

# Tuning lipase B from *Candida antarctica* C–C bond promiscuous activity by immobilization on polystyrene-divinylbenzene beads†

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Diana F. Izquierdo,<sup>a</sup> Oveimar Barbosa,<sup>b,c</sup> M. Isabel Burguete,<sup>a</sup> Pedro Lozano,<sup>d</sup> Santiago V. Luis,<sup>a</sup> Roberto Fernandez-Lafuente<sup>\*b</sup> and Eduardo García-Verdugo<sup>\*a</sup>

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Lipase B from *Candida antarctica* (CALB) is able to catalyze C–C bond formation. After immobilization onto a hydrophobic PS-DVB support, the activity increases when compared to that of the soluble or tan – the commercially available Novozyme 435 (being up to 6 fold more active). Our results show that although this activity is not related to the catalytic group, the promiscuous activity of CALB may be tuned via immobilization. In addition, we have show that the secondary structure of both immobilized enzymes is quite different, using FT-ATR-IR spectroscopy.

Lipase biocatalysts have been widely used as green and efficient catalysts for a wide range of synthetic transformations because of their high stability, catalytic efficiency, commercial availability and broad substrate specificity.<sup>1</sup> In recent years, novel synthetic applications related to non-conventional reactions catalyzed by biocatalysts have emerged.<sup>2</sup> Hence, synthetic C–C bond formation processes such as aldol reactions, Michael additions, Mannich reactions, Markovnikov additions, Henry reactions and others have been extensively documented.<sup>3,4</sup> In this context, the development of green protocols for the synthesis of  $\beta$ -nitroalcohols is highly attractive as these bifunctional compounds can be used as versatile precursors or intermediates for the preparation of a great variety of pharmaceutically active principles.<sup>5</sup> Indeed, different biocatalysts have been described to promote the Henry reaction in organic solvents, biphasic systems composed of water and organic solvents or in aqueous medium.<sup>6</sup> Although some enzymes, *e.g.*, hydroxynitrile lyase, are able to induce some degree of enantioselectivity,<sup>7,8,9</sup> in general, the use of lipases lead to quite poor enantiocontrol. Indeed, enantioselectivity is only obtained by coupling the biocatalytic Henry reaction to the enzymatic kinetic resolution of the racemic mixture.<sup>10</sup> However, even when low

enantioselection may be achieved, biocatalytic processes present clear advantages from an environmental point of view. The synthesis of *rac*-nitroalcohols usually requires the use of strong bases such as sodium methoxide, sodium hydroxide, LDA, butyl lithium, barium hydroxide or sodium carbonate to promote the condensation between aldehydes and nitroalkanes. In many cases, such conditions lead to the formation of undesired side products. Consequently, the development of new general, milder and efficient catalytic protocols for the catalysis of the Henry reaction has been pursued by replacing stoichiometric processes with cleaner catalytic alternatives. Moreover, a further step towards environmentally friendly synthetic routes is represented by the application of solid catalysts.<sup>11</sup> Thus, supported biocatalytic systems able to efficiently catalyze the Henry reaction are needed.

The nature of the support used for the immobilization of enzyme is a key element to define its catalytic properties (including activity, selectivity and specificity).<sup>12</sup> Thus, for different supports having different hydrophilic/hydrophobic surfaces significant differences on the catalytic efficiency can be found, even when the same immobilization protocol is used. The immobilization process may alter the conformational integrity of the enzyme leading to more or less efficient supported biocatalytic systems.<sup>13,14,15</sup>

Here we compared the performance of lipase B from *Candida antarctica* (CALB) immobilized onto PS-DVB beads<sup>16</sup> and the commercial preparation Novozyme 435 as promiscuous catalysts for the nitro aldol reaction between the nitromethane and 4-nitrobenzaldehyde. The immobilization of CALB on PS-DVB beads leads to a remarkable enhancement for CALB catalytic activity on different reactions.<sup>16</sup> Furthermore, we can explain the differences of our biocatalyst in comparison with commercial available Novozyme 435 by analyzing the differences of secondary structure of both immobilized enzymes by FT-ATR-IR

<sup>a</sup>Dpt. Química Inorgánica y Orgánica, Universidad Jaume I, Avda. Sos Baynat s/n, E-12071 Castellón, Spain. E-mail: [cepeda@uji.es](mailto:cepeda@uji.es); Fax: +34 964728214; Tel: +34 964728751

<sup>b</sup>Departamento de Biocatálisis, Instituto de Catálisis y Petrolquímica-CSIC, Campus UAM-CSIC, Madrid, Spain. E-mail: [rfl@icp.csic.es](mailto:rfl@icp.csic.es); Fax: +34 91 585 47 60

<sup>c</sup>Escuela de Química, Grupo de Investigación en Bioquímica y Microbiología (GIBIM), Edificio Camilo Torres 210, Universidad Industrial de Santander, Bucaramanga, Colombia

<sup>d</sup>Universidad de Murcia, Facultad de Química, Departamento de Bioquímica y Biología Molecular B e Inmunología, Campus de Espinardo, E-30100 Murcia, Spain. Fax: +34 86 888 41 48; Tel: +34 86 888 73 92

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spectroscopy. Our results suggest that even promiscuous activities of lipases may be tuned *via* immobilization on the adequate support.

## Results and discussion

As a part of our ongoing research efforts to develop green methods for C–C bond-forming reactions,<sup>16,17</sup> the catalytic behavior of CALB supported on polymeric supports (*i.e.* MCI GEL CHP20P (ref. 18) and Lewatit VP OC 1600) for the condensation between *p*-nitrobenzaldehyde (**1**) and nitromethane (**2**) as C–C bond (Henry) model reaction (Scheme 1) has been studied.

Fig. 1 depicts the time-course profile of 2-nitro-1-(4-nitrophenyl)ethanol (**3**) yield, as the Henry reaction product, by using the same amount of PS-DVD–CALB–1 or Novozyme 435 in water.

In both cases, the supported protein catalyzed the reaction with only traces of dehydrated product (<1%) detected by NMR. However, the immobilization of the CALB on PS-DVB seems to have a significant “*positive effect*” in terms of catalytic activity in this reaction. Indeed, after 10 hours of reaction the yield achieved in the reaction catalyzed by PS-DVD–CALB–1 is *ca.* 6-fold higher than the one obtained using Novozyme 435.

Table 1 summarizes the results obtained using water as the reaction medium and the supported CALB operating at different conditions. No adduct formation was observed in the absence of catalyst (Table 1, entry 1), while the supported

**Table 1** The catalytic Henry reaction between **1** and **2** in water carried out by PS-DVB–CALB–1<sup>a</sup>

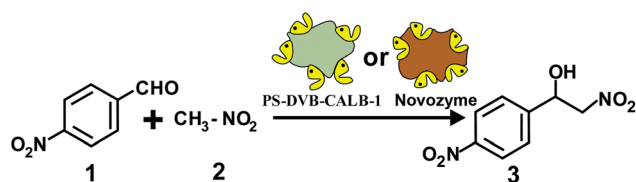
Entry	Catalyst	T (°C)	Time (h)	Yield <sup>b</sup> (%)
1	None	40	24	<5
2	PS-DVD–CALB–1	40	24	87
3	PS-DVD–CALB–1	30	24	54
4	PS-DVD–CALB–1	25	24	<5
5	PS-DVB	40	24	<5
6	Free-CALB <sup>c</sup>	25	24	<5
7	Free-CALB <sup>c</sup>	40	24	55

<sup>a</sup> 2.8 mL of a solution of 0.2 M of *p*-nitrobenzaldehyde in water, *p*-nitrobenzaldehyde : nitromethane 1 : 10 (molar ratio), **2** : PS-DVB–CALB–1 1 : 2 by weight. <sup>b</sup> Calculated by <sup>1</sup>H-NMR. <sup>c</sup> 1 mL of lipozyme in aqueous solution (6.9 mg mL<sup>-1</sup>) and 0.143 mmol of *p*-nitrobenzaldehyde, *p*-nitrobenzaldehyde : nitromethane 1 : 10 (molar ratio).

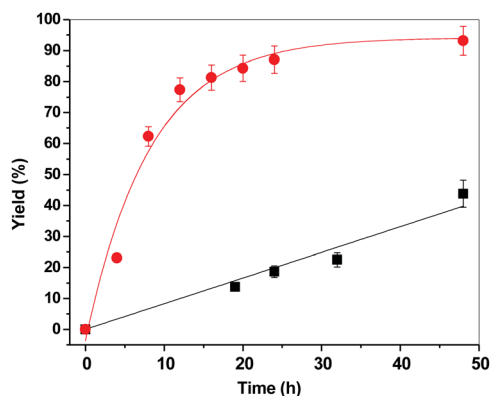
PS-DVD–CALB–1 catalyst led to a good conversion of the aldehyde **2** to the corresponding nitro-aldol, with a 87% yield at 40 °C (Table 1, entry 2). The possible catalytic effect of the polystyrene-divinylbenzene (PS-DVB) support and free enzyme were also studied as control experiments. The naked carrier did not catalyze the aldehyde transformation into the desired product (<5%, entry 6, Table 1), while the free protein was able to produce a 50% yield of nitro-aldol **4**, after 24 hours of reaction. These results clearly indicate a catalytic effect due to the protein, while the support itself is not able to catalyze the C–C bond formation.<sup>19</sup> It should be noted that reaction temperature has been found to be a key parameter for this reaction. Indeed, our biocatalyst (PS-DVD–CALB–1) led to less of 5% yield at 25 °C, while the yields were increased to 54 and 87% at 30 and 40 °C after 24 h. Similar temperature dependence was found for free CALB, meaning that the biocatalyst required some thermal activation for carrying out the nitro aldol reaction.

PS-DVD–CALB–1 biocatalyst was also tested to perform the reaction between the *p*-nitrobenzaldehyde and a different nitro-alkane such as nitroethane (**4**) at 40 °C during 8 h (see Scheme 2). The final adducts were isolated with good yield (72%), although, unfortunately, no diastereomeric induction was observed, as has been previously reported using other lipase.<sup>10</sup>

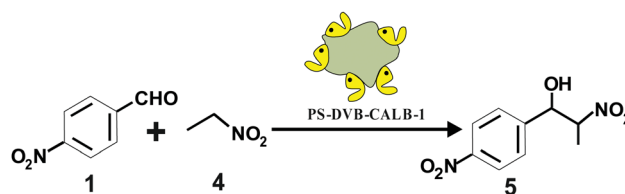
The catalytic behavior of both PS-DVD–CALB–1 and Novozyme 435 biocatalysts was also tested in different organic solvents, selected by their features as green solvents.<sup>20</sup> As can be seen in Table 2, the conversion of the aldehyde to the corresponding nitro aldol product was lower than those observed in water, which could be related to the acid–base catalytic



**Scheme 1** Immobilized lipase-catalyzed the 2-nitro-1-(4-nitrophenyl)ethanol (**3**) synthesis by condensation between *p*-nitrobenzaldehyde (**1**) and nitromethane (**2**).



**Fig. 1** Reaction profile of yield (%) vs. time for the Henry reaction catalyzed by PS-DVD–CALB–1 (●) and Novozyme 435 (■). 50 mg of biocatalyst, 10 eq. CH<sub>3</sub>NO<sub>2</sub> (80 μL) 1 eq. RCHO with 700 μL in water at 40 °C.



**Scheme 2** Immobilized PS-DVB–CALB–1-catalyzed 2-nitro-1-(4-nitrophenyl)propan-1-ol (**5**) synthesis by condensation between *p*-nitrobenzaldehyde (**1**) and nitroethane (**4**).

**Table 2** Immobilized lipases-catalyzed Henry reaction between *p*-nitrobenzaldehyde and nitromethane in different solvents, 24 hours<sup>a</sup> at 40 °C

Entry	Solvent	Biocatalyst	Yield <sup>b</sup> (%)
1	<i>tert</i> -Butanol	Novozyme 435	6
2	<i>tert</i> -Butanol	PS-DVD-CALB-1	22
3	Me-THF <sup>c</sup>	Novozyme 435	<5
4	Me-THF	PS-DVD-CALB-1	15
5	[Bmim][NTf <sub>2</sub> ] <sup>d</sup>	Novozyme 435	<5
6	[Bmim][NTf <sub>2</sub> ]	PS-DVD-CALB-1	40

<sup>a</sup> 2.8 mL of a 0.2 M solution of *p*-nitrobenzaldehyde in the corresponding dried solvent, *p*-nitrobenzaldehyde : nitromethane 1 : 10 (molar ratio); 2 : biocatalyst 1 : 2 by weight. <sup>b</sup> Calculated by <sup>1</sup>H-NMR. <sup>c</sup> Me-THF, 2-methyltetrahydrofuran. <sup>d</sup> [Bmim][NTf<sub>2</sub>], 1-butyl-3-methylimidazolium bistriflimide.

mechanism of the Henry reaction.<sup>6</sup> For all cases, the PS-DVB-CALB-1 derivative showed levels of activity higher than Novozyme 435, confirming the remarkable efficiency of the home-made biocatalyst in comparison with the commercial one. The best results were achieved when 1-butyl-3-methylimidazolium bistriflimide ([Bmim][NTf<sub>2</sub>]) ionic liquid were used as reaction medium. However, it is also relevant to mention that each solvent has a different effect for each of the biocatalysts. This way, the differences in activities, always favored the new catalyst, ranged from 3.5 times by using *tert*-butanol, to more than 8 times by using [Bmim][NTf<sub>2</sub>].

In order to rationalize the behavior of this supported PS-DVB-CALB-1 preparation, additional experiments were carried out comparing the results for two different catalytic reactions such as the kinetic resolution of 1-phenylethanol, which is a conventional biocatalytic lipase benchmark reaction, and the Henry reaction as example of a promiscuous biocatalytic activity. In the same context, the catalytic performance of this immobilized enzyme derivative was comparatively analyzed with respect to two different inactivated PS-DVB-CALB-1 derivatives, such as, a deactivated catalysts (obtained by the denaturalization of the supported protein by thermal treatment in water at 100 °C), and an irreversibly inhibited catalyst (obtained by treatment of the supported CALB with an excess D-pNP).<sup>21</sup> As it can be seen in Table 3, the kinetic resolution of 1-phenylethanol, which was chosen as a control reaction, ran properly when the dry and fresh PS-DVD-CALB-1 was used, leading to 50% conversion with a 99% e.e. for the acylated product. Furthermore, this reaction did not occur when either the thermally deactivated or the irreversible inhibited immobilized enzyme derivatives were used, showing that the active center of CALB had been somehow destroyed.

Table 3 also shows the results obtained for the model nitroaldol reaction by using the different PS-DVD-CALB-1 derivatives (*i.e.* fresh, deactivated and inhibited). As it can be seen, the obtained 2-nitro-1-(4-nitrophenyl)ethanol yield, for the inhibited CALB biocatalyst (entry 2) was similar to that resulted from using the fresh PS-DVD-CALB-1 preparation (entry 1). In the same way, the thermally deactivated immobilized enzyme derivative, which was shown as fully inactive for a classical transesterification reaction where the catalytic triad of the

**Table 3** Comparative study of PS-DVB-CALB-1-catalyzed kinetic resolution (KR) and Henry reaction after stress conditions<sup>a</sup>

Entry	Stress condition	KR <sup>a</sup>		Henry <sup>b</sup>
		Yield <sup>c</sup> (%)	e.e. <sup>c</sup> (%)	Yield <sup>d</sup> (%)
1	None	50	99	94
2	Inhibition <sup>e</sup>	<2	—	88
3	Thermal deactivation <sup>f</sup>	<2	—	53

<sup>a</sup> *rac*-1-Phenylethanol (18.6 μL, 0.150 mmol), vinyl propionate (34.3 μL, 0.315 mmol, 2.1 equiv.), and 20 mg of the corresponding biocatalyst were reacted in cyclohexane (1.5 mL) for 24 h. <sup>b</sup> 700 μL of a solution of 0.18 M of *p*-nitrobenzaldehyde in corresponding solvent, *p*-nitrobenzaldehyde : nitromethane 1 : 10 (molar ratio), and PS-DVB-CALB-1 (50 mg) for 24 h. <sup>c</sup> Determined by GC analysis. <sup>d</sup> Determined by <sup>1</sup>H NMR. <sup>e</sup> A solution of 1.45 mM of D-pNP was prepared with a solution 25 mM of BP; 10 mL of this final solution was added to 100 mg of PS-DVB-CALB and stirred during 16 h at room temp. <sup>f</sup> A mixture of water (10 mL) and PS-DVB-CALB-1 (100 mg) was heated and stirred at 150 °C for 1 h, then was filtered and dried under vacuum.

active-site is involved,<sup>22</sup> led to 53% yield in the Henry reaction product. These results should be explained by a catalytic mechanism not associated with the known natural catalytically active site of the CALB, suggesting that the Henry reaction is catalyzed by another site/s of the enzyme structure, a phenomenon coined as alternate-site enzyme promiscuity.<sup>23</sup> Obviously, the possibility of some unspecific catalytic groups with activity dependence on the enzyme structure can not be discarded. Promiscuous activity seems to depend on the enzyme structure (that may determine the activity of different groups on the enzyme surface), but do not seem to be associated with the active center of the lipase, or even with a defined proteins structure, as even the thermally inactivated enzyme exhibit a significant promiscuous activity.

In this context, it was reported how a suppression of the native hydrolytic activity occurred by substitution of one essential amino acid (Ser105Ala) in catalytic triad at the active site of CALB through rational design, favoring the promiscuous Michael addition activity of this “inactive” enzyme.<sup>24</sup> Moreover, a transglutaminase enzyme was also described as a biocatalyst suitable to catalyze Henry reactions of aliphatic, aromatic and hetero-aromatic aldehydes with nitroalkanes. In this case, it was reported how the reaction takes place in a specific fashion on the catalytic site of the enzyme by the essential assistance of an amine group of the protein backbone.<sup>25</sup>

The adsorption of CALB molecules by interfacial activation of the open form of the enzyme onto the poly-styrene-divinylbenzene support may result in the exposition of basic side-chains of amino acid (*e.g.* Lys, His, Arg) residues to the bulk reaction media or the generation of special environments that can generate an especially reactive group on the enzyme surface.<sup>12</sup>

On the other hand, there are reports stating that crude preparations of lipases may contain a significant number of other impurities, which may be responsible for the catalytic activity on certain reactions.<sup>26</sup> Hence, we performed the electrophoresis of both supported CALB derivatives to investigate

the presence of additional protein impurities (see Fig. 2) and also the content of CALB. The boiling of the enzyme derivative in SDS is enough to desorb the totality of the enzymes adsorbed on the support, which should be immobilized just by hydrophobic interactions. In both biocatalysts, the main protein band was observed at 32 kDa.

Noteworthy, the densitometry of this SDS-PAGE showed that the concentration of the protein is *ca.* of 2.5–3 folds larger in the dried Novozyme than in the dried PS-DVD-CALB-1. Furthermore, considering these results, this difference on protein loading, the specific activity of the PS-DVD-CALB-1 in comparison with the one of the Novozyme 435 should be multiplied by an additional 2.5–3 folds factor. For instance, in water, the CALB specific activity for PS-DVD-CALB-1 will be between 15 and 18 folds higher than for the Novozyme 435 as the difference on activity for total mass of biocatalyst is *ca.* of 6 fold.

The densitometry studies indicated that the concentrations of all protein bands are similar in both derivatives, although Novozyme 435 presents some additional protein bands that are not present in our new derivative. Summarizing, the electrophoresis results suggest that a catalytic effect due to the presence of a different protein impurity can, in principle, be excluded. Besides, it should also be noted that the immobilized derivative is much more active than the free enzyme used to prepare this immobilized biocatalyst (Table 1), where possible impurities can be also found. Thus, immobilization onto the hydrophobic surface seems to play a key role to tune the catalytic activity of supported lipases.<sup>27</sup> It is known that lipases present a peculiar mechanism of action that involves conformation changes of their overall structures.<sup>28</sup> Although CALB has a very short lid that does not fully block its active center,<sup>29</sup> CALB still suffers conformational changes due to the movement of this lid, changes that affect points very far from the enzyme active center. Indeed, it has been suggested that the functional

changes detected in lipases immobilized following different protocols are founded on controlling the conformational changes of the lipases.<sup>30</sup>

Thus, a study to elucidate if there are detectable changes on the CALB structure of both biocatalysts was performed. In this regard, the infrared spectra of the proteins, in particular the amide I region at 1700–1600  $\text{cm}^{-1}$ , can provide some valuable information about the protein's secondary structure in terms of  $\alpha$ -helix,  $\beta$ -sheets,  $\beta$ -turns and non-ordered or irregular structures.<sup>31,32</sup> Therefore, the FT-ATR-IR spectra of enzyme and support (PS-DVD-CALB-1 and Novozyme 435) were recorded and analysed by deconvolution of the amide I region (Fig. 2b, Table 4). The large peak at *ca.* 1740  $\text{cm}^{-1}$  is due to the C=O bonds present in the polymeric backbone of the Novozyme 435.

FT-ATR-IR spectra of both PS-DVD-CALB-1 and Novozyme 435 have been obtained with 2  $\text{cm}^{-1}$  resolution. The band of the amide I was deconvoluted in six main components and the relative area of each of them was determined by a Gauss-Newton curve fitting (Table 4). The different components appeared at similar defined frequencies and they can be assigned to protein secondary structure elements. Some significant differences can be observed in the contribution of these main bands. Hence, the components associated with  $\beta$ -sheets (1629 and 1637  $\text{cm}^{-1}$ ) are less important in the PS-DVD-CALB-1 than in the case of the Novozyme (*ca.* 12% vs. *ca.* 30%). The spectrum of both biocatalysts showed a band *ca.* 1649  $\text{cm}^{-1}$  having similar importance and that can be associated to the  $\alpha$ -helix structure of the protein, while peaks at 1657 and 1668  $\text{cm}^{-1}$ , characteristic respectively of  $\alpha$ -helix structure and  $\beta$ -turns non-associated by hydrogen bonds, are larger in the case of the PS-DVD-CALB-1 (*ca.* 54% vs. *ca.* 29%). Finally, both supported proteins seem to present a similar degree of aggregation, as the band at 1690  $\text{cm}^{-1}$  presents a similar intensity.<sup>33</sup> Those structural changes may well explain the high specific activity of our supported biocatalyst compared with Novozyme 435.<sup>34</sup> The combination of the hydrophilic structure and the enzyme loading clearly contributes to the differences observed for the structure of the protein in this two supported biocatalyst. These changes in the protein structure may modify the exposure of certain aminoacid such as L-histidine or L-arginine containing nonspecific active basic moieties able to catalyze the condensation between nitroalkene and the aromatic aldehyde.<sup>35</sup>

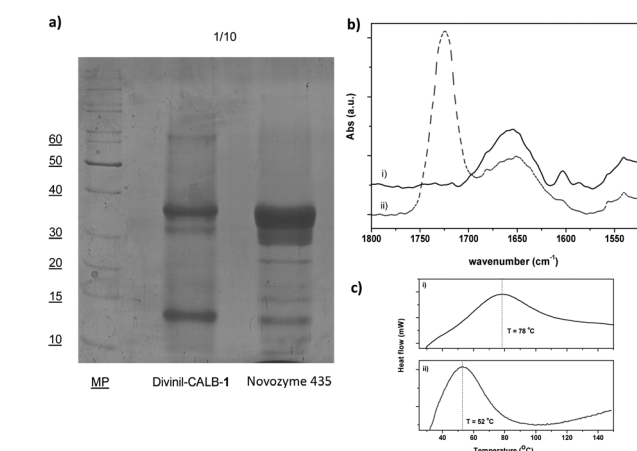


Fig. 2 (a) SDS-PAGE of the different immobilized preparations used on this paper. Experiments were performed as described in the Experimental section. Line 1: molecular weight markers. Line 2: PS-DVD-CALB-1, Line 3: Novozyme 435. (b) ATR-FTIR for the different biocatalysts tested; dashed line Novozyme 435, full line PS-DVD-CALB-1 (c) Differential Scanning Calorimetry (DSC) spectra for the corresponding supported enzyme (i) PS-DVD-CALB-1, (ii) Novozyme 435.

Table 4 Peaks and areas corresponding to deconvolution of the amide I bands for PS-DVD-CALB-1 and Novozyme 435<sup>a</sup>

Entry	PS-DVD-CALB-1		Novozyme 435	
	Peak ( $\text{cm}^{-1}$ )	Area (%)	Peak ( $\text{cm}^{-1}$ )	Area (%)
1	1629	3.2	1623	11.0
2	1637	8.7	1637	19.7
3	1647	18.8	1649	23.9
4	1657	26.0	1658	15.1
5	1668	28.9	1669	14.6
6	1682	14.3	1681	15.8

<sup>a</sup> Components and the relative area was determined by a Gauss-Newton curve fitting of the amide I band. The spectra were treated without subtraction of the support bands.

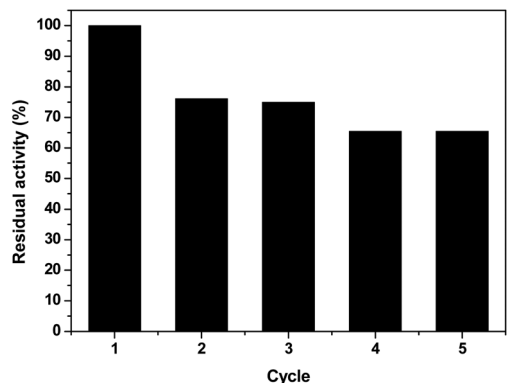


Fig. 3 Catalyst recycling experiments for the Henry reaction between *p*-nitrobenzaldehyde and nitromethane. 50 mg of biocatalyst, 10 eq.  $\text{CH}_3\text{NO}_2$  (80  $\mu\text{L}$ ) 1 eq. RCHO in 700  $\mu\text{L}$  of water at 40  $^\circ\text{C}$ , 24 h.

A study by differential scanning calorimetry (DSC) of the CALB immobilised onto PS-DVB and the commercially available Novozyme 435 supported onto Lewatit VPOC1600 was also carried out to characterise the effect of the immobilisation procedure on the thermal denaturation of the protein. The DSC traces showed a significant shift of the maximum temperature observed from 52  $^\circ\text{C}$  (Novozyme 435) to 78  $^\circ\text{C}$  (PS-DVD-CALB-1). Thus, the nature of the polymer contributes to the stabilization of the polymer structure of the supported protein. All together, these results suggest a significant effect of the immobilisation of CALB over the stabilization of the secondary structure of the protein, providing the PS-DVB highly hydrophobic polymer a “positive effect” in terms of specific activity and rigidity of the enzyme structure.

The possibility of reusing the biocatalyst was also evaluated. Fig. 3 shows the efficiency of the catalyst employed during five consecutive cycles to promote the reaction between *p*-nitrobenzaldehyde and nitromethane under the previous optimized reaction conditions. The residual activity of the system is reduced under continuous recycling. However, it seems that after the reduction observed for the first reuse, the activity tends to reach a stable value. Thus, after the fifth use the biocatalyst conserved 65% of the initial activity, being this value very similar to that for the fourth use.

## Conclusion

In this paper we have shown that the catalytic activity of the supported CALB for a non-conventional C–C bond biotransformation may greatly tune by the immobilization protocol and type of support used. Indeed, the immobilization of the enzyme in the highly hydrophobic PS-DVB material led to an increase of the enzymatic specific activity in water of *ca.* 15–18 folds in comparison with the results obtained for Novozyme 435. Although a certain positive effect on the enzyme the activity may be due to an increment in the local concentration of *p*-nitrobenzaldehyde around the enzyme compared to the bulk concentration of the substrate in water, this increment in activity could be found also in organic solvents (in some

instances evening with a higher increased of activity) suggesting that this effect was not very significant. A decrease in the bulk concentration of the substrates was neither found even using larger amounts of support.

The catalytic activity found is due to a mechanism not associated with the active site of the CALB as the enzyme maintained good levels of activity after its inactivation or after irreversible inhibition. Spectroscopic experiments suggest that the  $\alpha$ -helix structure and  $\beta$ -turns non-associated by hydrogen bonds of CALB are much larger in the case of the PS-DVD-CALB-1 (*ca.* 54% vs. *ca.* 15%) than on Novozyme 435. Thus, the increase on activity in this promiscuous reaction may be related with variations on the secondary structure of the enzyme induced during the immobilization protocol. Although changes on enzyme catalytic features *via* immobilization had been previously reported,<sup>27</sup> this is the first report where this modulation is shown on reactions not really related to the natural catalytic function of CALB but with a promiscuous reaction not involving the catalytic center of the enzyme.

## Experimental section

Free and immobilized (Novozyme 435) lipase B from *C. antarctica* were generously donated by Novo Nordisk (Denmark). MCI GEL CHP20P beads and *p*-nitrophenyl butyrate (*p*-NPB) were purchased from Sigma Chemical Company. Other reagents and solvents were of analytical or HPLC degree were purchased from Sigma or Scharlab and used without further purification.

### Preparation of PS-DVD-CALB-1 by immobilization of CALB

Immobilization was performed as previously described.<sup>7</sup> To fill the support pores with water, a sample of 10 g of MCI GEL CHP20P was suspended in 100 mL of acetonitrile for 1 h under mild stirring, and then 100 mL of water were added. After 1 additional hour of mild stirring, the beads were filtered and resuspended in 100 mL of distilled water for another hour under mild stirring. Finally, the resin was washed 5 times in a glass funnel using each time 5 volumes of water and stored at 4  $^\circ\text{C}$  in wet condition.

A sample of 19 g of washed wet support was suspended in 600 mL of 10 mM sodium phosphate adjusted at pH 7 with NaOH and then 152 mL of commercial enzyme (30 mg mL<sup>-1</sup>) solution were added. This suspension was submitted to mild stirring, and protein concentration of the supernatant was measured using Bradford's protocol.<sup>36</sup> Following this protocol, the enzyme loading was calculated to be 224 mg g<sup>-1</sup> of support. The activity of the supernatant was followed using the pNPB assay, which allowed to estimate an activity immobilization yield over 95%.

### Standard measure of enzyme activity

The enzyme activity assay was performed by measuring the increase in the absorbance at 348 nm produced by the released *p*-nitrophenol in the hydrolysis of 0.4 mM pNPB in 25 mM sodium phosphate at pH 7 and 25  $^\circ\text{C}$ , using a spectrophotometer with a thermostated cell and with continuous magnetic

stirring. To initiate the reaction, 0.1 mL of lipase solution or suspension were added to 2.5 mL of substrate solution. One international unit of pNPB activity was defined as the amount of enzyme necessary to hydrolyze 1  $\mu\text{mol}$  of pNPB  $\text{min}^{-1}$  (IU) under the conditions described above.

### SDS-PAGE experiments

SDS-polyacrylamide gel electrophoresis of the CALB preparations after 50 h of incubation in 50% acetonitrile was performed according to Laemmli<sup>37</sup> using a SE 250-Mighty small II electrophoretic unit (Hoefer Co.), 15% running gel in a separation zone of 9 cm  $\times$  6 cm, and a concentration zone of 5% polyacrylamide. Samples of each of the immobilized enzyme preparations (50 mg) were added to 1 mL of rupture buffer (2% SDS and 10% mercaptoethanol), boiled for 5 minutes and an aliquot of 20  $\mu\text{L}$  of the supernatant was used in the experiments. Gels were stained with Coomassie brilliant blue. Low molecular weight markers from Pharmacia were used (14 000–97 000 Da).

### General procedure for the preparation of nitroalcohols

A typical experiment procedure is as follows: over a suspension of the corresponding aldehyde (0.57 mmol) in water (2.80 mL) were added nitromethane (5.7 mmol) and supported enzyme (100 mg). The mixture was shaken at 40  $^{\circ}\text{C}$  and 250 rpm for the corresponding time. After that time, the reaction was quenched by adding  $\text{H}_2\text{O}$  (5 mL) and the aqueous phase extracted with  $\text{CH}_2\text{Cl}_2$  (3  $\times$  5 mL). The organic phases were combined, dried over  $\text{Na}_2\text{SO}_4$  and filtered, and the solvent was removed by distillation under reduced pressure. The reaction crude was analyzed by NMR to determine the yield.

**2-Nitro-1-(4-nitrophenyl)ethanol.**<sup>19</sup>  $R_f$  (20% EtOAc–hexane) 0.25;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300.13 MHz):  $\delta$  2.25 (brs, 1H), 4.58–4.61 (m, 2H), 5.63–5.68 (m, 1H), 7.64 (d, 3/HH) 6.5 Hz, 2H, 8.26 (d, 3/HH) 6.5 Hz, 2H;  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75.5 MHz):  $\delta$  70.4 ( $\text{CH}_2$ ), 81.0 (CH), 125.5 (2CH), 127.4 (2CH), 145.5 (C), 148.5 (C).

**2-Nitro-1-(4-nitrophenyl)propan-1-ol.**  $R_f$  (20% EtOAc–hexane) 0.29;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300.13 MHz):  $\delta$  1.35 (d, 3/HH) 7.1 Hz, (3H<sub>syn</sub>), 1.52 (d, 3/HH) 7.1 Hz, (3H<sub>anti</sub>), 3.20 (brs, 1H<sub>syn</sub> + 1H<sub>anti</sub>), 4.59–4.72 (m, 1H<sub>syn</sub> + 1H<sub>anti</sub>), 5.20 (d, 3/HH), 9.3 Hz, (1H<sub>syn</sub>), 5.55 (d, 3/HH) 3.5 Hz, (1H<sub>anti</sub>), 7.58–7.63 (m, 1H<sub>syn</sub> + 1H<sub>anti</sub>), 8.21–8.35 (m, 1H<sub>syn</sub> + 1H<sub>anti</sub>);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75.5 MHz):  $\delta$  anti 11.7 ( $\text{CH}_3$ ), 72.3 (CH), 86.6 (CH), 123.8 (2CH), 126.9 (2CH), 145.2 (C), 148.1 (C).  $\delta$  syn 16.1 ( $\text{CH}_3$ ), 74.9 (CH), 87.7 (CH), 124.0 (2CH), 127.8 (2CH), 145.5 (C), 148.1 (C).

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