g) is two times higher. The particle size distribution might be a reason for the higher amount of bound enzyme, whereas high porosity provides for a bigger surface for immobilization. Furthermore, the immobilization of endoglucanase by hydrophobic interactions on Sepabeads EC-BU is comparable to the conclusions drawn by Palomo et al. [34]. The experiments to saturate the carrier with enzyme summarized in Table 2 show a saturation above 100 mg/g Sepabeads EC.

Activity of Sepabeads EC-BU/endoglucanase

β -1,4-Endoglucanases show activities within the cleavage of the cellulose backbone due to an endocleavage. Oligosaccharides of different lengths as well as cellobiose and glucose are formed [36]. These enzymes attack the amorphous areas of the cellulose and the soluble cellulose derivatives [for example, carboxymethylcellulose (CMC)]. In the conversion of crystalline cellulose only low activities were observed [37]. Therefore, the activity was determined within the cleavage of CMC. Comparing the different preparations with different contents of endoglucanase (Fig. 3a) on the carrier (2, 4, 8, 12, and 24 mg/g) it is clear that the higher concentrated batches show a higher activity (Fig. 3b). In addition to the results obtained from the activity measurements the stability of the newly prepared biocatalyst was examined. After application via CMC-assay the Sepabeads EC-BU/endoglucanase were washed and used in a second batch within this assay. Thereupon, no activity was found. Regarding the long-term stability and in comparison, the free enzyme showed an activity of 75% of the initial activity after 30 min, and even an activity of 40% remained after 6 h, both measured at 70 °C.

The fact that there remains no activity of the catalyst after one washing step either means that the enzyme is

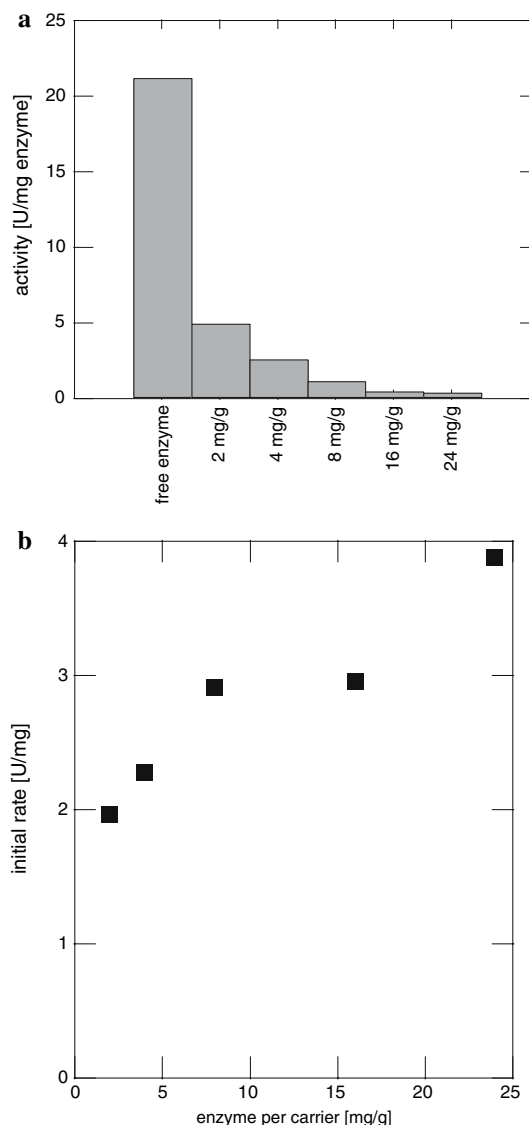


Fig. 3 a Specific activity of endoglucanase: Comparison of free enzyme solution and different adsorption approaches. b Initial rate of Sepabeads EC-BU/endoglucanase: Comparison of different adsorption approaches

inactivated or that it is removed by the washing. The removal by washing is a possibility because the hydrophobic interaction is a weak-binding type. Nevertheless, Palomo et al. [34] reported a strong interaction between a lipase and octadecyl-Sepabeads via hydrophobic interactions. This interaction is surely stronger than the interaction of butyl-Sepabeads.

Activity of Sepabeads EC-EA/BFD

The specific activity of Sepabeads EC-EA/BFD (enzyme loading 100 mg/g) and its temperature dependence in the range of 20–70 °C are illustrated in Fig. 4. It is clearly seen

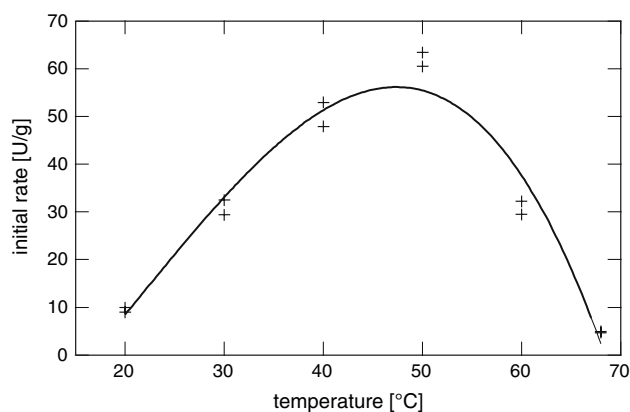
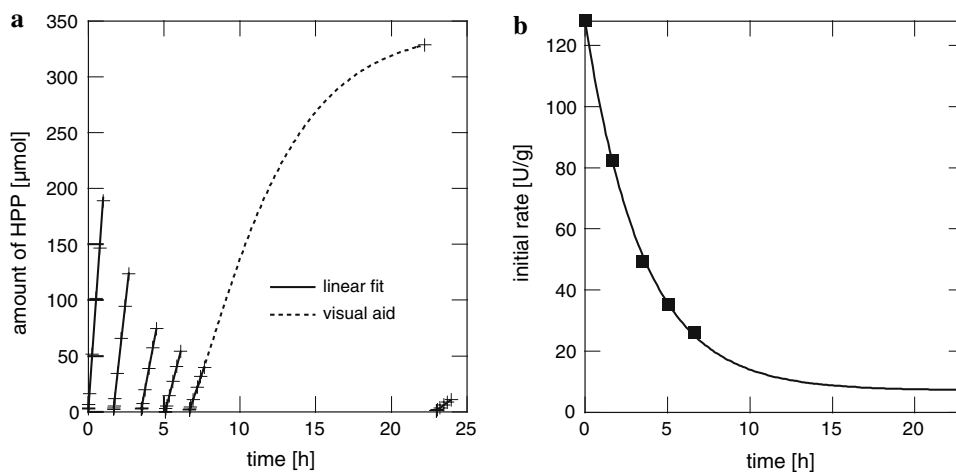


Fig. 4 Specific activity of immobilized wt BFD-His on Sepabeads EC-EA (50 mg/g) at different temperatures (reaction volume 5 mL; pH 8, 500 mM acetaldehyde, 50 mM benzaldehyde, 1 mM magnesium sulfate, 0.5 mM thiamine diphosphate)

that an activity maximum was found at 50 °C, showing a specific activity of around 62 U/g_{dry carrier}. The activity was determined for the reaction of acetaldehyde (500 mM) and benzaldehyde (50 mM) at pH 8. The corresponding activity of free BFD is 11 U/mg. At temperatures above 70 °C and lower than 20 °C the activity decreases below 10 U/g_{carrier}. In comparison to this temperature dependence of the activity of BFD/Sepabeads EC-EA the free enzyme reaches a maximum at ~50 °C [27].

Further experiments also showed that for an active biocatalyst in case of cofactor-dependent enzymes (BFD) it is essential for their activity to add the cofactors thiamine diphosphate and Mg²⁺ to all buffers [37]. The freeze-drying of immobilized Sepabeads EC-EA/BFD leads to an inactivation of the enzyme. Subsequent addition of cofactors slightly increased the activity. Therefore, Sepabeads EC-EA/BFD has not been dried but used as a suspension.

Fig. 5 Reaction course catalyzed by Sepabeads EC-EA/BFD. Deactivation of the biocatalyst Sepabeads EC-EA/BFD within repetitive batch use. Decrease of initial reaction rate vs. time. 3.2 U Sepabeads EC-EA; 30 °C; total batch volume: 50 mL; pH 8; benzaldehyde 45 mM; acetaldehyde 450 mM; magnesiumsulfate 1 mM; thiaminediphosphate 0.5 mM



Stability of Sepabeads EC-EA/BFD

The stability of Sepabeads EC-EA/BFD was determined by repetitive batch experiments while carrying out the synthesis of 2-hydroxy-1-phenylpropanone (Fig. 5). The reaction of benzaldehyde and acetaldehyde was carried out at 30 °C and after each batch the biocatalyst was washed. The decrease of the initial reaction rate was followed for six batches. The newly prepared biocatalyst (enzyme loading 320 mg/g) showed an initial reaction rate of 130 U/g with respect to the C–C-coupling reaction, whereby the rate decreased down to 10% (13 U/g) in the sixth batch (Fig. 5). As half-life time $\tau_{1/2} = 5.42$ h was determined.

When carrying out experiments in continuous stirred tank reactors a damaging of the carrier material was found, which in addition to leaching of the enzyme and thermal inactivation of the enzyme led to the decrease in activity over the different repetitive batches. The leaching can be overcome by cross-linking of the adsorbed enzyme. After 24 h of catalytic activity the immobilized biocatalyst Sepabeads EC-EA/BFD showed a remaining activity of 5% of its initial value.

Activity of Sepabeads EC-EP/CALB

The synthesis of polyglycerol laurate in a stirred tank reactor is an example for a biocatalytic production of a surfactant. This reaction exemplifies the application of Sepabeads EC-EP on a preparative scale. The CALB/Sepabeads EC-EP biocatalyst with an enzyme loading of ~100 mg/g exhibits high activity towards the direct esterification of polyglycerol and lauric acid (Fig. 6). The conversion of the lauric acid is at 80% after 4 h whereas the residual amount of polyglycerol is at 50% at this time.

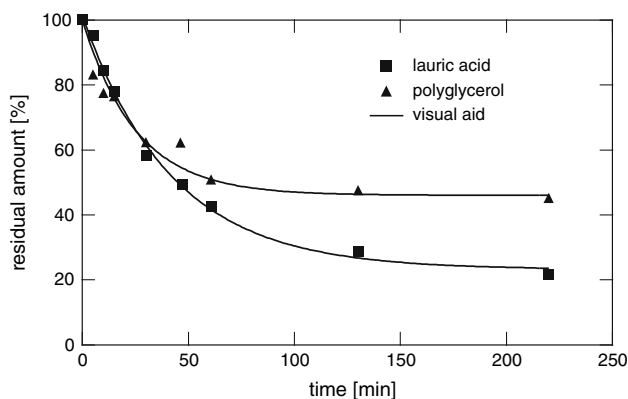


Fig. 6 Reaction course catalyzed by Sepabeads EC-EP/CALB. Equimolar amounts of lauric acid and polyglycerol for solvent free reaction (total 24 g); 5% (w:w) Sepabeads EC 100 mg/g; 75 °C; CSTR 400 rpm; vacuum

The initial reaction rate has been determined to be 0.40 kU/g, whereas the reaction rate catalyzed by Novozym 435 is 1.2 kU/g. Therefore, the initial rate of this biocatalyst as compared to the reaction catalyzed by Novozym 435 is lower by a factor of three.

The conversion of polyglycerol remains lower than the conversion of lauric acid, because of the multiple hydroxy functions within the mixture of different glycerol oligomers. The equilibrium of the reaction can be shifted to total conversion, however, by removal of water applying vacuum to the stirred tank reactor. Since the polyglycerol laurate consists of a hydrophilic and hydrophobic part, it is a good detergent. This is important regarding the leaching of the enzyme, because the product formed enables an easier removal of the enzyme in case of physical interaction. Therefore, the application of the biocatalyst in a repetitive batch mode will reveal the scale of leaching and indicate the type of binding. In case of a binding via the epoxy function a covalent binding is assumed [23].

The synthesis of myristyl myristate by Sepabeads EC-EP/CALB is an example for a biocatalytic surfactant production. Here both reactants form a hydrophobic two-phase-system, and their conversion by Sepabeads EC-EP/CALB leads to a yield of 92% after 4 h (Fig. 7). This means that there is a higher activity within the solvent-free esterification of 1.24 kU/g in comparison to the synthesis of polyglycerol laurate. This can be partly attributed to the effect of viscosity, which for myristyl myristate at 60 °C is only 5.8 mPa s in comparison to 2 Pa s in case of polyglycerol laurate. If this same reaction is catalyzed by Novozym 435, the initial rate of conversion is only 3.07 kU/g, i.e., lower by a factor of 2.5. Comparing the course of this reaction to that catalyzed by Novozym 435 the biocatalyst Sepabeads EC-EP/CALB shows similar activity.

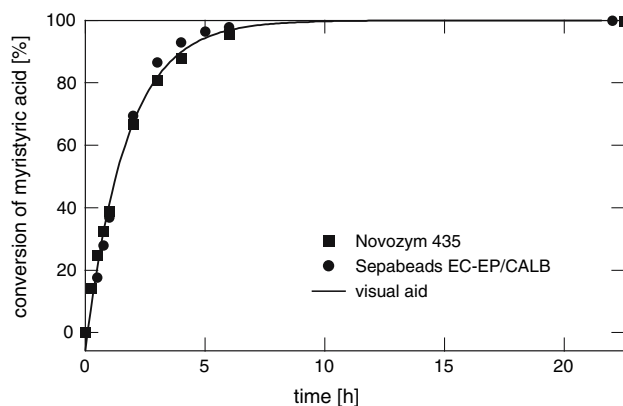


Fig. 7 Synthesis of myristyl myristate by Sepabeads EC-EP/CALB in comparison to Novozym 435. Reaction condition: equimolar amounts of reactants; total mass 996 g; 1.13% (w:w) Sepabeads EC-EP/CALB with an enzyme loading of 100 mg enzyme/g; 0.7% (w:w) Novozym 435; 60 °C; STR 400 rpm and vacuum

Stability of Sepabeads EC-EP/CALB

In addition to the activity, the stability of Sepabeads EC-EP/CALB was investigated within the solvent-free system polyglycerol/lauric acid (Fig. 8). The limiting factor of this system is its high viscosity and the different polarities of the starting materials. This causes a two-phase system, whereby the detergent formed as a product accounts for some leaching of the biocatalyst. These are challenges for the optimized use of the newly prepared biocatalyst.

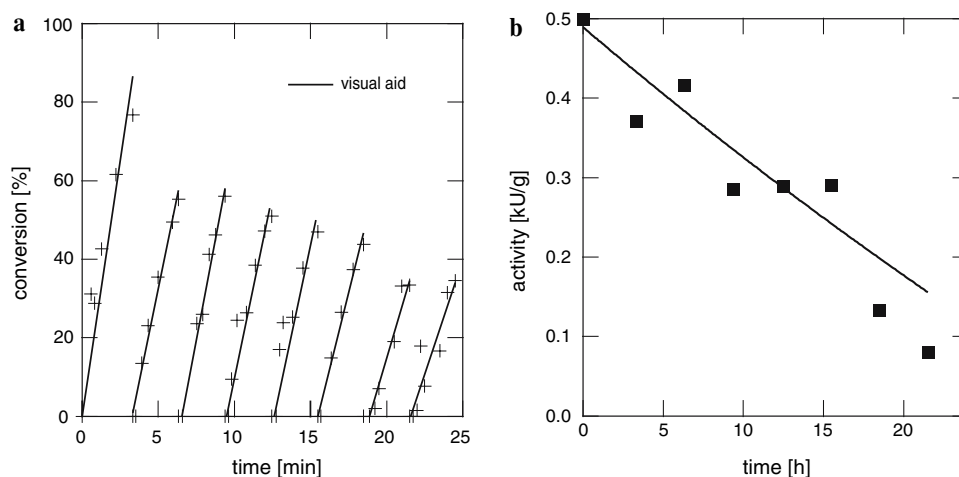
Using it in a repetitive batch mode gives information about the stability in the esterification reaction. The initial reaction rate was found to be 500 U/g and decreased within eight batches to 130 U/g (Fig. 8). As half-life time $\tau_{1/2} = 12.9$ h was determined.

When carrying out the experiments in a bubble column, the damaging of the carrier is not as pronounced as in a stirred tank reactor. However, stronger leaching of the enzyme is possible because of the detergent polyglycerol laurate formed. Hence, both thermal inactivation of the enzyme and leaching account for the decrease in activity over the different repetitive batches. After 24 h (exclusive standing time) the Sepabeads EC-EP/CALB typically retained 26% of their initial activity.

Conclusions

The immobilization of three industrially important biocatalysts has been investigated. These catalysts include an endoglucanase derived from a thermophile, an anaerobic mixed culture from the Azores, the synthetically important benzoylformate decarboxylase (BFD) from *Pseudomonas putida*, as well as lipase B from *C. antarctica* (CALB).

Fig. 8 Reaction course catalyzed by Sepabeads EC-EP/CALB. Equimolar amounts of lauric acid and polyglycerol for solvent free reaction (total 60 g). Deactivation of the biocatalyst Sepabeads EC-EP/CALB within repetitive batch use. Decrease of initial reaction rate vs. time. ~5% (w:w) Sepabeads EC 100 mg enzyme/g carrier; 75 °C; bubble column reactor 4.6 L/min pressurized air



They all were affixed onto the carrier supports Sepabeads EC-EP, Sepabeads EC-EA, and Sepabeads EC-BU, respectively. It could be shown that using the immobilized lipase B the synthesis of the bulk chemicals myristyl myristate and polyglycerol laurate can be carried out with an initial reaction rate of 0.40 and 1.24 kU/g, respectively. The corresponding half-life time was determined to be 12.9 h. The repetitive use of this biocatalyst in the production of polyglycerol laurate leads to an analytical yield of 272 g of this fine chemical, and the batch production of myristyl myristate to a yield of 996 g. This corresponds to productivity of 90 g/g in case of polyglycerol laurate and of 88 g/g in case of myristyl myristate. The synthesis of fine chemicals is illustrated by the syntheses of (*S*)-hydroxy phenyl propanone. The initial reaction rate achieved is 130 U/g. The benefit of the immobilization is illustrated by the repetitive use of this biocatalyst in a stirred tank reactor, whereupon a half-life time of 5.4 h was determined. A high stability of two biocatalysts was achieved and a reusability of up to eight times is possible. A comparison with Novozym 435 showed similar activity in case of on Sepabeads EC-EP immobilized CALB.

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References

- Buchholz K, Kasche V, Bornscheuer UT (2005) Biocatalysts and enzyme technology. Wiley-VCH, Weinheim
- Tischer W, Kasche V (1999) Immobilized enzymes: crystals or carriers? Trends Biotechnol 17(8):326–335
- Rao NN, Lütz S, Seelbach K, Liese A (2006) Basics of bioreaction engineering. In: Liese A, Seelbach K, Wandrey C (eds) Industrial biotransformations. Wiley-VCH, Weinheim, pp 115–145
- Schäfer T (2006) Screening nach industriellen Enzymen. In: Antranikian G (ed) Angewandte Mikrobiologie. Springer, Berlin, pp 173–188
- Thum O (2004) Enzymatic production of care specialties based on fatty acid esters. Tenside Surfactants Detergents 41(6): 287–290
- Hiltehaus L, Liese A (2007) Building blocks In: Sell D, Ulber R (eds) White biotechnology. Advances in biochemical engineering/biotechnology, vol 105. Springer, Berlin, pp 133–173
- Stillger T, Pohl M, Wandrey C, Liese A (2006) Reaction engineering of benzaldehyde lyase from *Pseudomonas fluorescens* catalyzing enantioselective C–C bond formation Organic Process Research and Development 10(6):1172–1177
- Lindstedt M, Schiott A, Johnsen CR et al (2005) Individuals with occupational allergy to detergent enzymes display a differential transcriptional regulation and cellular immune response. Clin Exp Allergy 35(2):199–206
- Niehaus F, Bertoldo C, Kahler M, Antranikian G (1999) Extremophiles as a source of novel enzymes for industrial application. Appl Microbiol Biotechnol 51(6):711–729
- Sunna A, Puls J, Antranikian G (1997) Characterization of the xylanolytic enzyme system of the extreme thermophilic anaerobic bacteria *Thermotoga maritima*, *T-neapolitana*, and *T-thermarum*. Comp Biochem Physiol 118(3):453–461
- Kube J, Brokamp C, Machielsen R, van der Oost J, Markl H (2006) Influence of temperature on the production of an archaeal thermoactive alcohol dehydrogenase from *Pyrococcus furiosus* with recombinant *Escherichia coli*. Extremophiles 10(3):221–227
- Bolivar JM, Wilson L, Ferrarotti SA, Fernandez-Lafuente R, Guisan JM, Mateo C (2006) Stabilization of a formate dehydrogenase by covalent immobilization on highly activated glyoxyl-agarose supports. Biomacromolecules 7(3):669–673
- Torres R, Mateo C, Fernandez-Lorente G, Ortiz C, Fuentes M, Palomo JM, Guisan JM, Fernandez-Lafuente R (2003) A novel heterofunctional epoxy-amino sepabeads for a new enzyme immobilization–stabilization protocol: immobilization–stabilization of beta-galactosidase from *Aspergillus oryzae*. Biotechnol Prog 19(3):1056–1060
- Segura RL, Palomo JM, Mateo C, Cortes A, Terreni M, Fernandez-Lafuente R, Guisan JM (2004) Different properties of the lipases contained in porcine pancreatic lipase extracts as enantioselective biocatalysts. Biotechnol Prog 20(3):825–829

15. Torres R, Pessela BCC, Mateo C, Ortiz C, Fuentes M, Guisan JM, Fernandez-Lafuente R (2004) Reversible immobilization of glucoamylase by ionic adsorption on sepabeads coated with polyethyleneimine. *Biotechnol Prog* 20(4):1297–1300
16. Alonso-Morales N, Lopez-Gallego F, Betancor L, Hidalgo A, Mateo C, Fernandez-Lafuente R, Guisan JM (2004) Reversible immobilization of glutaryl acylase on sepabeads coated with polyethyleneimine. *Biotechnol Prog* 20(2):533–536
17. Mateo C, Abian O, Fernandez-Lorente G, Pedroche J, Fernandez-Lafuente R, Guisan JM (2002) Epoxy sepabeads: a novel epoxy support for stabilization of industrial enzymes via very intense multipoint covalent attachment. *Biotechnol Prog* 18(3):629–634
18. Lopez-Gallego F, Betancor L, Hidalgo A, Dellamora-Ortiz G, Mateo C, Fernandez-Lafuente R, Guisan JM (2007) Stabilization of different alcohol oxidases via immobilization and post immobilization techniques. *Enzyme Microb Technol* 40(2): 278–284
19. Lopez-Gallego F, Betancor L, Hidalgo A, Alonso N, Fernandez-Lorente G, Guisan JM, Fernandez-Lafuente R (2005) Preparation of a robust biocatalyst of D-amino acid oxidase on sepabeads supports using the glutaraldehyde crosslinking method. *Enzyme Microb Technol* 37(7):750–756
20. Alonso N, Lopez-Gallego F, Betancor L, Hidalgo A, Mateo C, Guisan JM, Fernandez-Lafuente R (2005) Immobilization and stabilization of glutaryl acylase on aminated sepabeads supports by the glutaraldehyde crosslinking method. *J Mol Catal B Enzym* 35(1–3):57–61
21. Arroyo M, de la Mata I, Acebal C, Castillon MP (2003) Biotechnological applications of penicillin acylases: state-of-the-art. *Appl Microbiol Biotechnol* 60(5):507–514
22. Pollegioni L, Pilone M, Molla G, Cucchetti E, Verga R, Cabri W (2005) Cephalosporin C acylase mutants European Patent 1553175A
23. Hildebrand F, Lutz S (2006) Immobilisation of alcohol dehydrogenase from *Lactobacillus brevis* and its application in a plug-flow reactor. *Tetrahedron Asymmetry* 17(23):3219–3225
24. Palomo JM, Mateo C, Fernandez-Lorente G, Solares LF, Diaz M, Sanchez VM, Bayod M, Gotor V, Guisan JM, Fernandez-Lafuente R (2003) Resolution of (+/–)-5-substituted-6-(5-chloropyridin-2-yl)-7-oxo-5,6-dihydropyrrolo[3,4b]pyrazine derivatives—precursors of (S)-(+)-Zopiclone, catalyzed by immobilized *Candida antarctica* B lipase in aqueous media. *Tetrahedron Asymmetry* 14(4):429–438
25. Wilcocks R, Ward O, Collins S, Dewdney N, Hong Y, Prosen E (1992) Acyloin formation by benzoylformate decarboxylase from *Pseudomonas putida*. *Appl Environ Microbiol* 58:1699–1704
26. Wilcocks R, Ward O (1992) Factors affecting 2-hydroxypropionophenone formation by benzoylformate decarboxylase from *Pseudomonas putida*. *Biotechnol Bioeng* 39:1058–1063
27. Iding H, Dunnwald T, Greiner L, Liese A, Muller M, Siegert P, Grotzinger J, Demir AS, Pohl M (2000) Benzoylformate decarboxylase from *Pseudomonas putida* as stable catalyst for the synthesis of chiral 2-hydroxy ketones. *Chem Eur J* 6(8):1483–1495
28. Dunnwald T, Demir AS, Siegert P, Pohl M, Muller M (2000) Enantioselective synthesis of (S)-2-hydroxypropanone derivatives by benzoylformate decarboxylase catalyzed C–C bond formation. *Eur J Org Chem* (11):2161–2170
29. Gala D, DiBenedetto DJ, Clark JE, Murphy BL, Schumacher DP, Steinman M (1996) Preparations of antifungal Sch 42427/SM 9164: preparative chromatographic resolution, and total asymmetric synthesis via enzymatic preparation of chiral alpha-hydroxy arylketones. *Tetrahedron Lett* 37(5):611–614
30. Fang QK, Han ZX, Grover P, Kessler D, Senanayake CH, Wald SA (2000) Rapid access to enantiopure bupropion and its major metabolite by stereospecific nucleophilic substitution on an alpha-ketotriflate. *Tetrahedron Asymmetry* 11(18):3659–3663
31. Buchmann M (2006) Entwicklung eines skalierbaren Dialyseprozesses zur Hochzelldichtefermentation von *E. coli*. Dissertation, Hamburg University of Technology
32. Krahe M, Antranikian G, Maerkl H (1996) Fermentation of extremophilic microorganisms. *FEMS Microbiol Rev* 18(2–3): 271–285
33. Bradford MM (1976) A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
34. Palomo JM, Segura RL, Fernandez-Lorente G, Pernas M, Rua ML, Guisan JM, Fernandez-Lafuente R (2004) Purification, immobilization, and stabilization of a lipase from *Bacillus thermocatenuatus* by interfacial adsorption on hydrophobic supports. *Biotechnol Prog* 20(2):630–635
35. Ghazi I, De Segura AG, Fernandez-Arrojo L, Alcalde M, Yates M, Rojas-Cervantes ML, Plou FJ, Ballesteros A (2005) Immobilisation of fructosyltransferase from *Aspergillus aculeatus* on epoxy-activated Sepabeads EC for the synthesis of fructo-oligosaccharides. *J Mol Catal B Enzym* 35(1–3):19–27
36. Haki GD, Rakshit SK (2003) Developments in industrially important thermostable enzymes: a review. *Bioresour Technol* 89:17–34
37. Jaeger KE, Thiemann V, Antranikian G (2006) Biokatalyse. In: Antranikian G (ed) *Angewandte Mikrobiologie*. Springer, Berlin, pp 135–160