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Towards a cost-effective immobilized lipase for the synthesis of specialty chemicals

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ABSTRACT

Biocatalysis has the potential to provide the chemical industry with several advantages for the production of chemicals; but the use of this technology for the production of speciality and bulk chemicals is unfortunately limited due to the high costs related to the production of the biocatalyst. We have immobilized *Candida antarctica* lipase B (CALB) on different resins in order to obtain a more cost-effective biocatalyst, i.e. to find the cheapest preparation per catalytic activity, for one esterification and one amidation reaction. It was found that lipase immobilized on macroporous polypropylene (Accurel MP1000) and polymethylmethacrylate (Lewatit) provided biocatalyst preparations where the cost of enzyme and carrier was significantly less than the cost of commercially available Novozym®435, indicating a potential for decreased cost. Also the stability of Accurel MP1000 bound enzyme during repeated use matched that of Novozym®435. Finally the in-house biocatalyst was used in a packed-bed set-up, showing an excellent stability in repeated batches at 70 °C.

1. Introduction

Biocatalysis has become an established tool within the chemical industry for the production of fine chemicals [1,2]. The use of biocatalysis can offer a range of advantages such as purer products, lower energy consumption, reduced use of chemicals for derivatization and product purification, better working environment, etc. Hydrolases are the most commonly used biocatalysts in industry [3], amongst which lipases are known to be especially versatile and well suited for the conditions used in industrial synthetic chemistry, for example they have a good tolerance towards organic solvents and are relatively stable at elevated temperatures (up to 100 °C if anhydrous conditions are used) [4]. Several studies have shown the usefulness of lipases, not least the lipase B from *Candida antarctica* (CALB), which has been used for ester synthesis [5], peracid formation [6,7], polymerisation, etc. [8]. It has also recently been shown by us that immobilized CALB is highly efficient as catalyst for the amidation of lauric acid with ethanolamine in a solvent-free system at 90 °C [9].

However, despite the broad range of possibilities and immense academic interest, applications of lipases in industry are relatively limited. The commercially available immobilized CALB preparations: Novozym®435 (Novozymes A/S, Denmark) and Chirazyme L-2 (Roche Molecular Biochemicals, Germany), as well as similar preparations from SPRIN (Italy) and c-LEcta (Germany), are mainly used for the resolution of chiral intermediates for the pharmaceutical industry and production of other high priced fine chemicals. One notable exception is Evonik's process for the production of myristyl myristate (Essen, Germany) [10]. For industrial applications, both the performance and the cost of the biocatalyst are critical issues, which are even related. Enzymes are invariably used in immobilized form for the synthesis of chemicals. Immobilization is known to improve the stability, total productivities and scope of use of enzymes [12]. The properties of the carrier material (e.g. chemical composition, particle size, mechanical stability, etc.) used for immobilization (and the presence of any additives), has a considerable influence on the activity and stability of the biocatalyst preparation. Biocatalyst stability under the environmental conditions existing in the reactor, when translated into long-term operational stability or re-usability of the biocatalyst, can affect the total costs significantly.

It would seem that the primary factor limiting the wider industrial use of CALB for bulk and other lower priced chemicals is the relatively high price of the immobilized enzyme combined with its limited stability. In fact our calculations show that for the lipase-catalyzed production of lauric ethanolamides, the enzyme cost adds 35% to the total manufacturing cost, even if a fairly high stability of the enzyme preparation is assumed, thereby making enzymatic production more costly than conventional production using sodium methanolate as catalyst [11].

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Availability of a less expensive immobilized preparation would open up the possibilities for use in new applications such as the synthesis of alkanolamides, polyesters, biodiesel, and other low cost commodity chemicals used in bulk quantities. Efforts are ongoing in this area as indicated by several reports in the literature on immobilization of enzymes to different matrices, and even crosslinking of enzyme aggregates without involvement of any solid matrix [12,17]. Most studies focus on achieving the highest activity per gram of enzyme preparation, a factor that is important in industrial applications when the volume of the catalyst is limited by the reactor volume. To our knowledge, the topic of cost optimization for lipase preparations is not addressed in any publication. However, it is our contention that in order to stimulate the development of cost effective biocatalysts the choice of metrics is crucial. Therefore, in the current investigation, we suggest the use of activity per unit cost of biocatalyst to measure and direct the development of a cost-effective enzyme preparation by the choice of the carrier for immobilization and by determination of the optimal load of lipase to the carrier.

The added cost of the enzyme catalyst can be reduced in two principal ways: by reducing the cost of the enzyme preparation itself and/or by improving the activity and stability of the enzyme preparation (i.e. increasing the number of turnovers per amount of enzyme), or more specifically by optimizing the cost of the enzyme preparation per gram of product formed. The cost of the catalyst can be broken down into the costs for the enzyme, the matrix for immobilization, and finally the immobilization process (i.e. labor and equipment costs). One of the key factors in the development of a cost-effective biocatalyst is the selection of an appropriate enzyme loading on the carrier [13]. While high activity per gram of biocatalyst will most probably be obtained at excess enzyme loading, the optimal cost effectiveness will depend on the cost of both enzyme and carrier and the proportions at which they are mixed.

In this study, we have evaluated five different carriers for immobilization of CALB using an esterification reaction. The most cost-effective preparation was further evaluated for amidation of fatty acids, and tested both in a conventional batch stirred tank reactor (BSTR) and in a packed-bed coupled to a BSTR. The performance was compared to the commercially available Novozym®435 with regard to unit production cost and process stability.

2. Materials and methods

2.1. Materials

Novozym®435 (immobilized *C. antarctica* lipase B) and Lipozyme CALB L (liquid formulation of *C. antarctica* lipase B, 6.1 g CALB/L) were kindly provided by Novozymes A/S, Bagsvaerd, Denmark. Olein fatty acid (fatty acids from low erucic acid rapeseed oil; technical grade) and Accurel MP1000 were gifts from AkzoNobel Surfactants, Stenungsund, Sweden. Amberlite and Duolite carriers were gifts from Rohm&Haas, The Netherlands, while Lewatit VP OC 1600 was a gift from Bayer AB, Sweden. 2-Aminoethanol (monoethanolamine, 99%) and decanol (99%) were purchased from Aldrich and Sigma, respectively. All other chemicals of analytical grade were purchased from standard sources.

2.2. Immobilization of CALB

For immobilization of CALB on Accurel MP 1000, the resin was first sieved through meshes of 500 μm and 250 μm. The fractionated particles (50–250 mg, size range 250–500 μm and >500 μm) were wetted with 3 mL ethanol per gram of resin and then mixed with 2.0 mL sodium phosphate buffer (0.1 M, pH 7) and finally the desired amount of Lipozyme CALB L solution (6.1 mg protein/mL) was added. The suspended particles were moderately shaken horizontally up to 8 h during which time samples were withdrawn from the liquid phase to follow the decrease in total protein and lipase activity.

Subsequently, the Accurel MP 1000 particles were separated from the solution and immediately washed with buffer (2× 1.0 mL) to remove loosely bound lipase from the carrier. The obtained immobilizate was then dried overnight under vacuum (∼10 kPa) in an exicator. Alternatively the lipase was immobilized directly on to a packed bed of Accurel MP1000 particles, by alternatively recirculating ethanol, Lipozyme CALB L in 0.1 M phosphate buffer, pH 7, and finally only buffer over the bed, using the same volumes and times as described above. The bed was dried by applying a moderate air flow over the bed for approximately 12 h.

Immobilization on Amberlite and Duolite was done according to the manufacturers' recommendations and involved the same procedure as the one described above, excluding the sieving step and exchanging ethanol wetting for an extra buffer wash step. Lewatit was also not sieved, but was exposed to an extra overnight vacuum drying step prior to immobilization.

2.3. Enzymatic reactions on small scale

The immobilized enzyme preparation was weighed into a 4 mL reaction vial, followed by 2 mL of the reactants—olein fatty acid mixed with decanol in a 1:1 molar ratio for the esterification reaction, or olein fatty acid mixed with monoethanolamine in a 2:1 molar ratio in case of amidation reaction. The vials were incubated with shaking at the desired temperature in a temperature-controlled shaker (HLC Biotech, Bovenden, Germany) and samples were withdrawn for determination of activity of the enzyme preparations.

One unit of lipase activity was defined as the amount of enzyme required for conversion of 1 mmol substrate per minute. Specific activity was calculated as units expressed per gram catalyst preparation. Specific cost was defined as the sum of carrier and lipase cost divided by the activity units.

2.4. Stability of immobilized enzyme

The stability of the immobilized enzymes was investigated at temperatures between 60 and 90 °C by repeated amidation reactions as described above. The activity and final conversion obtained after the completed reaction were determined. After the reaction, the product was removed using a syringe and new reactants were added. For the stability studies using the packed bed reactor (PBR), the bed was first drained, then washed with 10–20 mL fatty acid, and thereafter drained again before running subsequent reactions.

2.5. Reactor set-up and preparative scale reaction

The preparative scale experiments were performed in a 250 mL batch stirred tank reactor (BSTR) submerged in a temperature-controlled oil bath. Olein fatty acid (40–50 g) was added to the reactor and heated to the desired temperature and then 40 mg/mL of the immobilized enzyme was added.

For the packed-bed experiments, the immobilized enzyme was packed in a glass column (10 mm i.d. × 150 mm length) stoppered by a steel mesh that was supported by a cylinder made from polyaryletheretherketone (PEEK) with a cavity to permit the even distribution of liquid across the mesh. Moving the end cylinders permitted some adjustment of the bed volume, and using a bed of 2.0 g of enzyme preparation resulted in a bed height of approximately 5 cm. The column packed with the immobilized enzyme was placed in the temperature controlled oil bath, and was connected through both ends to the stirred tank reactor with tubings allow-

Table 1
Different carriers investigated for the immobilization of CALB.

Name	Matrix	Type	Specific area (m^2/g)	Mean pore diameter (Å)	Mean diameter (μm)	Cost (€/kg)
Amberlite XAD4[a]	Polystyrene DVB	Hydrophobic	750	100	640	39
Amberlite XAD7HP[a]	Aliphatic acryl polymer	Hydrophilic	500	450	560	47
Duolite A568[a]	Crosslinked phenol–formaldehyde condensate	Hydrophilic	na	na	na	92
Accurel MP1000[b]	Polypropylene	Hydrophobic	70[c]	2000[c]	50–1000[d]	40
Lewatit VP OC 1600[e]	DVB-crosslinked methacrylate polymer	Hydrophobic	130	15	315–1000	41

[a] Manufactured by Rohm&Haas.
[b] Currently manufactured by Membrana Accurel Systems, Obernburg, Germany (previously by AkzoNobel).
[c] According to Al-Duri et al. [16].
[d] The particle size distribution of Accurel MP 1000 was sieved to obtain a more uniform preparation; 250–500 μm and >500 μm was used in the experiments.
[e] Manufactured by Bayer.

ing recirculation of the reactants using a peristaltic pump (Alitea, Stockholm, Sweden). The initial recirculation speed was approximately 7 g/min.

The reactions were started by adding to the BSTR half the molar amount of monoethanolamine with respect to the fatty acid. Additional amount of monoethanolamine was added in portions at different times to a final molar ratio of 1:1 compared to the fatty acid according to our earlier studies [9]. The reactants were mixed using an IKA Euroline mechanical stirrer (VWR International, Stockholm, Sweden) with a propeller. Water formed in the reaction was removed by applying a vacuum down to approximately 5–10 kPa using a vacuum pump (N810FT.18 from KNF-Neuberger, Stockholm, Sweden).

2.6. Analyses

The immobilization yield was quantified by determining the unbound protein content and lipase activity remaining in the supernatant before, during and after immobilization. Protein content was measured by the Bradford method [14], and lipase activity by determining the hydrolysis of *p*-nitrophenyl propionate (*p*NPP) according to the method of Gitlesen et al. [15]. To 2 mL phosphate buffer (20 mM, pH 7.0) in a 3-mL cuvette was added 10 μl solution of 100 mM *p*NPP in methanol and the reaction was started by adding 50 μL an aliquot of the enzyme solution. The formation of *p*-nitrophenol was followed by measuring the change in absorbance over 2 min at 405 nm.

The lipase catalyzed amidation and esterification were analyzed by acid titration using 50 mM KOH and phenolphthalein, which measured total acid conversion to amide as well as the diacylated amide ester, as observed by comparison with HPLC results of amidation of lauric acid. The conversion based on titration was calculated as:

$$\text{Conversion}\quad (\%) = \frac{[\text{Acid}]_{t0} - [\text{Acid}]_t}{[\text{Acid}]_{t0}} \times 100$$

where Acid_{t0} and Acid_t are the acid number at time zero and time *t*, respectively.

The amide end-products were analyzed using HPTLC, where samples were applied to a normal phase silica plate and developed with cyclohexane:*t*-butanol:acetic acid:2-propanol:2-pentanol (80:10:5:2.5:2.5) for 7.5 cm followed by cyclohexane:2-propanol (8:2) for 2 cm and then visualized by fluorescein (0.2% in ethanol). Standards were prepared by repeated crystallization and dissolution in a cyclohexane:2-propanol system.

3. Results and discussion

3.1. Immobilization of C. antarctica lipase

Five different carriers commonly used for enzyme immobilization were evaluated for immobilization of *C. antarctica* lipase B (see Table 1). Different loadings of Lipozyme CALB L were applied on the carriers and the resulting preparations were used for the esterification of olein fatty acid with decanol without solvent at 60 °C. Based on these results, the specific activity was plotted (Fig. 1). Accurel MP 1000 (also referred to as MP1000), an irregular shaped macroporous polypropylene resin with large pore size and Lewatit, a methacrylate–styrenedivinylbenzene resin were found to have the best immobilization performance. It can be seen that already at a loading of 2–3 wt.% these carriers are saturated with lipase and only a moderate increase in specific activity is seen if the loading is increased to 5 wt.%. As expected, the specific activity of MP1000 particles was also dependent on the particle size, the smaller particles exhibiting higher (about 1.4-fold) activity than >500 μm particles. Lewatit® VP OC 1600 (Lewatit) is the carrier used for the commercially available Novozym®435, and MP1000

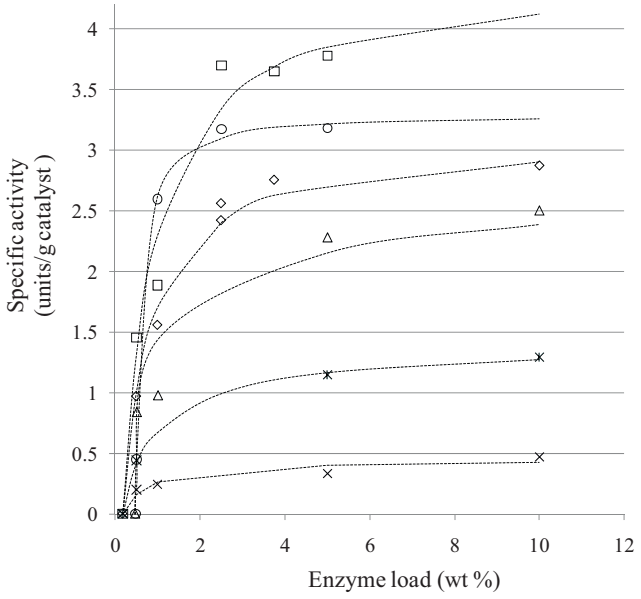

Fig. 1. Specific activity (units/g catalyst) of the different immobilized preparations for esterification of olein fatty acid with decanol at increasing loading of Lipozyme CALB L on the carrier. The data were fitted to the Langmuir adsorption isotherm using least square regression [21] (dotted lines). Diamond: MP1000 >500 μm, square: MP1000 200–500 μm, triangle: Duolite A568, cross: Amberlite XAD4, star: Amberlite XAD7HP, circle: Lewatit.

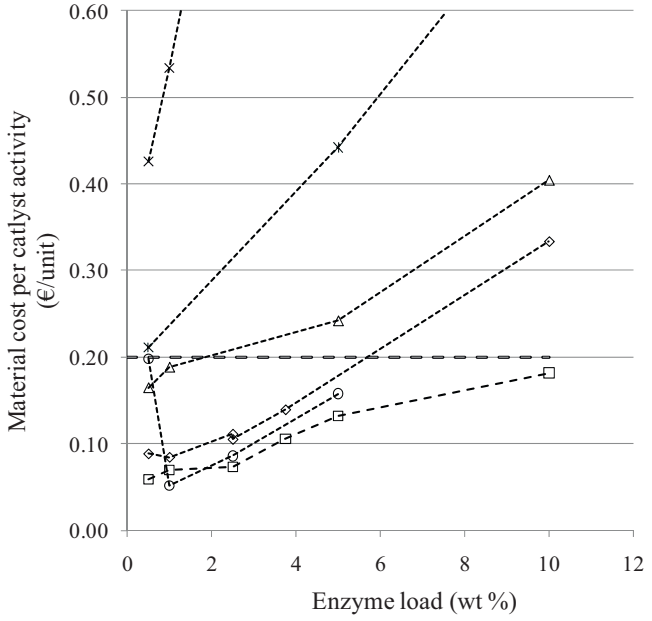

Fig. 2. Specific cost (€/unit) of immobilized lipase versus the enzyme load, for the esterification of olein fatty acid with decanol at 60 °C using CALB immobilized on different commercially available carriers. Diamond: MP1000 >500 μm, square: MP1000 200–500 μm, triangle: Duolite A568, cross: Amberlite XAD4, star: Amberlite XAD7HP, circle: Lewatit VP OC 1600. The horizontal interrupted line indicates the approximate cost of commercially available CALB preparations per catalyst activity.

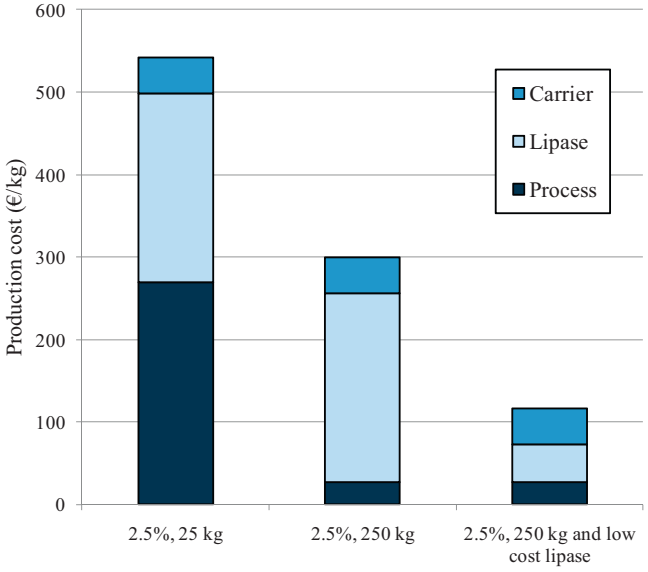

Fig. 3. Estimated total cost (€/kg) of production of immobilized *Candida antarctica* lipase B at different production volumes. The rightmost column assumes the lipase cost to be 1/5 of the normal cost.

has previously been reported to be a suitable carrier for many different lipase applications [17–20], and is considered to be relatively inexpensive compared to other common carrier materials [13].

The time course of the immobilization of CALB on MP1000 (>500 μm) and Lewatit using loadings up to 10 wt.% was also studied to ascertain that equilibrium of adsorption had been reached in the above studies. It was seen that the adsorption of the lipase on the carrier was fast; after 6–8 h of moderate shaking at room temperature no further decrease in protein content or lipase activity in the supernatant was seen.

To determine which carrier and loading held the best economical potential, the material cost per catalytic activity (the cost of carrier and enzyme divided by the activity) was also calculated and plotted versus lipase loading (Fig. 2). It could be expected that when using an enzyme loading lower than ca. 1 wt.%, the impact of carrier cost will predominate, and at loadings higher than 2–3 wt.% the extra lipase is wasted since it is not adsorbed on to the carrier and is thus washed away after the immobilization step. Comparing the loading and material for the preparations, the lowest unit production cost was obtained for 1 wt.% lipase loading on MP1000 and Lewatit, calculated to be about 0.05 €/unit of lipase activity on the immobilized preparation. The in-house preparation of 2.5 wt.% lipase loading on Lewatit had a specific cost of 0.09 €/unit of lipase activity (corresponding to a preparation cost of 275 €/kg) and showed the same initial activity as Novozym®435 (3.2 unit/g catalyst). The corresponding cost for the commercially available CALB on Lewatit is considerably higher, 0.2–0.3 €/unit, calculated from the market price of commercially available CALB on Lewatit (~800–1200 €/kg depending on purchased amount and supplier).

In the above calculations, processing costs and profit margin are not included as these costs are hard to estimate and strongly dependent on production volume. However, assuming a total labor input of 32 man hours, at a cost of 60 €/h, including overhead, and 48 h equipment use at a cost of ca. 100 €/h, the processing costs add up to about 7000 €. Assuming a production volume of 25 kg per batch, the added processing cost amounts to 270 €/kg, however, for a production volume of 250 kg this amounts to less than 30 €/kg, significantly reducing the total cost of the preparation. An increase in production volume would most probably also lead to a reduction in the cost of the biocatalyst [22]. Assuming a decrease of the cost of the enzyme to 20% of current cost would reduce the total cost of the preparation significantly (see Fig. 3). This would also affect the optimum loading of enzyme to carrier to a slightly higher value (not shown).

Based on the promising results obtained for the in-house preparation of MP1000, it was decided to study this preparation in more detail for the acylation of ethanolamine, and to use the commercial preparation Novozym®435 as a benchmark. The MP1000 fraction retained by a mesh of 500 μm was selected for further immobilization experiments. The rationale for choosing a rather large particle size was to facilitate separation after reaction, and to simplify the use of the preparation in a packed-bed reactor without excessive back-pressure.

3.2. Amidation of olein fatty acid using CALB immobilized on Accurel MP1000

The preparations of immobilized CALB on MP1000 were used for studying the amidation of olein fatty acid with monoethanolamine at different temperatures. Fig. 4 shows a plot of the specific activity at 70–90 °C versus the enzyme loading used for immobilization. As was expected, the graph has the same shape as in Fig. 1, i.e. after saturating the particles with lipase (at approximately 2.5 wt.% lipase loading) no further increase in reaction rate is seen.

Similar to the calculations above, the proportion of carrier material and lipase that would be most economical for the amidation reaction was determined by plotting the material cost per activity against the loading of lipase added. It can be seen from Fig. 5 that the lower loadings (<2.5 wt.%) give the lowest material cost per activity. The production costs at 80 °C were 0.2 and 0.4 €/unit activity using CALB loadings of 0.5 and 2.5 wt.%, respectively. This is also significantly lower than the 0.7–0.9 €/unit activity for the commercially available preparations, although additional costs for processing, marketing, etc. should be considered. As discussed earlier, the stability of the preparation during reaction is equally important for the total added cost of the enzyme to the production cost.

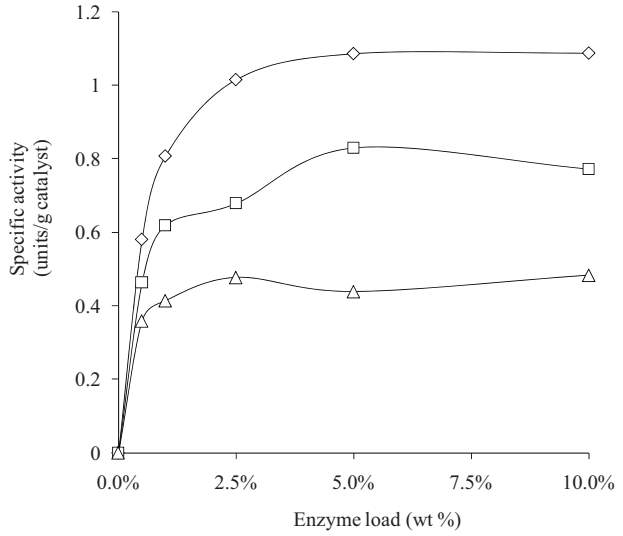

Fig. 4. Specific activity (unit/g) versus CALB loading (wt.%) on MP1000 (>500 μm) for the amidation reaction of olein fatty acid with monoethanolamine in 2 mL volume at (△) 70 °C, (□) 80 °C and (◊) 90 °C, respectively.

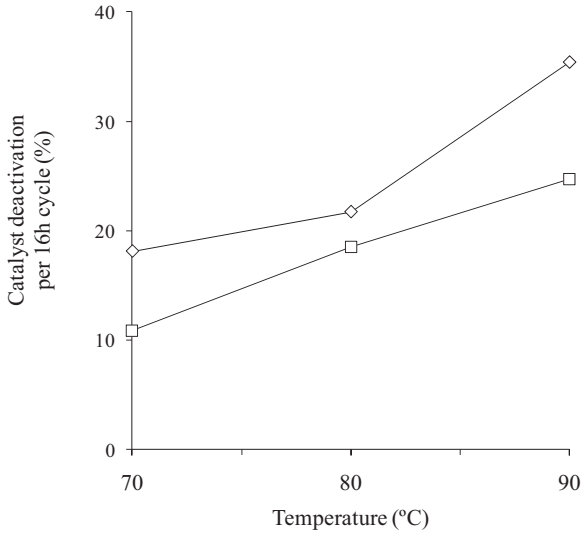

Fig. 6. Activity loss of Novozym®435 (◊) and immobilized CALB (2.5 wt.%) on MP1000 (>500 μm) (□) during repeated 16 h amidation reactions between olein fatty acid and monoethanolamine in 2 mL volume at 70–90 °C.

The preparation with a loading of 2.5 wt.% was selected for stability studies at 70, 80 and 90 °C for the amidation reaction, and was benchmarked with the stability of Novozym®435 (which has a reported loading of 8.2 wt.% [23]). As can be seen from Fig. 6, the stability of the MP1000 preparation was slightly higher than that of the commercial preparation. Not unexpectedly, the rate of inactivation was higher at elevated temperatures.

3.3. Amidation using the immobilized lipase in different reactor set-ups

Two different reactor set-ups were considered for evaluation of the immobilized enzyme: a batch stirred tank reactor (BSTR) with the immobilized enzyme dispersed in the reactants and a BSTR connected to a packed bed loop containing the enzyme. In both cases, a vacuum was applied to the BSTR for removal of water.

The different set-ups were compared for the amidation reaction at 70 and 80 °C. At 80 °C, both reactor types produced similar results giving a conversion of fatty acid of >97% in 9 h (Fig. 7), whereas at 70 °C, the reaction was stopped after 10 h when the conversion had reached almost 90% because the product started to solidify in the unheated tubes connecting the packed bed reactor with the substrate reservoir. The results were also compared to a BSTR using Novozym®435 at 80 °C and were found to give equally good yields although requiring a slightly longer reaction time.

The packed bed option was selected as the preferred set-up as this facilitates separation of the immobilized enzyme after reaction and also avoids shear stress on the particles caused by stirring [24]. This aspect is especially important for the MP1000 preparation as the carrier is quite brittle and tends to break down into microparticles that could contaminate the product and lead to loss of activity when re-using the preparation. It was noted that the packed bed seemed to operate without significant mass-transfer limitations despite the uneven character of the particles.

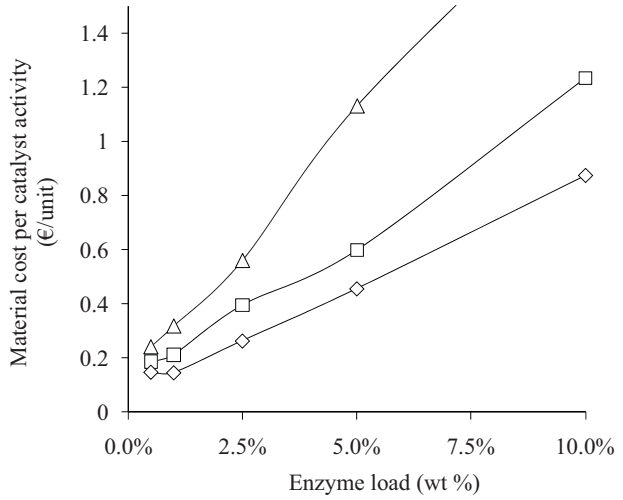

Fig. 5. Specific cost (€/unit) of immobilized lipase at varying enzyme load on MP1000 >500 μm for the amidation of olein fatty acid with monoethanolamine at (△) 70 °C, (□) 80 °C and (◊) 90 °C, respectively.

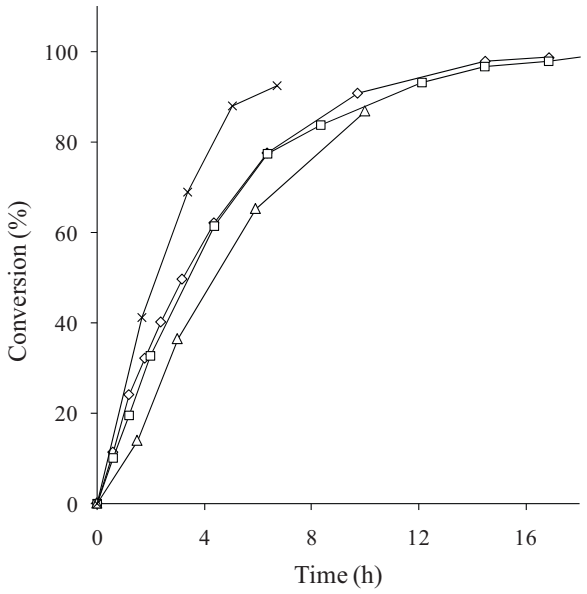

Fig. 7. Conversion of olein fatty acid during amidation catalyzed using different preparations and process set-ups. The different symbols represent Novozym®435 in BSTR at 80 °C (×), and CALB immobilized on MP1000 (2.5%, w/w, >500 μm) in BSTR at 80 °C (◊), and in PBR at 80 °C (□), and in PBR at 70 °C (△).

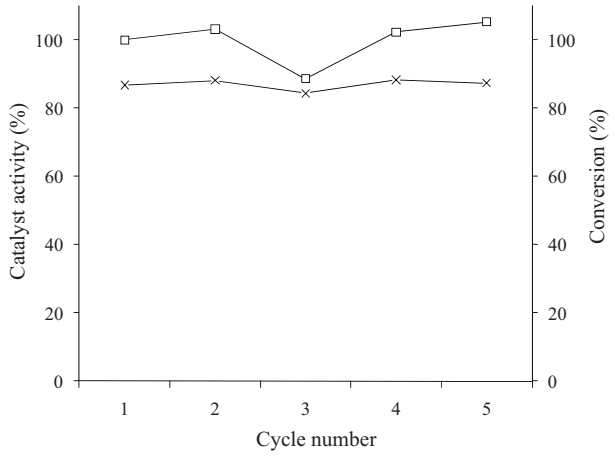

Fig. 8. Process stability during repeated batches of amidation of olein fatty acid in 10 h reaction at 70 °C using immobilized CALB on MP1000 (2.5%; >500 μm) in a packed bed reactor-loop set-up. The symbols represent: catalyst activity expressed as percentage of the initial reaction rate retained (□), and conversion to amide obtained after 10 h (×).

Finally the process stability of the packed bed set-up was investigated by running repeated amidation reactions at 70 °C (10-h cycles). As can be seen in Fig. 8, no decrease in catalyst activity was observed after five consecutive batches. Even the carrier material retained its form during the long-term use in the PBR set-up. Since the total economy of the process is dependent on the stability, this opens up the possibility for this biocatalyst to be considered for application at industrial scale for the production of amides.

4. Conclusions

This study shows that immobilization of CALB on Accurel MP 1000 and Lewatit has the potential to provide a cost-efficient biocatalyst preparation for esterification and amidation reactions. MP1000 is cheaper than most other materials used for immobilization of enzymes and furthermore, the immobilization procedure was very simple. Even immobilization in a PBR was possible, which would decrease costs for processing, etc. This study also shows that the cost of the immobilized enzyme could be minimized by balancing the amount of CALB added to the resin and that a larger market for the product would enable scale benefits that could lead to a significantly cheaper biocatalyst, something that should be considered when making cost evaluations for future lipase catalyzed processes. Furthermore, the stability of the developed enzyme preparation was similar to that of the commercial preparation. It could be used both in a normal batch stirred tank reactor as well as in a packed bed reactor although the latter format is preferred. Such an immobilized enzyme preparation, with a unit cost of one quarter of the presently available preparations, is very promising from an industrial perspective for present and new applications and would certainly improve the economic feasibility of many suggested processes.

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