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Evaluation of the lipase from *Bacillus thermocatenulatus* as an enantioselective biocatalyst

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Abstract—The lipase from *Bacillus thermocatenulatus* (BTL2) has been immobilized using different techniques, and used in the resolution of different chiral substrates in hydrolytic reactions. This enzyme acts as an interesting biocatalyst for these types of reactions, whose catalytic properties can be further improved via the use of different immobilization techniques and reaction conditions. In this way, once the best immobilization techniques and reaction conditions has been chosen, the enzyme can be used to resolve 2-*O*-butyryl-2-phenylacetic acid (±)-**5** with high enantiomeric excess of the release product (ee>99%, *E*>100) in the hydrolysis catalyzed by octadecyl-Sepabeads-BTL2 preparation (at 4°C pH 7 or 9) or glyoxyl-BTL2 preparation (at 4°C pH 7), yielding (*R*)-mandelic acid. The immobilized preparations of BTL2 presented high sensitivity to the experimental conditions [e.g., BTL2 immobilized on glyoxyl-agarose improve *E* value from 11 (37°C) to >100 (4°C)]. In the resolution of 2-hydroxy-4-phenyl-butyric acid ethyl ester (±)-**12** the use of octadecyl-Sepabeads-BTL2 in the presence of 20% acetonitrile gave 2-hydroxy-4-phenyl ethyl butyrate (*S*)-**12** by hydrolyzing the racemic ester to 60% (ee of remaining ester over 90%).

1. Introduction

Lipases (glycerol-ester-hydrolase, EC 3.1.1.3) are enzymes which catalyze the hydrolysis of fatty acid ester bonds in triacylglycerol to give fatty acids, diacylglycerols, monoacylglycerols and glycerol. These enzymes are widely used in organic chemistry because of their high specificity, recognizing very different substrates, sometimes with high regio and enantioselectivity; as a consequence they are used in the resolution of many racemic compounds (acids, alcohols, amines, etc.).[1–9] However, many lipases are only moderately stable at high temperature, drastic pHs, etc. can reduce their usefulness in some interesting reactions. This can be solved by using lipases from thermophilic microorganisms, whose resistance to drastic conditions has been developed by nature.[10–13]

At present, the majority of the thermophilic lipases that has been purified and characterized are obtained from *Bacillus*,[14,15] e.g. lipases from *B. thermocatenulatus*. The thermophile *B. thermocatenulatus* produces two lipases, BTL1 and BTL2.[16a] The gene for the BTL2 lipase has been cloned and expressed in *Escherichia coli*.[16b] The BTL2 lipase is also a 43 kDa protein (predicted from the DNA sequence) and shows high stability at medium temperatures (50°C), alkaline pH (9.0–11.0) and in organic solvents (2-propanol, acetone, methanol). These properties suggest that it can be used as a sturdy biocatalyst for organic synthesis. In the literature, data about its selectivity towards several chiral substrates showed[17] that this hydrolase gives moderate enantioselectivity toward primary alcohols, in spite of its high enantioselectivity with aryl-secondary alcohols. Thus, taking into account these advances, we herein report the selectivity of this interesting lipase, in resolutions of racemic mixtures of different compounds, key intermediates in the synthesis of drugs, in aqueous media, which until now had not been studied with this lipase, e.g. (±)-2-hydroxyphenylacetic acid methyl ester (±)-**4** or *O*-butyryl-2-phenylacetic acid (±)-**5**, direct precursors of the (*R*)-isomer of mandelic acid **3**, a key intermediate in the synthesis of the styryllactone (+)-goniodiol [(1′*R*,2′*S*,5*R*)-5-(1′,2′-dihydroxy-2-phenyl-

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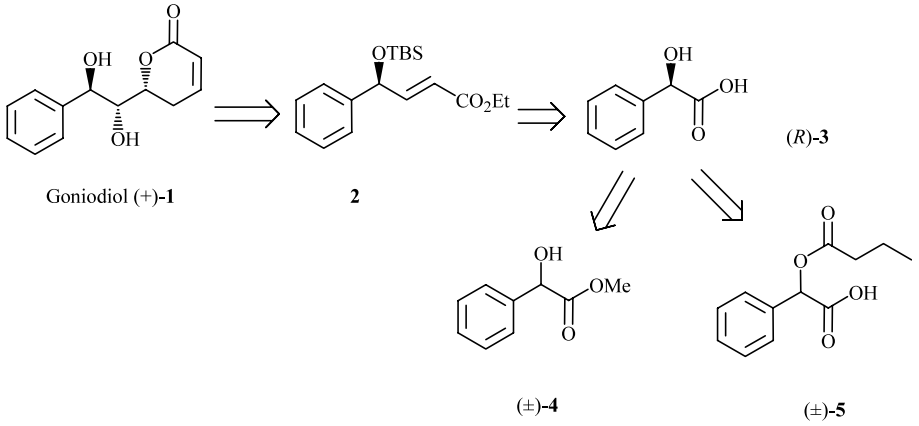
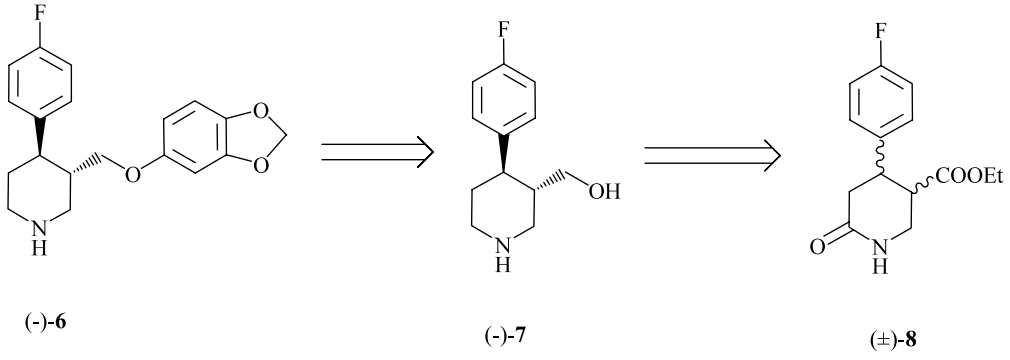
ethyl)-pent-2-eno-5-lactone)] (+)-**1**, which exhibits a potent and selective cytotoxicity against human lung carcinoma with no significant toxicity against brine shrimp[18] (Scheme 1); (±)-*trans*-4-(4′-fluorophenyl)-6-oxopiperidin-3-ethyl carboxylate (±)-*trans*-**8** an intermediate in the synthesis of (−)-Paroxetine, used in the treatment of depression, obsessive compulsive disorder and panic disorder[19] (Scheme 2); (±)-6-(5-Chloropyridin-2-yl)-7-*oxo*-5-(vinyloxycarboyloxy)-5,6-dihydropyrrolo[3,4*b*]pyrazine (±)-**10**, which is an interesting intermediate in the synthesis of (+)-Zopiclone (4-methyl-1-piperazinecarboxylic acid 6-(5-chloro-2-pyridinyl)-6,7-dihydro-7-oxo-5*H*-pyrrolo-[3,4*b*]pyrazin-5-yl ester) (+)-**9**[20], a hypnotic agent (Scheme 3). 2-Hydroxy-4-phenylbutyric acid ethyl ester (±)-**12** was also used, because it is a very important intermediate in the synthesis of (*S*)-Enalapril, (*S*)-1-[*N*-[1-ethoxycarbonyl-3-phenylpropyl]-L-alanyl]-L-proline (*S*)-**14**, a prodrug that presents an important relevance in the control of hypertension and in the treatment of cardiac deficiency (Scheme 4).

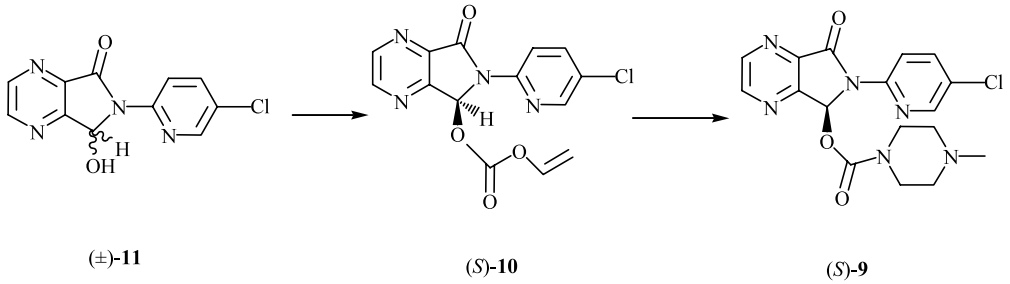
Special interest has also been shown regarding the likely modulation of the properties of the lipase by using different enzyme immobilized preparations that have been used successfully with mesophilic lipases.[21–26] This modulation is based on the alteration of the dramatic conformational changes in the enzyme structure that lipases suffer during catalysis[27–30] due to the different orientation of the enzyme on the support, the degree of rigidification or the alteration of the enzyme environment.



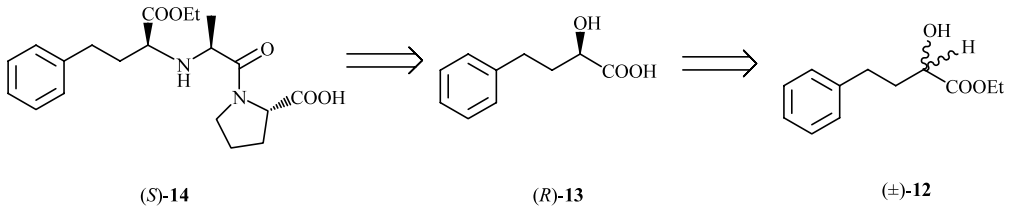
Scheme 1. Retrosynthetic pathway of (+)-Goniodiol.



Scheme 2. Retrosynthetic pathway of (−)-Paroxetine.



Scheme 3. Synthetic pathway of (*S*)-(+)-Zopiclone.



(*S*)-**14** (*R*)-**13** (±)-**12**

Scheme 4. Retrosynthetic pathway of (*S*)-Enalapril.

Table 1. Specific activity of different immobilized preparations of BTL2 in the hydrolysis of different compounds at pH 7. Specific activity: (μmol/h mgprot). The relative error was estimated by ±3%

Immobilized preparation	Ethyl butyrate	*p*-NPP	(±)-**4**	(±)-**5**	(±)-**8**	(±)-**10**	(±)-**12**
Octadecyl-BTL2	0.76	0.24	0.0217	0.038	–	–	2.38×10^{-3}
Glyoxyl-BTL2	0.1676	0.137	5.48×10^{-3}	6.52×10^{-3}	–	–	0.0236
PEI-BTL2	0.266	0.14	nd	5.08×10^{-3}	–	–	nd

2. Results

2.1. The screening of activity of BTL2 on different compounds precursors of drugs

The catalytic activity of BTL2 against different esters at pH 7 and 25°C was analyzed (Table 1). The lipase, immobilized on different supports catalyzed the hydrolysis of ethyl butyrate and pNPP and gave better activities when using the chiral substrates (±)-**4**, (±)-**12** (with the stereogenic centre on the acyl-donor) and (±)-**5** (with the stereogenic centre on the acyl chain). However, the lipase could not hydrolyze compounds (±)-**8** and (±)-**11**, even though they have been successfully tested with other enzymes.[29–33]

For all enzyme preparations, the highest activity was found when using ethyl butyrate as the substrate whilst the lowest was found against compound **4**. The octadecyl-Sepabeads-BTL2 preparation presented the highest activity against most of the compounds, being three times lower with pNPP and 20 times lower with compound **4** with respect to the activity with ethyl butyrate. Furthermore, the octadecyl-Sepabeads-BTL2 preparation was three times more active than PEI-BTL2 preparation and five times more active with respect to the glyoxyl-BTL2 preparation against ethyl butyrate. However, when using pNPP as the substrate, the activity of the octadecyl-Sepabeads-BTL2 was only a two fold-factor higher than the other immobilized preparations which presented similar catalytic activity between them. With substrates (±)-**4** and (±)-**5**, the octadecyl-Sepabeads-BTL2 preparation was around five times more active than the other immobilized preparations, which presented similar activities. However, when using compound **12** the glyoxyl-BTL2 was ten times more active than octadecyl-Sepabeads-BTL2 in the standard conditions analyzed.

Therefore, it would be interesting to study the selectivity of this thermophilic lipase as a biocatalyst in the hydrolytic resolution of compounds (±)-**4**, (±)-**5** and (±)-**12**.

2.2. Enantioselective resolution of (±)-4 and (±)-5, key intermediates in the synthesis of (+)-Goniodiol, catalyzed by immobilized BTL2

Table 2 shows the results obtained in the resolution of (±)-**4** catalyzed by different BTL2 preparations at pH 7 and 25°C. The glyoxyl-BTL2 preparation presented an enantiomeric excess (ee) of 64% at 10% conversion, giving the highest enantiomeric ratio ($E=4.5$), while the octadecyl-sepabeads-BTL2 only showed a low ee of 16% ($E=1.03$) at 11% of conversion.

Table 2. Hydrolytic resolution of (±)-**4** catalyzed by BTL2 immobilized at pH 7 and 25°C. The relative error was estimated by ±4%

Immobilized preparation	Conversion (%)	SP	ee (%)	*E*
Octadecyl-Sepabeads-BTL2	11	R	16	1.03
Glyoxyl-BTL2	10	*R*	64	4.5

SP: stereochemical preference; ee = enantiomeric excess of the product; E = value of enantioselectivity.

Table 3 shows the analysis of the enantioselectivity of different immobilized preparations of BTL2 at pH 7 and different temperatures with substrate (±)-**5** (Scheme 5). At 25°C, the glyoxyl-BTL2 immobilized preparation was the most enantioselective ($E=24$) towards the (*R*)-enantiomer. Interestingly, the PEI-BTL2 preparation presented a lower E value (9), but hydrolyzed the (*S*)-enantiomer quicker.

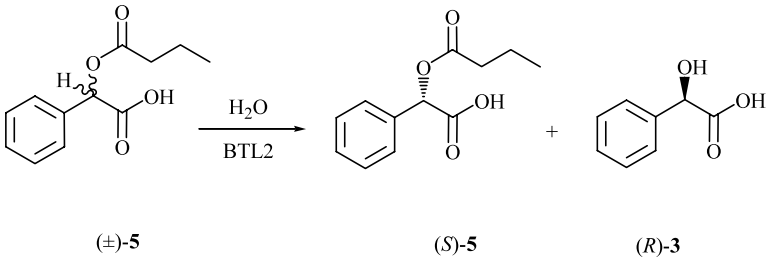
At 4°C and pH 7, the octadecyl-Sepabeads-BTL2 and glyoxyl-BTL2 preparations showed a very high enantioselectivity ($E>100$), hydrolyzing only the (*R*)-enantiomer, while the PEI-BTL2 preparation decreased the E value (2) but still preferring the opposite isomer.

At 37°C and pH 7, the octadecyl-Sepabeads-BTL2 behaved as at 25°C, while the other immobilized preparations decreased their selectivity or even disappearing as in the case of the PEI-BTL2 preparation.

Table 3. Enantioselective resolution of (±)-**5** catalyzed by different BTL2 preparations at pH 7 and different temperatures

Immobilized Preparation	Sp	4°C		25°C		37°C	
		ee	*E*	ee	*E*	ee	*E*
Octadecyl-BTL2	*R*	>99	>100	82	15	88	16
Glyoxyl-BTL2	*R*	>99	>100	92	24	84	11
PEI-BTL2	*S*	37	2	80	9	0	1

SP: stereochemical preference; ee = enantiomeric excess of product at 15% of conversion; *E* = enantiomeric ratio.



(±)-**5** (*S*)-**5** (*R*)-**3**

Scheme 5. Enzymatic resolution of compound (±)-**5** catalyzed by BTL2 immobilized preparation.

Moreover, slight changes in the pH of the reaction provoked differences in the selectivity of the different immobilized preparations of BTL2. Thus, when the pH of the reaction solution was decreased from 7 to 5, a decrease in the enantioselectivity of all the immobilized preparations of BTL2 was observed (Table 4). In this case, at 25°C the octadecyl-Sepabeads-BTL2 was the most enantioselective preparation ($E = 6.5$), hydrolyzing the (*R*)-isomer quicker while PEI-BTL2 was the lowest ($E = 1.5$), but curiously maintaining the preference towards the (*S*)-enantiomer. At 4°C, an increase in the *E* value in all cases was observed, especially in the octadecyl-Sepabeads-BTL2 preparation (*E* value from 6.5 to 17).

At pH 9, the octadecyl-Sepabeads-BTL2 presented a high enantioselectivity ($E = 56$), being again the most enantioselective between all the immobilized preparations of BTL2, being 13-fold more enantioselective than the glyoxyl-BTL2. The PEI-BTL2, maintained the enantiopreference towards the (*S*)-enantiomer with an *E* value of 2 (Table 5). When the temperature was decreased to 4°C, an improvement in the *E* value for all BTL2 immobilized preparations was observed, in which the octadecyl-Sepabeads-BTL2 only hydrolyzed the (*R*)-enantiomer ($E > 100$); PEI-BTL2 immobilized preparation increased the *E* value almost five times ($E = 9.3$), hydrolyzing the opposite enantiomer faster.

Therefore, it seems that using some immobilization techniques and different experimental conditions it is possible to resolve the racemic mixture of compound **5** with very high enantioselectivity ($E > 100$), with an ee> 99% from 8% until 50% of conversion (Fig. 1).

Table 4. Enantioselectivity of immobilized preparations of BTL2-catalyzed hydrolysis of (±)-**5** at pH 5

Immobilized preparation	Preferred enantiomer	25°C		4°C	
		ee	*E*	ee	*E*
Octadecyl-Sepabeads-BTL2	*R*	72	6.5	89	17
Glyoxyl-BTL2	*R*	46	2.7	49	2.9
PEI-BTL2	*S*	21	1.5	58	3.8

Ee = enantiomeric excess of the product at 15% of conversion; *E* = value of enantioselectivity.

Table 5. Enantioselectivity of immobilized preparations of BTL2-catalyzed hydrolysis of (±)-**5** at pH 9

Immobilized preparation	Preferred Enantiomer	25°C		4°C	
		ee	*E*	ee	*E*
Octadecyl-Sepabeads-BTL2	*R*	96	56	>99	>100
Glyoxyl-BTL2	*R*	62	4.3	70	6.4
PEI-BTL2	*S*	33	2	81	9.3

Ee = enantiomeric excess of the product at 15% of conversion; *E* = value of enantioselectivity.

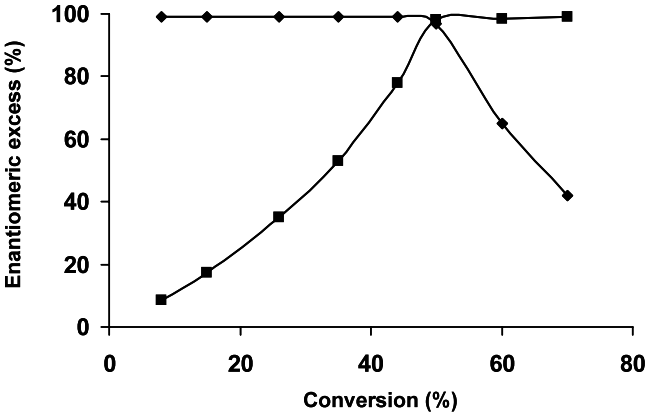


Figure 1. Graphic of evolution of enantiomeric excess (ee) versus conversion in the hydrolysis of (±)-**5** catalyzed by octadecyl-Sepabeads-BTL2 immobilized preparation. Experiments were performed using concentration of substrate of 0.5 mM at pH 7 and 4°C. ee of remaining substrate (*S*)-**5** (squares), ee of released acid (*R*)-**3** (rhombus).

Table 6. Specific activity of different BTL2 immobilized preparations in the hydrolysis of (±)-**12** at different temperatures. Specific activity: (μmol/h mgprot).

Immobilized preparation	4°C	25°C
Octadecyl-Sepabeads-BTL2	0.0262	0.143
Glyoxyl-BTL2	1.85	3.77
Glutaraldehyde-BTL2	0.52	1.42

2.3. Enzymatic resolution of compound (±)-12 as precursor of (*S*)-Enalapril

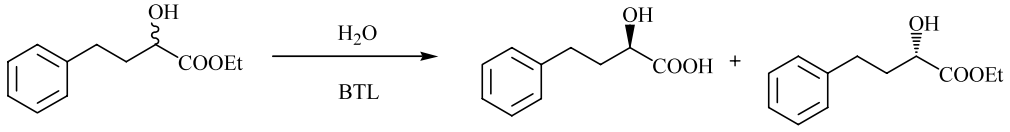
The enzymatic activity of different immobilized preparations of BTL2 was also assayed in the hydrolysis reaction of compound (±)-**12** at different temperatures (Table 6). At 25°C, the glyoxyl-BTL2 preparation presented the highest activity against (±)-**12**, twenty six times higher than octadedyl-Sepabeads-BTL2 one. When the temperature was decreased, the immobilized preparations suffered a decrease in activity, more drastic than when the enzyme was immobilized on octadecyl-Sepabeads support (up to a 6-fold factor).

The hydrolytic resolution of (±)-**12** catalyzed by different immobilized preparations of BTL2 was performed (Scheme 6). First, the enantioselectivity at pH 7.5 and different temperatures in aqueous media was studied (Table 7). At 25°C, significant differences for the different immobilized preparations were not observed, with enantiomeric excesses of the release product between 37 and 49% at 15% of conversion (Table 7), being obtained and the (*R*)-enantiomer hydrolyzed faster. However, at 4°C, it was possible to see differences in the *E* value, with the octadecyl-Sepabeads-BTL2 preparation being the most enantioselective ($E=4.7$), up to more than 2 times higher *E* value that glyoxyl-BTL2 preparation. Thus, the octadecyl-Sepabeads-BTL2 was chosen as the best biocatalyst to perform the resolution.

As a result, the effect of the co-solvent on the enantioselectivity of this biocatalyst in the hydrolysis of (±)-**12** in aqueous media in order to improve the selectivity of this lipase was studied. Three different solvents were selected to perform the study (Table 8).

In the presence of a co-solvent, the activity of the octadecyl-Sepabeads-BTL2 was slightly increased and the enantioselectivity of the biocatalyst improved, being slightly higher in the presence of 10% acetonitrile ($E=8$). Table 9 shows the results obtained using increasing concentrations of acetonitrile.

The octadecyl-Sepabeads-BTL2 preparation gave the highest *E* value in 20% of acetonitrile ($E=13$). The organic co-solvent did not provoke this increase in the



(±)-**12** (R)-**13** (S)-**12**

Scheme 6. Enantioselective hydrolysis of compound (±)-**12** catalyzed by BTL2 immobilized preparation.

Table 7. Enantioselective resolution of (±)-**12** catalyzed by immobilized BTL2 at different temperatures. Experiments were performed as described in Section 4

Immobilized Preparation	Preferred enantiomer	4°C		25°C	
		ee	*E*	ee	*E*
Octadecyl-Sepabeads-BTL2	*R*	62	4.7	49	3
Glutaraldehyde-BTL2	*R*	54	3.6	49	3
Glyoxyl-BTL2	*R*	32	2.1	37	2.2

Ee: enantiomeric excess of the product at 15% of conversion (%); $E=$value of enantioselectivity.

Table 8. Effect of the presence of co-solvent on the enantioselectivity of octadecyl-Sepabeads-BTL2 preparation. Experiments were performed with 10% of co-solvent and 2 mM of substrate at pH 7.5

Solvent	Conversion (%)	Specific Activity	Ee (%)	E (R/S)
Dioxane	16	0.2	73	7.3
Acetonitrile	15	0.21	75	8
Diglyme	15	0.23	72	6.8

Specific activity: (μmol/h mgprot); ee = enantiomeric excess of the product; E = value of enantioselectivity.

selectivity of the enzyme immobilized on glutaraldehyde-agarose support or glyoxyl-agarose support (E value remaining around 2).

In this way, by hydrolyzing the racemic ester with this immobilized preparation (octadecyl-Sepabeads-BTL2), a drastic decrease in the enzyme activity at hydrolysis yields over 60% has been observed showing the great enantioselectivity of this enzyme for the (R)-enantiomer. Thus, the combined use of this enzyme and reaction conditions permitted to have quite pure (S)-**12** at 60% (ee of remaining ester over 90%) (Fig. 2).

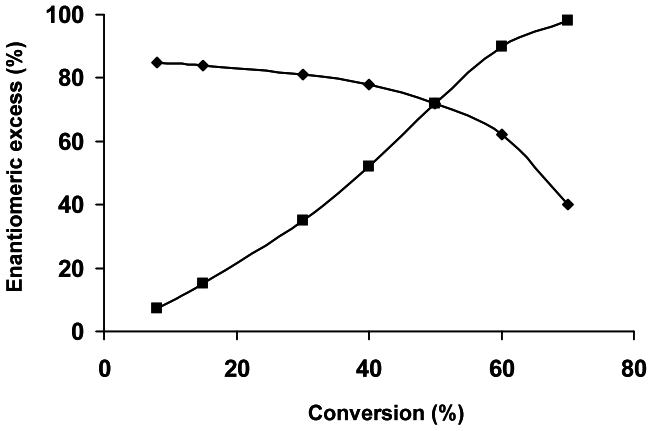


Figure 2. Graphic of evolution of enantiomeric excess (ee) versus conversion in the hydrolysis of (±)-**12** catalyzed by octadecyl-Sepabeads-BTL2 immobilized preparation. Experiments were performed using concentration of substrate of 2 mM with 20% (v/v) acetonitrile pH 7.5, 25°C, as described in Section 4. Ee of remaining substrate (S)-**12** (squares), ee of released acid (R)-**13** (rhombus).

3. Conclusion

This enzyme has been able to catalyze the hydrolysis reaction of esters of very different natures, in some instances with good enantioselectivity. This enantioselectivity may be strongly modulated by the immobilization protocol used and by the reaction conditions. In this way, different methods of immobilization gave very different results in the resolution of compound (±)-**5**, even observing a selective inversion of the enzyme after the ionic exchange on agarose support coated with polyethyleneimine.

Furthermore, the obtained results suggest the important influence of slight changes in the experimental conditions on the selectivity of this lipase. Thus, in the resolution of compound (±)-**5** it was possible to improve the E value from 11 (at 37°C) to E value of >100 (at 4°C) for glyoxyl-BTL2 preparation, whereas in the resolution of compound (±)-**12** interesting results for the E value using octadecyl-Sepabeads-BTL2 immobilized preparation were obtained, from 3 (at 25°C pH 7) to E value of 13 (by adding with 20% acetonitrile), only by modification of the experimental conditions.

Therefore, this enzyme has been able to resolve compound (±)-**5** with high enantiomeric excess (ee>99%, E>100) in the hydrolysis reaction catalyzed by octadecyl-Sepabeads-BTL2 preparation (at 4°C pH 7 or 9) and glyoxyl-BTL2 preparation (at 4°C pH 7), yielding only the (R)-enantiomer of mandelic acid, a key intermediate in the synthesis of (+)-Goniodiol, an interesting drug which has been resulted using different chemical routes.[33–36]

With this resolution, the use of immobilized BTL2 and reaction conditions permitted to have quite pure (S)-**12** by hydrolyzing the racemic ester to 60% (ee of remaining ester over 90%), comparable with some resolutions reported in the literature.[37]

4. Experimental

4.1. General

Lipase from *Bacillus thermocatenulatus* (BTL2) cloned in *E. coli* was produced as previously described.[16b] Glyoxyl-agarose 6BCL and 10 BCL was kindly donated by the company Hispanagar SA (Burgos, Spain). Octadecyl-Sepabeads was generously donated by Resindion Srl (Mitsubishi Chem. Coorp.) (Milan, Italy). Octyl-agarose 4BCL was purchased from Pharmacia

Table 9. Effect of the co-solvent concentration on the enantioselectivity of different BTL2 preparations at 25°C and pH 7.5. The percentages show the concentration of acetonitrile used (v/v)

Immobilized preparation	10%		20%		30%	
	ee	E (R/S)	ee	E (R/S)	ee	E (R/S)
Octadecyl-Sepabeads-BTL2	76	8.8	84	13	81	11
Glutaraldehyde-BTL2	20	1.5	10	1.2	–	1
Glyoxyl-BTL2	–	1	32	2	18	1.4

Ee = enantiomeric excess of the product at 15% of conversion; E = value of enantioselectivity.

Biotech (Uppsala, Sweden). Polyethyleneimine (PEI) (Mr 25000), Triton X-100, p-nitrophenyl propionate (pNPP), butyric acid ethyl ester were from Sigma. Glyoxyl-agarose,[38] and PEI-agarose[39,40] were prepared as previously described. (±)-α-Hydroxy-phenylacetic acid methyl ester (±)-**4** were purchased from Sigma. 2-*O*-butyryl-2-phenylacetic acid (±)-**5** was synthesized as previously described.[41] (±)-*trans*-4-(4′-fluorophenyl)-6-oxopiperidin-3-ethyl carboxylate (±)-**8** and 2-hydroxy-4-phenylbutyric acid ethyl ester (±)-**12** were kindly donated by Vita Invest S.A. (Barcelona, Spain), (±)-6-(5-Chloropyridin-2-yl)-7-oxo-5-(vinyloxycarboyl-oxy)-5,6-dihydropyrrolo[3,4*b*] pyrazine, (±)-**10** was donated by Vicente Gotor (University of Oviedo, Spain). Other reagents and solvents used were of analytical or HPLC grade.

4.2. Enzymatic activity assay determination

This assay was performed by measuring the increase in absorbance at 348 nm produced by the release of *p*-nitrophenol (pNP) in the hydrolysis of 0.4 mM pNPP in 25 mM sodium phosphate buffer at pH 7 and 25°C. To initialize the reaction, 0.05 mL of lipase solution or suspension was added to 2.5 mL of substrate solution. One international unit of pNPP activity was defined as the amount of enzyme that is necessary to hydrolyze 1 μmol of pNPP per minute (IU) under the conditions described above.

4.3. Purification of the enzyme

To purify the lipase from any other contaminant proteins (e.g. esterases), the enzyme preparation was incubated in the presence of octyl-agarose at low ionic strength, following a previously described procedure.[42] Periodically, activity of suspensions and supernatants was assayed by using the pNPP assay. After immobilization, adsorbed lipase preparation was abundantly washed with distilled water. To desorbe the enzyme, the adsorbed lipase was washed with Triton X-100 0.2% in 5 mM sodium phosphate buffer at pH 7 and 25°C.

Following these protocols, a quantitative immobilization of lipase activity was observed and the SDS-PAGE analysis of the adsorbed protein preparation only showed a single band with a molecular weight corresponding to that of the native lipases. Final yield was near to 100%. Protein concentration was measured using Bradford's method.[43] The calibration curve was obtained with bovine serum albumin (BSA) for determining protein concentrations in the range of 0.1 to 1.5 mg/mL.

4.4. Immobilization of lipases on different supports

Different immobilized preparations were prepared following the procedures previously described:

(i) ionically adsorbed lipase on solid supports coated with PEI[39] (ionic microenvironment surrounding large areas of the protein) at pH 9 and 25°C.
(ii) covalent immobilization on amine–agarose beads activated with glutaraldehyde[44] at pH 7 and 25°C.
(iii) multipoint covalent immobilization on glyoxyl-agarose beads (through areas with the highest density of lysine (Lys) groups)[45] at pH 10.5 and 25°C.
(iv) interfacial adsorption on a hydrophobic support, Sepabeads resin with the surface covered by octadecyl groups.[46] To immobilize the purified lipase on octadecyl-Sepabeads support, the Triton was diluted 500-fold with 5 mM sodium phosphate buffer at pH 7.

A schematic representation of the different immobilization protocols is shown in Scheme 7. Enzyme load was 1 mg pure lipase /mL of support (that is approx. 1–2% of the maximum load) and 23 mg/mL (with compound **12**) (maximum capacity of octadecyl-Sepabeads) and in all cases more than 95% of the lipase became immobilized on all different supports offered. Protein concentration was determined by the Bradford method.[43]

4.5. Enzymatic hydrolysis of esters

The activities of different immobilized preparations of lipase from *B. thermocatenulatus* were analyzed in the hydrolysis reaction of different esters. Ethyl butyrate was dissolved in a 100 mL solution of 25 mM sodium phosphate buffer at pH 7 to 10 mM of substrate concentration and 0.1 g of biocatalyst were added. pNPP was dissolved in a 10 mL solution of 25 mM sodium phosphate buffer at pH 7 to 0.4 mM of substrate concentration and 0.1 mL of biocatalyst were added.

Substrate (±)-**4** was dissolved in a 3 mL solution of 10 mM sodium phosphate buffer to 2 mM of compound concentration at 25°C and pH 7 under different conditions (pH, T) and 0.6 g of immobilized preparation were added.

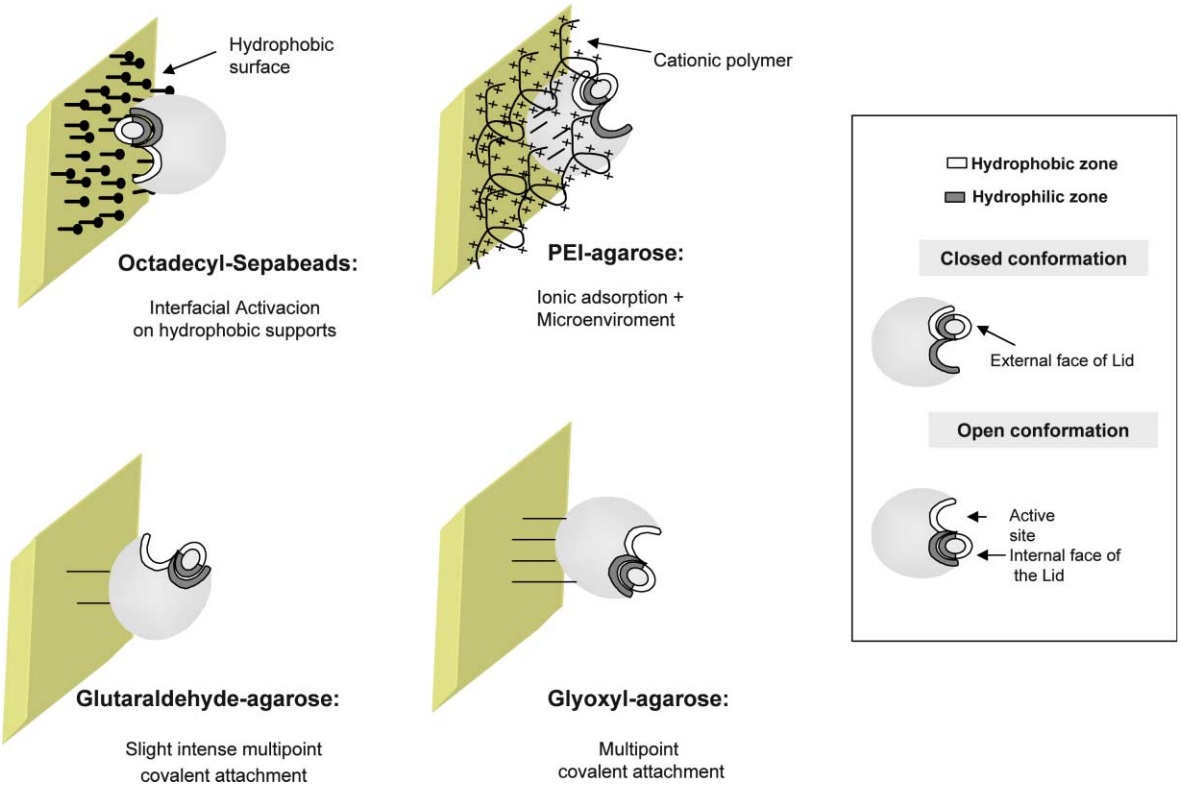
Substrate (±)-**5** was dissolved in a 3 mL solution of 10 mM sodium phosphate buffer to 0.5 mM of compound concentration under different conditions (pH, T) and 0.5 g of immobilized preparation were added.

Substrate (±)-**8** was dissolved in a 3 mL solution of 10 mM sodium phosphate buffer to 2 mM of compound concentration at 25°C and pH 7 under different conditions (pH, T) and 0.1 g of immobilized preparation were added.

Substrate (±)-**10** was dissolved in a 3 mL solution of 10 mM sodium phosphate buffer to 2 mM of compound concentration at 25°C, pH 7 and 50% dioxane and 0.3 g of immobilized preparation were added.

Substrate (±)-**12** was dissolved in a 3 mL solution of 10 mM sodium phosphate buffer to 2 mM of compound concentration at 25°C and pH 7.5 under different conditions (pH, T) and 0.1 g of immobilized preparation were added.

During the reaction, the pH value was maintained constant using a pH-stat Mettler Toledo DL50 graphic



Scheme 7. Different immobilized preparations from *B. thermocatenulatus* lipase.

and the enzymatic activity was defined as μmol of substrate hydrolyzed per minute per mg of immobilized protein. The degree of hydrolysis was analyzed by reverse-phase HPLC (Spectra Physic SP 100 coupled with an UV detector Spectra Physic SP 8450). For these assays a Kromasil C_{18} (25×0.4 cm) column was used, mobile phase acetonitrile–10 mM ammonium phosphate buffer at pH 2.95 (35:65, v/v) for compound **5**, (30:70, v/v) for compounds **4** and **8**, (40:60,v/v) for compound **12**, (50:50, v/v) form compound **10**, at 1.5 mL/min (or 1 mL/min to **8**) and UV detection was performed at 254 nm (compounds **4** and **5**) and 270 nm (compounds **10** and **8**).

4.6. Determination of enantiomeric excess and enantioselectivity

The enantiomeric excess (ee) of the released acid (at conversions between 10 and 15% (initial rate in first order) was analyzed by Chiral Reverse Phase HPLC. The column was a Chiracel OD-R, mobile phase an isocratic mixture of acetonitrile and $NaClO_4/HClO_4$ 0.5 M, (5:95 v/v) for compounds (±)-**4** and (±)-**5** and (20:80 v/v) for compound (±)-**12** with a final pH of 2.3, at a flow of 0.5 mL/min and UV detection was performed at 225 nm The enantiomeric ratio (*E*) was calculated using the equation reported by Chen et al.[47]

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