Journal of Molecular Catalysis B: Enzymatic 130 (2016) 32–39

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Improving enantioselectivity of lipase from *Candida rugosa* by carrier-bound and carrier-free immobilization

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ARTICLE INFO

Article history:
Received 21 January 2016
Received in revised form 8 April 2016
Accepted 8 April 2016
Available online 12 May 2016

Keywords:
Immobilization
Enantioselectivity
CLEA
Lipase

ABSTRACT

The enantioselectivity of carrier-bound and carrier-free immobilized lipase from *Candida rugosa* (CRL) was studied. CRL was immobilized in six agarose-based carriers functionalized with different reactive groups and in two different CRL cross-linked aggregates. Both, activity and enantioselectivity of all the immobilized lipase preparations were evaluated with different racemic esters under different reaction conditions (temperature, pH and solvent polarity). A strong effect of reaction media and immobilization protocol on enzyme activity and selectivity was found. Enzyme immobilization and reaction engineering allowed us obtaining the best immobilization protocol and reaction conditions to achieve high activity and enantioselectivty of CRL as heterogeneous catalyst. CRL immobilized on an agarose-based carrier activated with primary amino groups preferentially hydrolyzed (*S*)-phenylethyl acetate with $E > 200$ under pH 7, 4 °C and 30% of acetonitrile. On the other hand, CRL aggregated and cross-linked through their carboxylic groups preferentially hydrolyzed the (*S*)-isomer of ethyl 2-hydroxy-4-phenylbutyrate with an $E = 39$ under pH 5, 4 °C and 30% of acetonitrile. This work demonstrates the success of the combinatorial enzyme engineering for the production of highly enantioselective heterogeneous biocatalysts by screening different immobilization protocols and reaction media conditions.

1. Introduction

Enantioselective biotechnological processes have gained interest because of exquisite enzyme selectivity [1]. The growing global market of chiral technology was estimated in $5.3 billion dollars in the year 2011, and its projection for 2016 is nearly $6.5 billion dollars [2]. In this scenario, enzymes have been intensively exploited in kinetic resolution and asymmetric synthesis of optically pure compounds with high impact on pharmaceutical, agricultural and food industries [3–6].

Enzyme enantioselectivity is a multi-variable dependent catalytic feature which can be altered either by directly engineering the biocatalysts (protein engineering or enzyme immobilization) [7,8] or by reaction engineering controlling different conditions such as temperature [9], presence of co-solvents [10] and additives [11,12], substrate concentration [13,14], water [15], etc. In the last decade, enzyme immobilization has been revealed as a powerful technique to enhance enzyme enantioselectivity beyond its capacity to turn enzyme into re-usable and stable biocatalysts [16–20]. Therefore, an optimal combination of immobilization techniques and reaction conditions allows easy, fast and low cost preparation of enantioselective heterogeneous biocatalysts.

There are many classifications for immobilization protocol; one of the most frequent is based on whether the insoluble enzyme is either carrier-bound or carrier-free. Among carrier-bound techniques, there are diverse types of carriers driving enzyme immobilization by adsorption, entrapment, encapsulation or covalent-binding on pre-existing matrixes [21]. Carrier-free immobilization is based on the formation of protein-protein aggregates covalently cross-linked (CLEA). Carrier-free immobilization is a less expensive option since no matrix cost contributes to the biocatalyst price. Moreover, CLEA technology yields biocatalysts with higher volumetric productivities because the major part of

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http://dx.doi.org/10.1016/j.molcatb.2016.04.006
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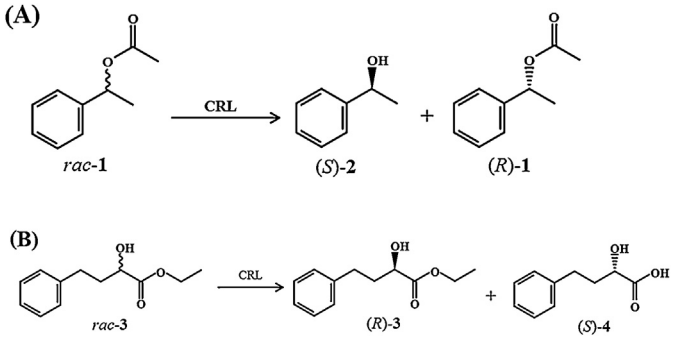

Scheme 1. (A) Enantioselective hydrolysis of *rac*-**1** for the lipase catalyzed resolution of (*S*)-**2**. (B) Lipase catalyzed enantioselective hydrolisis of *rac*-**3** for the resolution of (*R*)-**3**.

insoluble biocatalyst mass belongs to the enzyme [22]. However, to select the best immobilization protocol for a certain enzyme, both carrier-free and carrier bound must be tested in order to fit the best biocatalyst properties to the optimal chemical process configuration.

In this work, activity and enantioselectivity of *Candida rugosa* lipase (CRL) were evaluated after combinatorial screening of both immobilization protocols and reaction media conditions. A survey of immobilized biocatalysts was assayed towards different esters under different reaction conditions controlling temperature, pH and media polarity. As model chiral reactions, two esters with different acyl substituents; an alcohol and hydroxyacid ester were evaluated for enantioselective hydrolysis (Scheme 1). Chiral alcohols and α-hydroxyacids play an important role as building blocks in fine chemistry [23–25]. Therefore, we studied the enantioselectivity of different CRL derivatives towards the resolution of (*S*)-1-phenylethanol (*S*-**2**) starting from racemic 1-phenylethyl acetate (*rac*-**1**). Besides, we also tested the steroselective hydrolysis of racemic ethyl 2-hydroxy-4-phenylbutyrate (*rac*-**3**) to yield ethyl (*R*)-2-hydroxy-4-phenylbutyrate (*R*-**3**); a key precursor in the synthesis of angiotensin-converterting enzyme inhibitors [26] like enalapril, benazepril, cilazapril, ramipril and quinapril applied in the treatment of hypertension and heart diseases [27,28]. This study is one of the scarcely works which compares the performance of carrier-free and carrier-bound heterogeneous lipases towards different enantioselective resolutions.

2. Materials

The reagents racemic 1-phenylethyl acetate (*rac*-**1**), racemic 1-phenylethanol (*rac*-**2**), (*R* or *S*)-1-phenylethanol (*R* or *S*-**2**), ethyl 2-hydroxy-4-phenylbutyrate (*rac*-**3**), ethyl (*R* or S)-2-hydroxy-4-phenylbutyrate (*R* or *S*-**3**), 1-ethyl-3-[3-dimethylaminopropil]carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were purchased from Pierce Scientific (Mexico City, Mexico). Glutaraldehyde (GA) (25% in water solution), sodium borohydride, polyethyleneimine (PEI) molecular weight of 1300 g/mol, polyethylenglycol (PEG) molecular weight of 600 g/mol, bovine serum albumin (BSA), Triton® X-100, ethanolamine, ethylenediamine, trioctanoin, *p*-nitrophenyl butyrate (*p*NPB) were acquired from Sigma (St. Louis, MO, USA). All other reagents were analytical or HPLC grade. Supports as cyanogen bromide activated agarose (CNBr) and octyl-sepharose 4BCL (octyl) were purchased from GE Healthcare (Uppsala, Sweden). Crosslinked agarose beads 6 BCL were purchased from Agarose Beads Technologies (Madrid, Spain). The enzyme *Candida rugosa* lipase (CRL) TVII (with 20 mg of protein/g of powder) was purchased from Sigma (St. Louis, MO, USA).

3. Methods

3.1. Carrier-free immobilization of CRL

CRL was immobilized as carboxyl-activated cross-linked enzyme aggregates (c-CLEA) and by amino cross-linked enzyme aggregates (a-CLEA) as described previously [29]. EDC-NHS 28 mM and GA 14 mM were employed for c-CLEA and a-CLEA respectively. In both cases, PEI at 3 g/L and 10 mg of BSA as protein feeder were employed.

3.2. Carrier-bound immobilization

3.2.1. Immobilization of CRL on cyanogen bromide activated agarose (CNBr-CRL)

According with a previous report [30], the immobilization procedure was conducted by mixing 10:1 (v/w) the enzyme solution (1 mg of enzyme powder per mL of 25 mM phosphate buffer pH 7, 0.05% w/v Triton® X-100) with CNBr-activated agarose (detergent avoids the attachment of lipase dimmers [31]) and maintained under gentle agitation at 4 °C for 7 min. The immobilization was stopped by filtration (30% of enzyme was loaded to the support) and 10 mL of ethanolamine 1 M at pH 7 were added and let them to react 2 h at 4 °C. Then, the immobilized preparation was filtered and washed with 10 vol of water and finally rinsed with 25 mM phosphate buffer pH 7 and stored at 4 °C.

3.2.2. Immobilization of CRL on octyl-sepharose (octyl-CRL)

Based in a previous described methodology [32,33], hydrophobic adsorption of CRL on octyl-sepharose was carried out as follows: ten millilitres of enzyme solution (1 mg of enzyme powder per mL of 25 mM phosphate buffer pH 7) were mixed with 1 g of octyl-sepharose and were maintained under gentle agitation at 25 °C. The immobilization curse was followed by measuring the activity in the supernatant and in the suspension every 30 min. Once no activity was detected in the supernatant, the immobilization was stopped by filtration through POEBEL Buchner funnel with perforate plate No. 2, then rinsed with 10 vol of 25 mM phosphate buffer pH 7 and stored at 4 °C.

3.2.3. Immobilization of CRL on naphthalene-agarosa (naphthyl-CRL)

First, the preparation of the naphthalene-activated agarose (naphthyl) was conducted. The procedure consisted in two steps: 1) preparation of epoxy-activated agarose as described elsewhere [34]; and 2) naphthalene-activated epoxy-agarose. Based on a previous report [35], a suspension of 10 g of epoxy-agarose in 100 mL of 15 mM tionaphtalene in water/dimethylsulfoxide (20:80) was prepared. The suspension was maintained under gentle agitation for 2 h at room temperature. Afterwards, the support was filtered and rinsed with 10 vol (100 mL each time) of water/acetone (20:80). Once naphthyl-agarose was obtained, the immobilization of CRL on this support was carried out following the methodology above described for octyl-sepharose.

3.2.4. Immobilization of CRL on glyoxyl-agarose (glyoxyl-CRL)

Fully glyoxyl-activated agarose (glyoxyl) was prepared as described elsewhere [36]. Afterwards, the immobilization was conducted by mixing 10 mL of enzyme solution (1 mg of enzyme powder per mL of 100 mM bicarbonate buffer pH 10 with 40% PEG [37] and 0.05% Triton® X-100 p/v) with 1 g of glyoxyl-agarose. The suspension was maintained under gentle agitation at 4 °C. The immobilization curse was followed by measuring the activity in both the supernatant and the suspension as far as no activity was detected in the supernatant. The attachment of the 100% of the enzyme on the support was done in 15–30 min; however,

Table 1
Carrier-bound and carrier-free immobilization of CRL.

Type	Derivative	Enzyme-support interaction	Probably enzyme orientation	Y(%)	rA_e(%)
Carrier-bound	CNBr	Covalent unipoint binding	Binding of amino *N-terminus*	54	15
	GA	1st – ionic adsorption2nd – covalent binding	1st – negatively charged area2nd – lysine abundant area	100	8
	Glyoxyl	Covalent multipoint binding	Lysine abundant area	99	7
	MANAE	Ionic adsorption	Negatively charged area	83	14
	Octyl	Hydrophobic adsorption	Hydrophobic area	100	53
	Naphthyl			41	24
Carrier-free	c-CLEA	none	Aspartic and glutamic acids abundant area	55	22
	a-CLEA	none	Lysine abundant area	81	15

In all cases the activity was measured with *p*NPB as substrate.
Immobilization yield (Y) = (initial activity – activity in the supernatant)/initial activity.
Relative expressed activity (rA_e) = [final expressed activity of the immobilized enzyme/(initial activity – activity in the supernatant)].

the immobilization was maintained in curse through 24 h at 4 °C in order to promote the formation of multiple enzyme-support bonds. Subsequently, a reduction was done by addition of sodium borohydride (1 mg/mL of suspension) followed by soft agitation for 30 min at room temperature. Once the reduction was finished, the immobilized derivative was abundantly washed with water and was equilibrated for 5 min with 10 mL of 25 mM phosphate buffer pH 7, filtered and stored at 4 °C.

3.2.5. Immobilization of CRL on MANAE-agarose (MANAE-CRL)

Monoamino-N-aminoethyl activated agarose (MANAE-agarose) was prepared as previously reported [38]. The immobilization was done by mixing 10 mL of enzyme solution (1 mg of enzyme powder per mL of 25 mM phosphate buffer pH 7 and Triton® X-100 0.05% w/v) with one gram of the support. The suspension was maintained under gentle agitation at 4 °C until no activity was present in the supernatant (around 30 min). The immobilization was stopped by filtration with POEBL Buchner funnel with perforate plate No. 2, and rinsed with 10 vol of 25 mM phosphate buffer pH 7 and stored at 4 °C.

3.2.6. Immobilization of CRL on glutaraldehyde-agarose (GA-CRL)

Glutaraldehyde-activated agarose (GA-agarose) was prepared as described elsewhere [39]. The immobilization of CRL on GA-agarose was carried out according a described methodology [40]. Ten millilitres of enzyme solution (1 mg of enzyme powder per mL of 25 mM phosphate buffer pH 7 with 0.05% w/v Triton® X-100) were mixed with one gram of fresh GA-agarose. The suspension was maintained under gentle agitation at 4 °C until no activity was detected in the supernatant (around 30 min). The immobilization was stopped by filtration in a POBEL Buchner funnel with perforate plate No. 2, rinsed with 10 vol of 25 mM phosphate buffer pH 7 in order to remove remaining glutaraldehyde and detergent, and stored at 4 °C.

3.3. Polymer coating of immobilized CRL (PEI-coating)

Based on a reported methodology [41], a suspension of CLEA (30–40 μg of protein/mL) or 1 g of support-immobilized CRL with 16 mL of PEI (25 kDa) solution (25 mg/mL adjusted at pH 7) was maintained at soft agitation at 4 °C for 16 h. Afterwards, the PEI-coated derivatives were filter and washed with abundant 25 mM phosphate buffer pH 7. The physically modified derivatives were stored at 4 °C.

3.4. Activity assay

Lipase activity during immobilization procedures was determined by the hydrolysis of *p*-NPB as substrate. To start the reaction, 50–100 μL of enzyme solution or suspension properly diluted were added to a reaction mixture containing 20 μL of *p*NPB (50 mM in acetonitrile) in 2 mL of 25 mM phosphate buffer pH 7 in a magnetic stirred cell at 25 °C. The released *p*-nitrophenol was monitored at 348 nm during 5 min of reaction. Calibration curve of *p*-nitrophenol in the same conditions was carried out. One unit of lipase activity was defined as the amount of enzyme required for release 1 μmol of *p*-nitrophenol per minute.

3.5. Enzymatic hydrolysis of racemic 1-phenylethyl acetate (*rac*-**1**)

The hydrolysis of *rac*-**1** was carried out by adding CLEA (300–400 μg of protein) or immobilized derivative (500 mg) to 3 mL of 5 mM of *rac*-**1** in buffered solution (10 mM of Tris or sodium acetate, for pH 7 or pH 5, respectively). The reaction mixture was maintained under gentle agitation (at 25 or 4 °C, as required), up to attain 10–15% of hydrolysis degree of the substrate. For the hydrolysis degree determination, samples of the reaction mixture were withdrawn and analyzed in an HPLC (Spectra System P4000) coupled to an UV-diode array detector (Spectra System SN4000) and eluted with a Kromasil C18 (25 cm × 0.4 cm) column. The elution of compounds was conducted with a mobile phase of ammonium phosphate buffer (10 mM and pH 7.0) in acetonitrile/water (35:65, v/v), with an isocratic flow of 1.0 mL/min. The compounds were detected at 205 nm. Retention times for *rac*-**2** and *rac*-**1** were 4.6 and 22.0 min, respectively. One unit of activity was defined as the amount of enzyme needed for release one μmol of (*R*) or (*S*)-**2** per minute. The conversion degree was estimated after relationship of the peak's area and a calibration curve in the same elution conditions.

The enantiomeric excess (*ee*) of released (*S*)-**2** was determined by reverse chiral HPLC (Spectra System P4000) coupled to an UV-diode array detector (Spectra System SN4000) with a Chiralcel OD-R (250 mm × 4.6 mm) column. To assure a first-order enzyme kinetic, samples with 10–15% of substrate's hydrolysis degree were employed. The separation of **2** enantiomers was done with an isocratic mobile phase of ammonium phosphate buffer (10 mM at pH 7.0) in an acetonitrile/water mixture (35:65% v/v), with a flow of 0.5 mL/min. The enantiomers were detected at 205 nm, with a retention times of 13.5 and 14.7 min for (*S*)- and (*R*)-**2**, respectively. Enantioselectivity (*E*) was calculated with the Chen equation [42].

3.6. Enzymatic hydrolysis of racemic ethyl 2-hydroxy-4-phenylbutyrate (*rac*-**3**)

The enzymatic hydrolysis of *rac*-**3** was carried out according to a previous report [43]. A suspension of CLEA (300–400 μg of protein) or immobilized derivative (500 mg) in 3 mL of 5 mM of *rac*-**3** in 5 mM of buffered solution (10 mM of sodium acetate or sodium phosphate, for pH 7 or pH 5, respectively) was prepared. The reaction mixture was maintained under gentle agitation (at 25

or 4 °C) until the achievement of 10–15% of substrate hydrolysis degree. Hydrolysis degree was determined by HPLC (Spectra System P4000) coupled to an UV-diode array detector (Spectra System SN4000) with a Kromasil C18 (25 cm × 0.4 cm) reverse phase column. The elution of compounds was done with an isocratic mobile phase of ammonium acetate buffered solution (10 mM and pH 2.3) in an acetonitrile/water mixture (40:60 v/v) and a constant flow of 1.0 mL/min. Analytes were detected at 225 nm with retention times of 3.2 and 10.2 min for the product, racemic hydroxy-phenylbutyric acid (*rac*-**4**) and the substrate (*rac*-**3**), respectively. One unit of activity was defined as the amount of enzyme needed for release one μmol of (*R*)- or (*S*)-**4** per minute. The conversion degree was estimated after relationship of the peak's area and a calibration curve in the same elution conditions.

The *ee* of released (*R*)-**3** was determined by reverse chiral HPLC (Spectra System P4000) coupled with an UV-diode array detector (Spectra System SN4000) and a Chiralcel OD-R (250 mm × 4.6 mm) column. Samples with 10–15% of substrate's hydrolysis degree were analyzed in order to ensure a first-order enzyme kinetic. HPBE enantiomers were eluted with an isocratic mobile phase of ammonium phosphate buffer (10 mM at pH 2.3) in an acetonitrile/water mixture (20:80, v/v) and a flow of 0.5 mL/min. Enantiomers were detected at 225 nm with retention times of 23.1 and 25.3 min for (*S*)- and (*R*)-**3**, respectively. Enantioselectivity was calculated with Chen equation [38].

4. Results and discussion

4.1. Immobilization of CRL by both carrier-bound and carrier-free techniques

Immobilization is frequently associated to the loss of enzymatic activity and the alteration of some catalytic properties. Hence, the study of a variety of matrixes functionalized with different reactive groups enabled to orient CRL through different enzyme regions. Both enzyme orientation and immobilization chemistry directly affect the catalytic properties of the immobilized biocatalyst [20]. As model carrier we used agarose beads because of its versatility to be functionalized with many different reactive groups, its compatibility with biomolecules and its mechanical stability to be used in a plethora of reactor designs [36,44]. On the other hand, the commercial preparation of CRL used in this work is not pure since is mainly composed by the isoforms Lip1 and Lip3 [45]. However, industrial biocatalysts must meet the cost requirements regarding to their large scale applications; hence, purified enzymes represent a very high expensive choice for such purpose. Besides, available commercial CRL preparations contain more than one isoform of this enzyme and they have been widely applied in the successful resolution of racemic mixtures [17,46–48] thus confirming its potential as robust industrial biocatalyst.

In order to find a highly enantioselective insoluble CRL, we screened six immobilization chemistries on pre-existing supports and two cross-linking chemistries on enzyme aggregates. Table 1 shows the immobilization chemistry, the proposed enzyme orientation and the resulting immobilization parameters for each immobilized preparation. Among overall immobilization chemistries, the adsorption on hydrophobic supports achieved the highest expressed activity of CRL, while multipoint covalent ones drives the least active biocatalysts.

CNBr-activated support immobilizes CRL through its *N-terminus* establishing only one (or very few) enzyme-carrier bond. Consequently, the enzyme suffers low modification degree, exhibiting nearly the same catalytic properties as the free enzyme [49,50]. This immobilization chemistry drives the quantitative immobilization of CRL expressing only 15% of the specific activity of the soluble enzyme. On the other hand, CRL covalently immobilized on agarose activated with aldehyde groups -glutarladehyde (GA) and glyoxyl-carriers- under alkaline conditions gave rise to inactive enzymes. These types of immobilization chemistry enhance enzyme stability towards temperature, pH, and solvents, among others [39,40,44,51]. However, immobilization conditions (pH 10 and sodium borohydride) on carriers activated with aldehyde groups are deleterious for several enzymes [52]. To address such inactivation issue during the immobilization, CRL was immobilized on glyoxyl-agarose in the presence of 40% PEG allowing the stabilization of the enzyme under alkaline conditions [37]. Under such conditions, 99% of CRL was immobilized expressing only 7% specific activity of the soluble enzyme. Alternatively, CRL was immobilized on GA-agarose under neutral pH conditions. In this immobilization chemistry, the negatively charged enzyme regions are firstly adsorbed to positively charged surface of the carrier, this ionic immobilization is followed by the covalent binding between GA groups and lysine residues located at the carrier and enzyme surface. In spite of the mild immobilization conditions, CRL immobilized on GA-agarose only expressed 8% specific activity of the soluble enzyme although the immobilization yield was 100% (Table 1). Likewise, reversible immobilization of CRL on agarose activated with primary amine groups (MANAE-agarose) was carried out under soft conditions (neutral pH, low ionic strength and short immobilization times). This immobilization chemistry orients CRL through its most basic regions (rich in Asp and Glu). On this aminated support, 83% of CRL was immobilized expressing 14% of the enzyme specific activity in solution. When we carried out the same immobilization protocol with lower enzyme loads (ten times less), 83% of CRL was immobilized but the specific activity of the immobilized enzyme was nearly 42% of its soluble counterpart. This result points out that the activity loss after the immobilization process is mainly due to transfer limitations rather than an intrinsic inactivation caused by the enzyme-carrier interaction. Beyond the ionic interaction, CRL was also reversibly immobilized through hydrophobic forces. In this regard, the enzyme was immobilized on agarose activated with either octyl (octyl-sepharose) or naphtyl (naphtyl-agarose) groups. This immobilization chemistries orient CRL through its hydrophobic active site by hydrophobic interaction between hydrophobic residues on the vicinity of the catalytic pocket and the either alkyl chain or aromatic rings on the carrier surface, resembling an oil-water interface at low ionic strength [53]. Adsorption of CRL on both octyl- and napthyl-agarose resulted in the highest expressed specific activity among all the immobilized preparations herein studied (Table 1). Besides both derivatives presented the higher expressed specific activity of the enzyme, octyl-sepharose immobilized CRL was much more active than napthyl-agarose. In conrast, 100% of CRL was immobilized on octyl-sepharose while only 41% of CRL could be immobilized on napthyl-agarose. These differences might be attributed to a higher affinity of CRL for alkyl chains than for aromatic rings. This fact may be explained because a better geometric congruence between the CRL active site and the octyl chains, since CRL exhibits a long, narrow hydrophobic tunnel which accommodates the acyl moiety of the substrate [54]. Alternatively, CRL was aggregated and covalently cross-linked by using two different cross-linking chemistries 1) using glutaraldehyde as cross-linking agent that covalently bridge inter- or intraprotein lysine residues (a-CLEA) or 2) using carbodiimide as carboxy-activating agent followed by polyethyleneimine cross-linking that covalently bridges intra- or intermolecular bridges between two carboxylic groups from either Asp or Glu residues (c-CLEA). Cross-linking with glutaraldehyde irreversibly immobilized a higher yield of CRL than cross-linking with carbodiimide. However, both CLEA showed similar CRL specific activities; around 20% of the soluble enzyme. Carrier-free immobilized derivatives of CRL drove to 2-fold more

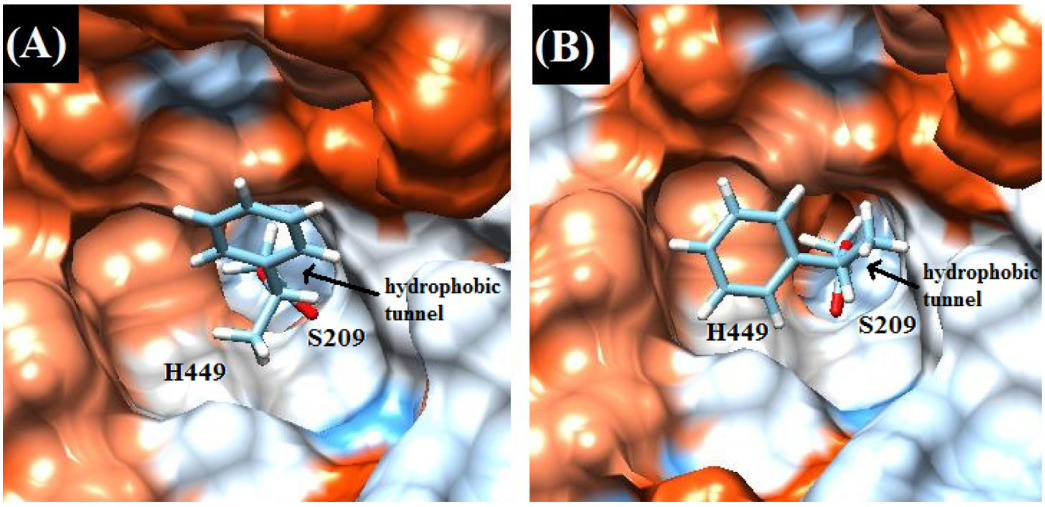

Fig. 1. Molecular substrate binding models for enantiomers of *rac*-**1** suggested by molecular modeling done through SwissDock server (http://www.swissdock.com.ch/). CRL 3D structure was PDB 1CRL. (A) (*S*)-**1** with ΔG = −6.03 kcal/mol, (B) (*R*)-**1** with ΔG = −6.15 kcal/mol. Lighted in orange and blue the hydrophobic and the hydrophilic enzyme zones, respectively. In black, two amino acids of the catalytic triad (serine 209 and histidine 449). Models A and B represent the lowest energy binding models for each enantiomers. Data and adjustment visualization were obtained with UCSF Chimera program. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Effect of temperature and co-solvent addition in the hydrolysis of *rac*-**1** catalyzed by CRL derivatives.

Biocatalysts	25 °C, pH 7		4 °C, pH 7		4 °C, pH 7, acetonitrile (30%)	
	Specific activity (U/g)[1]	*E* (*S*)[2]	Specific activity (U/g)[1]	*E* (*S*)[2]	Specific activity (U/g)[1]	*E* (*S*)[2]
CNBr	277^{c}	6^{a}	5^{d}	7^{a,b}	0.4^{d}	41^{e}
GA	56^{d}	2^{b}	1^{a}	6^{a}	0.1^{a,b}	19^{b}
glyoxyl	176^{e}	6^{a}	2^{a,b}	6^{a}	0.1^{b}	27^{c}
octyl	752^{f}	7^{a}	19^{e}	10^{c}	0.3^{c}	34^{d}
naphthyl	45^{g}	6^{a}	3^{b,c}	8^{b,c}	0.1^{b}	106^{f}
MANAE	602^{h}	10^{b}	4^{c,d}	5^{a}	0.5^{e}	>200^{g}
c-CLEA	161^{a}	5^{a}	2^{a,b}	6^{a}	0.1^{b}	14^{a}
a-CLEA	20^{b}	6^{a}	2^{a,b}	5^{a}	0.05^{a}	13^{a}

Reaction conditions were 5 mM of *rac*-**1** in buffer Tris (10 mM, pH 7). ^{a,b,c,d,e,f,g}Mean values with different letters are statistically different ($P < 0.05$). Values represent the average of two independent assays.

[1] Specific activity was expressed as units per gram of immobilized protein.

[2] *E* was calculated with Chen equation.

active immobilized enzymes than their multipoint covalent carrier-bound counterparts, demonstrating lower inactivating effects on CLR's activity.

The difference of the immobilization parameters among the different CRL immobilized preparation suggests that the enzymatic properties of this lipase may be quite different from one preparation to others. Therefore, here we present a library of CRL heterogeneous catalysts with different orientation and immobilization chemistries that may alter the CRL performance in different biotransformations. This library will be the starting point to screen for high lipase enantioselectivity towards two different racemic esters.

4.2. Enantioselective hydrolysis of racemic 1-phenylethyl acetate (*rac*-**1**)

We used eight immobilized CRL preparations to hydrolyze *rac*-**1** in order to produce the corresponding enantiomer of the secondary alcohol (Scheme 1A). Firstly, this biotransformation was carried out in aqueous media at two different pH values. Generally, all CRL derivatives presented very low selectivity towards the *S* enantiomer (Supporting information Table 1S). However, the activity strongly varied among the immobilized biocatalysts, being the CRL immobilized on MANAE-agarose and octyl-sepharose the most active preparations, while the a-CLEA and naphtyl-derivative presented the lowest activity. Besides, more acid pH decreased the lipase activity towards *rac*-**1**, but the activity trend for each immobilized preparation was kept regarding to pH 7, being the CRL immobilized on both MANAE-agarose and octyl sepharose the most active ones. This effect is supported by the optimum activity of CRL, which was reported at pH 6.5, maintaining 80% of its activity at pH 7, whilst at pH 6 only the 55% was retained [55]. Under acidic conditions, CRL presented a poor selectivity towards the *S*-enantiomer as well as under pH 7. In the tested enantioselective resolution, CRL was not positive affected by the pH of the media, unlike its enhanced enantioselectivity in the resolution of mandelic acid esters at acidic pH conditions [18].

The effect of temperature on both activity and enantoselectivity was evaluated at pH 7 with and without addition of acetonitrile (Table 2). Although, for all the immobilized biocatalysts, the activity is strongly reduced (10–60 times) at 4 °C, the enantioselectivity at 4 °C and 25 °C is similar. The enantioselectivity was not improved by reducing the reaction temperature as it has been observed for this enzyme in the resolution of L-lactic acid [56] and in the esterification of 1-phenylethanol with caproic acid [57] and also for other enzymes [9,43]. In order to improve the enantioselectivity, acetonitrile was added to the reaction media as co-solvent and the reaction was carried out at 4 °C. As expected, the presence of acetonitrile in the reaction media decreased the enzyme activity by 1–2 orders of magnitude, mainly because of the enzyme

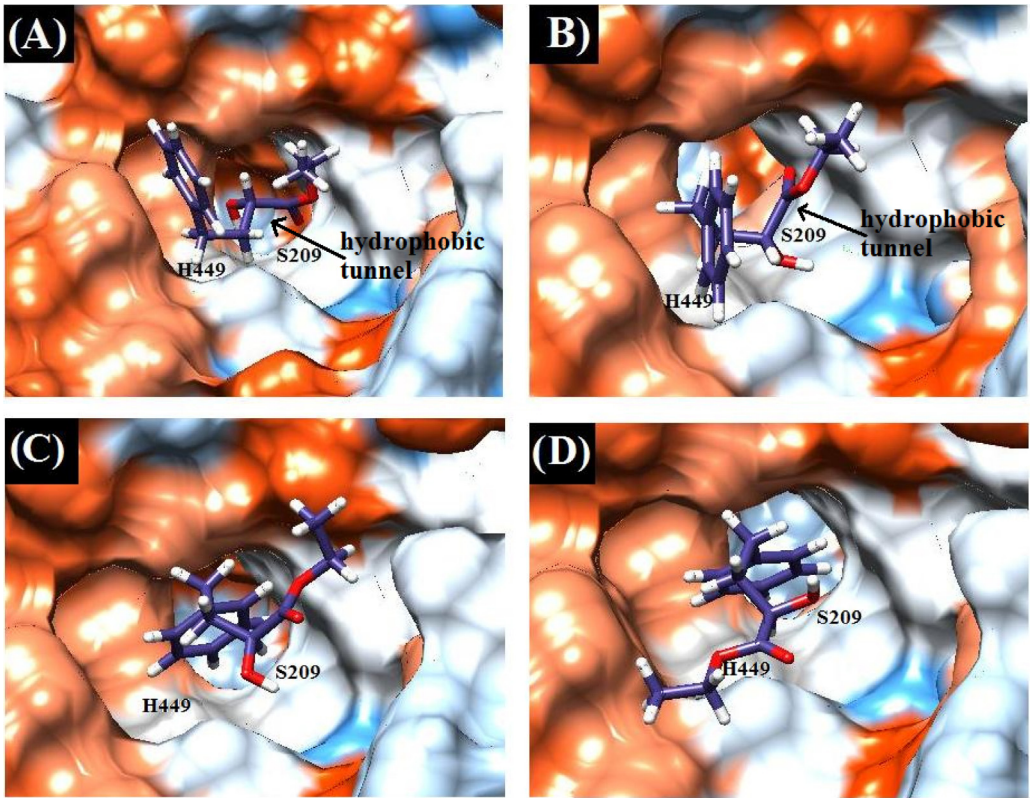

Fig. 2. Molecular substrate binding models for enantiomers of *rac*-**3** suggested by molecular modeling done through SwissDock server (http://www.swissdock.com.ch/). CRL 3D structure was PDB 1CRL. Models A and B represent the lowest energy binding models for each enantiomers. Models C and D represent the lowest energy binding models for each enantiomers in which the substrate's ring is oriented towards the hydrophobic tunnel. A) (*S*)-**3** with $\Delta G = -6.26$ kcal/mol, B) (*R*)-**3** with $\Delta G = -6.23$ kcal/mol, C) (*S*)-**3** with $\Delta G = -5.58$ kcal/mol and D) (*R*)-**3** with $\Delta G = -5.57$ kcal/mol. Lighted in orange and blue the hydrophobic and the hydrophilic enzyme zones, respectively. In black, two amino acids of the catalytic triad (serine 209 and histidine 449). Data and adjustment visualization were obtained with UCSF Chimera program. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Effect of co-solvent addition in the hydrolysis of *rac*-**3** catalyzed by CRL derivatives.

Biocatalysts	4 °C		4 °C, acetonitrile (30%)	
	Specificactivity(U/g)[1]	*E* (*S*)[2]	Specific activity (U/g)[1]	*E* (*S*)[2]
CNBr	87^{f}	$8^{a,b,c}$	2.0^{c}	$12^{b,c}$
GA	35^{d}	$9^{b,c}$	3.1^{d}	$14^{c,d}$
glyoxyl	16^{c}	11^{c}	0.4^{a}	9^{b}
octyl	87^{f}	5^{a}	4.3^{e}	4^{a}
naphthyl	7^{b}	$8^{a,b,c}$	0.9^{b}	1^{a}
MANAE	49^{e}	$9^{b,c}$	1.1^{b}	3^{a}
c-CLEA	9.5^{b}	10^{c}	$1.3^{b,c}$	39^{e}
a-CLEA	1.5^{a}	$6^{a,b}$	1.1^{b}	18^{d}

Reaction conditions were 5 mM of *rac*-**3** in acetate buffer (10 mM, pH 5) at 4 °C. [a,b,c,d,e,f]Mean values with different letters are statistically different ($P < 0.05$). Values represent the average of two independent assays.

[1] Specific activity was expressed as units per gram of immobilized protein.

[2] *E* was calculated with Chen equation.

inactivation/inhibition triggered by the acetonitrile molecules [58]. However, under such conditions enantioselectivity of CRL was highly increased in all cases. The organic solvent presence is an important factor which might modify the enzyme's enantioselectivity. However, a general correlation between the physiochemical properties of the organic solvent (log P value or dielectric constant) and a higher enantioselectivity was not found [10]. Noteworthy, the highest enantioselective ($E > 200$) was obtained with the CRL immobilized on MANE-agarose. We consider that the active site of this immobilized biocatalyst is re-shaped to more selectively bind the *S*-enantiomer of **1** by the cooperative effect of the CRL orientation through its most basic regions and the new solvatation sphere of the active site promoted by the acetonitrile molecules. Therefore, this artificial molecular environment seems to be only stable under low temperature conditions because addition of acetonitrile to the reaction media at 25 °C had a deleterious effect on both activity and enantioselectivity (results not shown). Probably, that temperatures above 4 °C may alter the optimal local conformation of CRL due to some negative molecular vibrations thermally triggered.

With the aim of understanding the molecular binding of *rac*-**1** to the active site, we docked this substrate into the CRL active site (PDB 1CRL) by the UCSF Chimera program [59]. We observed a different orientation of the aromatic ring for each enantiomer (Fig. 1A and B). In the case of (*S*)-**1**, the aromatic ring is aligned to the hydrophobic tunnel locating the carbonyl group very close to the catalytic serine (S209). Contrarily, the aromatic ring of the (*R*)-**1** is stuck into

one hydrophobic cavity, resulting in a still possible but less catalytically productive conformation (Fig. 1B). These slight differences in the substrate binding may explain the slight enantiopreference of CRL towards *S*-enantiomer. Thereby, a little change in the molecular vicinity of the catalytic pocket caused by one specific enzyme orientation, as well as substrate solubility differences promoted by the acetonitrile, might build a more *S*-preferential active site as was experimentally observed.

4.3. Enantioselective hydrolysis of racemic ethyl 2-hydroxy-4-phenylbutyrate (rac-**3**)

Besides alcohols, enantiopure carboxylic acids constitute a wide field of potential application of enantioselective lipases. Thereby, in this study we also evaluated the aqueous hydrolysis of *rac*-**3** catalyzed by the eight lipase immobilized preparations (Scheme 1B).

Initially, the effect of pH on the biocatalysts's enantioselectivity and activity was evaluated. All biocatalysts showed major preference for the hydrolysis of the (*S*)-enantiomer at the two tested pH values (Table 2S). However, observed *E* values were relatively low (E_{max} pH 7 < 4 and E_{max} pH 5 < 9). Regarding the activity, five biocatalysts showed higher activity at pH 7 than at pH 5. As in the resolution of (*S*)-**2**, this effect might be attributed to the major activity that CRL exhibits at pH 7. Nonetheless, the activity of the MANAE-CRL (aminated support) was higher at pH 5 than at pH 7. The latter results might be related to a weak enzyme-support interaction, since in this support most of the salt bridges formed by the enzyme-carboxyl groups and the support-amino groups would be cut off at pH 5. Therefore, the immobilized enzyme on this aminated support at pH 5 expresses more activity due a less rigidification effect.

Despite most biocatalysts presented higher activity at pH 7 than at pH 5, all the immobilized preparations were more enantioselective at pH 5; however, in all cases the obtained *E* values were too low for practical utilization.

In order to improve the enantioselectivity of the immobilized biocatalysts, temperature and co-solvent addition were evaluated (Table 3). All biocatalysts showed lower activity (around 4-times less) when temperature was decreased to 4 °C. However, at this lower temperature enantioselectivities were 1.2-times higher.

Then, we added acetonitrile to the reaction medium, which promoted the decreasing of the enzymatic activity for all the immobilized preparations; likewise it occurred for *rac*-**1**. The variation of the enantioselectivity was unpredictable since glyoxyl-, octyl-, naphthyl- and MANAE-CRL derivatives presented lower enantioselectivity in the presence of acetonitrile than in full aqueous media. Conversely, CNBr-, GA-CRL and both CLEA preparations were more enantioselective in presence of acetonitrile, achieving the highest enantioselectivity value of this study with the c-CLEA ($E = 39$). The improved enantioselectivity of c-CLEA might be related to the combination of tree effects: 1) conformational changes of the catalytic site, as a result of the enzyme-enzyme interactions, 2) the reaction medium conditions (4 °C and 30% acetonitrile); and 3) the positive charged microenvironment in the surroundings of the enzyme, which was provided by the PEI.

To explain the positive effect of PEI on the enantioselectivity of CRL, we coated with PEI all the derivatives where CRL was immobilized on pre-existing carriers and tested them for the stereoselective hydrolysis of (*S*)-**3**. This physical modification provides an increase of hydrophylicity and charge microenvironment of the enzyme. The latter modification was not performed in CLEA since these derivatives are prepared in the presence of PEI. Immobilized preparation coated with PEI- showed negligible variations in enantioselectivity; *E* values were indeed lower when PEI was coating the enzyme (data not shown). Therefore, we discard the effect of PEI as major contributor to enhance the CRL enantioselctivity in the c-CLEA preparation. Another insight that reinforces the negligible role of PEI is that, a-CLEA showed lower enantioselectivity than c-CLEA even though both were prepared in the presence of PEI. Hence, we suggest that such enantioselectivity increase might be due to conformational changes of the catalytic pocket of the enzyme, as a result of the merged effect of carboxyl-cross-linking of the enzyme and the reaction conditions. For a better understanding, we obtained the molecular binding models of the catalytic pocket of CRL and the enantiomers of *rac*-**3** with different orientations of the substrate's ring [facing outward (Fig. 2A and B) or inward (Fig. 2C and D) of the hydrophobic tunnel]. Such modeling revealed that (*S*)-**3** is equally favorable as (*R*)-**3** since both present almost the same formation energies in the different fitting models, which is in agreement with the low observed *E* values. Therefore, the enhanced enantioselectivity of c-CLEA, might be more related to the resulted enzyme 3D configuration after its immobilization in combination with the reaction medium conditions, and not for a different orientation of the substrate's moieties within the enzyme's hydrophobic pocket.

5. Conclusions

We have constructed a library of different carrier-bound and carrier-free insoluble preparation of CRL. By using a combinatorial approach that involved immobilization techniques and reaction engineering we have been able to screen 8 different immobilized CRL variants in 4 different reaction conditions, controlling parameters such as temperature, pH and media polarity. We have found different heterogeneous biocatalysts able to enantioselectively hydrolyze the *S*-enantiomer of two different esters with high *E* values. The immobilization of CRL on MANAE agarose hydrolyzed *rac*-**1** with the highest rate and highest enantioselectivity ($E > 200$) under pH 7, 4 °C and 30% (v/v) acetonitrile. Likewise, aggregated CRL and cross-linked with cardodiimide hydrolyzed *rac*-**3** with the highest enantioselectivity ($E = 39$) under pH 5, 4 °C and 30% (v/v) of acetonitrile. Therefore, low temperatures and presence of co-solvents enhance the CRL enationselectivity towards the *S*-isomer, while pH had negligible effect for the hydrolysis of *rac*- **1** but notorious for the hydrolysis of *rac*-**3**. Finally, in this work we have demonstrated the importance of the immobilization protocol in the final catalytic properties of the heterogeneous biocatalyst as result of the variety of orientations and structural conformation of the immobilized enzyme. Moreover, combinatorial preparation of heterogeneous biocatalysts can efficiently merge to other strategies as reaction medium engineering to discover highly enantioselective heterogeneous biocatalysts. Nowadays, several works have demonstrated the effectiveness of carrier-bound immobilization techniques as suitable and feasible tool for the enhancement and modulation of enantioselectivity. However, herein we demonstrated that carrier-free immobilization is also available for the same purpose, with the advantage that is a less expensive choice. Thereby, this work contributes to the knowledge and understanding of lipase enantioselectivity behaviour.

Declaration of interest

The authors declare no competing financial interest.

Acknowledgements

This work was supported by the Mexican Council for Science and Technology (CONACyT) by the project No. 154004. S. Velasco is grateful for the scholarship received from CONACYT. We would like to thank IKERBASQUE, Basque foundation for Science for the funding to PhD Fernando López Gallego.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2016.04.006.

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