Improved racemateresolution of pentan-2-ol and trans-(Z)-cyclooct-5-ene-1,2-diol by lipase catalysis.

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Abstract

Lipases are important catalysts in chiral synthesis due to their wide substrate recognition combined with a high stereoselectivity. We demonstrate here that the state, free or immobilized, of *Candida antarctica* lipase B (CaLB) affects enantioselectivity and also alters the temperature dependancy of the enzyme. This indicates that CaLB undergoes various conformations induced by its interaction with the different immobilization supports studied. Molecular imprinting experiments, using immobilized enzyme co-dried with mimic substrate molecules, enhanced the enantiomeric ratio two-fold or three-fold, depending on the immobilization support. The structure of the acyl donor has a pronounced effect on CaLBcatalyzed resolution, due to the proximity of the acyl and alcohol moieties during catalysis. When the acylation of pentan-2-ol was examined, we found that the 3C methyl propanoate donor afforded the highest resolution.Trans-(Z)-cyclooct-5-en-1,2-diolwas used as

a modelracemicsubstrate to study the ability of lipase to catalyze the resolution of difunctionalized compounds. There was a clear enhancement in the enantiomer selectivity of the biotransformation of the diol when vinyl butanoateis used as the acyl donor.The conversion and enantiomeric excess of (1*R*,2*R*)-monoacetates were enhanced, using immobilized CaLB, when the chain length of the donors increased from C2 to C4.

Keywords: Biocatalysis; Kinetic Resolution; Lipase; Immobilized Enzymes; Substrate Engineering; Bioreactors.

1.Introduction

Chiral synthesis is a key process in synthetic organicchemistry and, in particular, in medicinal chemistry because the different enantiomers or diastereomers of a molecule frequently have substantially different biological activity.Enzymes with very high regio- and stereo- selectivity are catalysts of choice for chiral resolution and asymmetric synthesis. The requirement forcompounds with high stereochemical purity is increasing and there is a growing need for enzymes and reaction conditions with improved and preciseperformance. Lipases are often used because they are available from various sources, they do not need cofactors and they arerelatively thermostable. In addition,they also accept a broad range of substrates, yet exhibit high selectivity(Bornscheuer and Kazlauskas, 2005). *Candida antarctica* Lipase B (CaLB) (Noël et al., 2004; Léonard et al.,2004)and *Burkholderiacepacia* Lipase (BC)are relatively thermostable enzymes that are often used as catalysts in various biotransformations in non-aqueous media and demonstrate high enantioselectivity toward many chiral substrates.

Catalytic activity, selectivity, specificity and enzyme stability are key factors affecting the efficiency of biocatalysts and enzyme immobilization may improve these properties. In addition, immobilization of lipases confers good thermal and operational stabilities, enabling the development of continuous processes.Immobilization of lipases by sol-gel encapsulation is an easy and effective method that can also increase enzyme activity and selectivity (Reetz et al., 2003; Hellner et al., 2011). Another successful strategy for enhancing the activity and selectivity of lipases involves the tuningof the enzyme active site by molecular imprinting. The "molecular imprinting effect" theorywas proposed by several authors to explain the modification of enzyme selectivity or activity in the presence of molecules thatmimic substrates(Hellner et al., 2011; Yan et al., 2009; Yang et al., 2010, Chaput et al., 2012). The theory is that the enzyme slightly adjusts its active site structure around the imprint molecule and remains "trapped" in this conformation until the substrate enters.For example, pretreatment of a lipase with a chiral substrate such as (*R*)-2-octanol results in enantioselective activation (Furukawa et al., 2002). The dualuse of sol-gel entrapment and imprinting has been successfully performed with lipases,to enhancetheir activity or selectivity(Reetz et al., 2003; Hellner et al., 2011; Cao et al., 2009). In these studies the enzyme is brought together with an "imprinting" molecule and then co-immobilized by sol–gel encapsulation.

The reaction efficiency can be enhanced by modifying the biocatalysts andalso by changing the reaction conditions. The influenceof temperature on selectivity is one of the major concerns in enzymatic reactions;in most cases, stereoselectivitydecreases with increasing temperature. Very few groupshave studied the temperature effect on lipase-catalyzed kinetic resolutions in continuous-flow mode;most datause batch mode(Abahazi et al., 2014).

Acyl donors also have the ability tochange lipase activity and selectivity during kinetic resolution of chiral molecules through acylation (Devendran and Yadav, 2014).By changing the acyl group of acylation agents, the lipase is transformed into various acyl-enzyme

intermediates, with structurally different active sites. This exertsa variety of chiral discrimination abilities duringthe enantioselective acylation of chiral molecules (Ema et al., 1996).

In the present study, different strategies -engineering of the substrate molecule, reaction temperature, use of imprinting molecules andimmobilization of the enzyme- have been investigated toexplore lipase activity and enantioselectivity toward pentan-2-ol, through the acylation reaction. (*S*)-pentan-2-ol is a chiral intermediate in the synthesis of several potential anti-Alzheimer drugs that inhibit β-amyloid peptide release and/or its synthesis (Audia et al., 1998). In addition, lipase-mediateddesymmetrization of trans-(Z)-cyclooct-5-ene-1,2-diols(Rouillard et al., 2014) was examined. Indeed,enantiomerically pure vicinal diols are interesting building blocks for the production of flavours and fragrances(Rouillard et al., 2014).Resolution of pentan-2-ol experiments were performed in a continuous solid/gas reactor that allowsthe control and independent variation of thethermodynamic activity of substrates and other components. The solid/gas reactor offers the possibility to modulate, and to study independently, the effect of each component present in the reaction medium or the effect of each reaction parameter, all other things being equal (Lamare et al., 2004).

2. Materials and Methods

2.1. Materials

Lipases from*Candida antarctica* (immobilized on acrylic resin 077K1155, 10 000 PLU^{-1}Novozym 435®)and from*Burkholderiacepacia* (free and immobilized on diatom MKBB3465, 500 PLU^{-1})were purchased from Sigma Aldrich (Sigma-Aldrich ChemieS.a.r.l., Saint Quentin Fallavier,France). For pentan-2-ol resolution, free CaLB (Lipozyme®) was used. It was a gift from NovozymesA/S (Bagsværd, Denmark) and was used after purification by gel filtration

on PD 10 column (Health Care, Danemark), using ultra-pure water as elutant and then freeze-dried. For diol resolution, free CaLB was purchased from Sigma Aldrich. All enzymes were stored in a vacuum dessicator containing P_2O_5before use.

All other chemicals were purchased from Sigma Aldrich and were used without further purification except in the case of vinyl acetate which was used after fresh distillation.

2.2. Preparation of lipase co-dried with imprinting molecules

In order to prepare Novozym435® co-dried with imprinting molecules, 0.5g of immobilized biocatalyst was mixed in 3mL of hexane at room temperature during two hours with (*R*)-2-aminohexane, (*S*)-2-aminohexane or 1-aminohexane at different concentrations. The aim was to obtain a ratio of 1, 10, 100 or 1000 moles of amine per mole of enzyme for each amine, assuming that the amount of active lipase on the carrier was equal to 1000nmole/g carrier (Hedfors et al., 2010).

2.3. General procedure for Sol-Gel Immobilization of Lipase

Sol-gel immobilized CaLB in presence of imprinting molecules or not, was prepared according to previous works (Hellner et al.,2011; Weiser et al., 2012). The following compounds were mixed in a 5mL glass vial: 390µL Tris-HCl buffer (0.1M, pH 7.5), 200µL propan-2-ol, possibly the imprinting molecule ((*R*)-2-aminohexane, (*S*)-2-aminohexane or 1-aminohexane at a ratio of 1or 1000 moles of amine per mole of enzyme, assuming a quantity of enzyme of 50mg), 100µL NaF 1M, 200µL polyethylene glycol 1000(4% w/v) at room temperature for 10 min. The silane precursors TEOS(tetraethoxysilane):OcTEOS(octyltriethoxysilane):PhTEOS(phenyltriethoxysilane)(10: 7:3mmol) (in the given molar ratio) and 50mg of purified free CaLB were then added under stirring, submitted to sonication during 15 min and agitation during 12 hours at room temperature in order to obtain complete polymerization. The resulting solid was washed with

propan-2-ol (7mL), distilled water (5mL), propan-2-ol (5mL) and hexane (5mL). The residual white powder was dried in a vacuum desiccator.

2.4. Instrumentation

IR spectra were performed with a Perkin–Elmer Spectrum 100 IRFT-ATR instrument. ^{1}H and ^{13}C NMR were obtained with a JEOL JNM LA400 (400 MHz) spectrometer. Chemical shifts (δ) are given in parts per million (ppm) downfield from internal standard tetramethylsilane (TMS). Coupling constants J are given in Hz. The high resolution mass spectra (HRMS) were obtained with a Varian MAT311 spectrometer in the Centre Régional de Mesures Physiques de l'Ouest (CRMPO, University of Rennes, France).

2.5. General procedure for enantioselective acetylation of racemic pentan-2-ol using lipase

For pentan-2-ol resolution reactions, initial rates of reaction were calculated at different temperatures in a solid-gas reactor (Scheme 1). In this type of reactor,the enzyme in the solid state, is packed between two layers of glass wool, to form a packed bed, which is percolated by a carried gas, transporting gaseous substrates. A full description of this type of reactor was given previously (Lamare et al., 2004). Thermodynamic activities for ester and alcohol substrates were respectively a_{ester}=0.1 and$a_{alcohol}$=0.05. Reactions were carried out under anhydrous conditions. Pentan-2-ol commercial solutions was dried byrefluxing with magnesium and then distilled. Methyl esterscommercial solutions were dried with Na_2CO_3anddistilled from P_2O_5. The dried substrates were stored under argon atmosphere and over molecular sieves.The amount of enzyme in the fixed-bed of the reactor was equal to 2.5 mg of Novozym 435® or 0.15 mg of free CaLB. The total gaseous flow was equal to 2000 µmol.min^{-1}.

For experiments with Novozym435® co-dried with imprinting molecules, the mass of enzyme used was 1 mg and for sol-gel immobilized CaLBit was equal to 2mg, with a total flow rate

equal to 1400µmol.min^{-1}.In these conditions, conversion level ofsubstrates was comprised between 2 and 5% and allowed initial reaction rates to be calculated.

For the solid-gas system analysis, the vapor phase leaving thebioreactor was injected in a gas chromatograph (Agilent model6890N Series) with the column Chirasil-Dex CB (25m×0.25mmi.d.×0.25µm β-cyclodextrin; Chrompack,France), as previously described (Létisse et al., 2003).The column temperaturewasprogrammed to hold between 5 and 20 min at 55°C then to increase at different rates from 55 to 85 to 160°C, depending on the ester used as substrate.The external calibration of the two substrates (pentan-2-ol and methyl esters) was carried out by programminga range of their partial pressures in the bioreactor andby analyzing with the gas chromatograph. For the productspent-2-yl esters, an external calibrationwas carried out with pure esters synthesized as described below, by using gas chromatography (Agilent 7890A) equipped with an auto-sampler (7688B), in the same analytical conditions as described above.For accurate determination of *E* values the vapor phaseleaving the bioreactor was re-condensed if necessary and was then partiallyevaporated in order to enhance pent-2-yl esters detection andquantification.The enantiomeric ratio was calculated using the equationfrom Wescott and Klibanov(Wescott and Klibanov, 1993):$E = Vi^R / Vi^S$, $Vi^R$and $Vi^S$are the initial rates of (*R*)-pent-2-yl ester and (*S*)-pent-2-yl estersynthesis respectively.The enantiomeric ratios were calculated by taking the average of three different experiments. The associated standard deviation was calculated for each result.

2.6. General procedure for the synthesis of pent-2-yl esters ***(1-5)***

To a solution of pentan-2-ol (1eq) in pyridine (0.5 eq), anhydride (1.1eq) was added and the mixture was kept at room temperature for 16 h. The mixture was poured into ice cold water and extracted with ethyl acetate (3×20 mL). The ethyl acetate extract was washed with water (2×10 mL), 1 N HCl (2×10 mL), and finally with water (3×10 mL), dried over $MgSO_4$,

filtered, and the solvent was removed under vacuum. The product was purified by column chromatography (silica gel 200–400 mesh, usually using 9:1 petrol ether/ethyl acetate).

*Pent-2-yl propanoate**(1)***

Compound ***(1)*** was obtained as colorless oil. ν_{max} (cm^{-1}): 2961, 2937, 1733, 1463, 1377, 1190. δ_H NMR (400 MHz, $CDCl_3$): 4.80-5.03 (m, 1H, C<u>H</u>-O), 2.12-2.32 (m, 2H, C<u>H</u>$_2$), 1.19-1.67 (m, 10H, 2xC<u>H</u>$_3$, 2xC<u>H</u>$_2$), 0.92 (m, 3H, C<u>H</u>$_3$).

*Pent-2-yl butanoate **(2)***

Compound ***(2)*** was obtained as colorless oil. ν_{max} (cm^{-1}): 2962, 2935, 1731, 1458, 1379, 1184, 1121. δ_H NMR (400 MHz, $CDCl_3$): 4.81-5.00 (m, 1H,C<u>H</u>-O), 2.26 (t, *J* = 7.4 Hz, 2H, C<u>H</u>$_2$), 1.28-1.77 (m, 6H, 3xC<u>H</u>$_2$), 1.29 (d, *J*=6.4 Hz, 3H, C<u>H</u>$_3$), 0.98-0.86 (m, 6H, 2xC<u>H</u>$_3$).

*Pent-2-yl hexanoate**(3)***

Compound ***(3)*** was obtained as colorless oil. ν_{max} (cm^{-1}): 2958, 2933, 1732, 1459, 1377, 1245, 1176. δ_H NMR (400 MHz, $CDCl_3$): 4.85-5.01 (m, 1H,C<u>H</u>-O), 2.27 (t, *J* = 7.4 Hz, 2H, C<u>H</u>$_2$), 1.67-1.19 (m, 13H, C<u>H</u>$_3$, 6xC<u>H</u>$_2$), 0.93-0.88 (m, 6H, 2xC<u>H</u>$_3$).

*Pent-2-yl isobutanoate**(4)***

Compound ***(4)*** was obtained as colorless oil. ν_{max} (cm^{-1}): 2964, 2935, 1730, 1468, 1384, 1260, 1194, 1160. δ_H NMR (400 MHz, $CDCl_3$): 4.80-4.92 (m, 1H, C<u>H</u>-O), 2.35-2.50 (m, 2H, C<u>H</u>$_2$), 1.53-1.04 (m, 14H, 4xC<u>H</u>$_3$, C<u>H</u>$_2$), 0.85(t, *J*=7.2 Hz, 3H, C<u>H</u>$_3$).

*Pent-2-yl decanoate**(5)***

Compound ***(5)*** was obtained as colorless oil. ν_{max} (cm^{-1}):2924, 2854, 1732, 1458, 1376, 1175, 1121. δ_H NMR (400 MHz, $CDCl_3$): 4.79-4.95 (m, 1H, C<u>H</u>-O), 2.26 (t, *J* = 7.4 Hz, 2H, C<u>H</u>$_2$), 1.63-1.20 (m, 21H, C<u>H</u>$_3$, 9xC<u>H</u>$_2$,), 0.93-0.86 (m, 6H, 2xC<u>H</u>$_3$).

*2.7. General procedure for the synthesis of diol **(7)** using mesoepoxyde**(6)** as intermediate:*

(Z)-(1S,8R)-9-oxa-bicyclo[6.1.0]non-4-ene ***(6).*** This compound***(6)*** was synthesized and analysed by IR and NMR as previously described (Rouillard et al., 2014). Trans-*(Z)-cyclooct-5-en-1,2-diol* ***(7)***was thenobtainedfrom***(6)*** andanalyzed as previouslydescribed(Rouillard et al., 2014).

2.8. General procedure for enantioselective acetylation of racemic trans-(Z)–cyclooct-5-en-1,2-diol ***(7)*** *using lipase*

To a solution of racemic diol ***(7)*** (0.2 g, 1.41 mmol) solubilized in 2.5 mL of THF and under inert atmosphere, the corresponding vinyl ester (ethanoate, propanoate, butanoate, 14 mmol) and free or immobilized lipase were added. The mixture was stirred at room temperature and then filtrated, extracted and purified as previously described (Rouillard et al., 2014) to give the esters:*(1R,2R)-(Z)-1-hydroxy-cyclooct-4-enylethanoate****(8a)***, *(1S,2S)-(Z)-1-hydroxy-cyclooct-4-enyl ethanoate****(8b)***, *(1R,2R)-(Z)-1-hydroxy-cyclooct-4-enyl propanoate****(9a)***, *(1S,2S)-(Z)-1-hydroxy-cyclooct-4-enyl propanoate****(9b)***, *(1R,2R)-(Z)-1-hydroxy-cyclooct-4-enyl butanoate****(10a)*** and*(1S,2S)-(Z)-1-hydroxy-cyclooct-4-enyl butanoate****(10b)***.

Compounds***(8a)*** and ***(8b)***were analysed by IR and NMR as previously described (Rouillard et al., 2014).

*(Z)-1-hydroxy-cyclooct-4-enyl propanoate****(9a*** and ***9b)***

Compounds***(9a*** and ***9b)*** were obtained as colorless oil. ν_{max} (cm^{-1}): 3413, 2942, 1718, 1356, 1272, 1183, 1028.δ_H NMR (400 MHz, $CDCl_3$):5.54 – 5.81 (2H, m, C<u>H</u>=C<u>H</u>), 4.96 (1H, td, *J* = 8.7, 3.8 Hz, C<u>H</u>-CO), 3.90 (1H, td, *J* = 8.4, 3.8 Hz, C<u>H</u>-COH), 2.33 – 2.46 (4H, m, 2xC<u>H_2</u>), 2.01 – 2.26 (5H, m, 2xC<u>H_2</u>, C-O<u>H</u>), 1.66 – 1.79 (2H, m, C<u>H_2</u>), 1.34 – 1.03 (3H, m, C<u>H_3</u>).

(Z)-1-hydroxy-cyclooct-4-enyl butanoate ***(10a*** and ***10b)***

Compounds***(10a*** and ***10b)*** were obtained as colorless oil. ν_{max} (cm^{-1}): 3414, 2962, 1718, 1255, 1177, 1042.δ_H NMR (400 MHz, $CDCl_3$): 5.60-5.73 (1H,m, C<u>H</u>=C<u>H</u>), 4.97 (1H, td, *J* =

8.7, 3.8 Hz, CH-CO), 3.91 (1H, td, *J* = 8.5, 3.9 Hz, CH-COH),2.30-2.48(4H, m, 2xCH_2)2.03-2.22 (5H, m 4xCH_2, C-OH), 1.60 – 1.83 (m, 4H, 2xCH_2), 0.96 (3H, t, J = 8.0 Hz, CH_3).

For the determination of enantiomeric excess (*ee*) of monoesters gas chromatography (Agilent 7890A) was used as previously described (Rouillard et al., 2014). The enantiomeric excesses and yields were calculated by taking the average of three different experiments. The associated standard deviation was calculated for each result.

For the determination of enantiomeric excess of remaining diols, high performance liquid chromatography was carried out in a Waters 600s combined with an auto-sampler (Waters 717 plus) as previously described (Rouillard et al., 2014).

3. Results and Discussion

3.1. Enantioselective acylation of pentan-2-ol using free andimmobilized CaLB

Novozym 435® (CaLB immobilized on acrylic resin) was used here as a biocatalyst. The effect of acyl donor chain length on resolution was studied by measuring the enantiomeric ratio (*E*) in the kinetic resolution of racemic pentan-2-ol through acylation with different methyl esters (Figure 1), at different temperatures ranging from 50 to 100°C, in a continuous solid-gas reactor.

When CaLB is immobilized on acrylic resin, the highest *E* values are obtained with methyl propanoate as an acyl donor, at each temperature tested. *E* values decrease when the chain length of the acyl donor increases from C3 to C4 and to C6, at each temperature tested. *E* value is very low when methyl isobutanoateis used as an acyl donor (Figure 2A). The same trend was obtained for enzyme activity at 60°C, but the valueswere divided by 3 and 5 going

from a C3 acyl chainto C4 andC6 acyl chains, respectively, and divided by 10 when methyl isobutanoateis used as an acyl donor.

The enantiomeric ratiosand enzyme activitieswere then measured with free CaLB instead of immobilized lipase, under the same conditions and using a longer acyl donor(C10 instead of C6). The highest enzymatic activities were obtained with the shortest acyl chain tested (C3), both at 60 and 80°C. Three fold higher values of enantiomeric ratio *E*wereobtained compared to immobilized enzyme on acrylic resin (Figure 2A and 2B).Interestingly,in previous studies, it was shown by Jacobson *et al.* that free and immobilized CaLBon acrylic resin presented the same enantioselectivity toward other secondary alcohols such as 1-phenoxy-propan-2-ol or 3-chloro-1-phenoxy-propan-2-ol, through acylation with vinyl butanoate in hexane (Jacobsen et al., 2005).

The effect of acyl donor lengthonthe resolution of pentan-2-ol with free CaLBdemonstrates that the highest *E* valueisobtained at 50°C with aC3 acyl chain, and at 80°C with a C10 acyl chain (Figure 2B).CaLB has a deep narrow active site into whichthe substrate ester binds in a hairpin structure(Uppenberg et al., 1995).The acyl and pentan-2-ol moieties arethus broughtspatially close together during catalysis;this could explainlipase sensitivity in relation to the length of the acyl chain. Each different acyl-enzyme intermediate formed leads to different active site structures, with different enantioselectivity toward the secondary alcohol. Furthermore CaLB exhibits a funnel-like scissile fatty acid binding site, inside which fatty acids with chain lengths as long as 13 carbons can bind completely (Pleiss et al., 1998);this means that amino acid residues in the acyl binding site that are at some distance from the point of chirality also affectenantioselectivity(Haeffner et al., 1998). Finally, it is known that immobilization of a protein may produce distortion of the active site, therefore, it is not surprising that enantioselectivityof CaLBvaries both with the acyl chain length of the donor and with immobilization. Recently two different conformations, open and closed, were

observed for crystal structure of CaLB at very high resolution (Stauch et al., 2015). The shift from the open form to the closed form involves the unfolding of the α-helix 5, that allows the formation of a salt bridge between Asp145 and Lys290, thus closing the catalytic cavity. Consequently, changes in both dielectric constant and ionic strength of the medium are able to affect interfacial activation of CaLB (Stauch et al., 2015). These recent findings give new insights into the possible effects of substrates, media and immobilization on CaLB fold and activity. These features make it extremely difficult to predict *a priori* and explain CaLBenantioselectivity.

The resolution of 3-methyl-2-butanol through acylation with vinyl esterswas studied by Ottosson and Hultand it was found thatlong alkyl chains like vinyl octanoate afforded the highest resolution.A decrease in resolution was obtained with shorterchain lengths for vinyl hexanoate and vinyl butanoate.Shortening the acyl chain to vinyl propanoategave a slightincreasein resolution(Ottosson and Hult, 2001). There is no general rule concerning the effect of acyl chain length on CaLB-catalyzed resolution of secondary alcohols as it differs from one combination of acyl donor/alcohol to another. In conclusion, the choice of an achiralacyl donor strongly influences the efficiency of the resolution ofa chiral alcohol in acylation reactions with CaLB.

We then wished to ascertain the conditions necessary to obtain a high enantioselectivityin acylation reactions of pentan-2-ol usingimmobilized lipase, compared with free CaLB. Methyl propanoate was chosen as acyl donor as it gives the best resolution compared to other acyl donors and also has the highest enzyme activity. 70°C was chosen as reaction temperature as it gives the best compromise between high enzyme activity and long-term stability.

The effect ofco-drying CaLBNovozym 435 with imprinting molecules in hexane, before performing the acylation reaction on immobilized enzyme activity and enantioselectivity was first studied.

The different amines ((*R*)-, (*S*)-2-aminohexane and 1-aminohexane)have a similar effect on enantioselectivity: *E*doubles for 1 mole of amine per mole of enzymebut the increase of *E* is lower at higher concentrations of amines (Figure 3A).Enzymatic activity is increased 1.4 times to 1.8 times for 1 mole of amine per mole of enzyme and by lower amounts at higher concentrations of amines (1.5 to 1.7 times for 10 molesof amine per mole of enzyme, 1.3 to 1.6 times for 100 moles of amine per mole of enzyme and 1.15 to 1.5 times for 1000 moles of amine per mole of enzyme; Figure 3B). In all cases no amides were detectable in the reaction product. This is in agreement with the fact that alcohols deacylate the acyl-enzyme intermediate faster than amines (Öhrner et al., 1996).

The analysis of these different results is complex. The fact that the amines have a significant effect on enantioselectivity at a ratio 1/1 with the enzyme supports the hypothesis of an imprinting effect, but this is called into question by the fact that the three amines, with different structures, have an almost similar effect. The decrease in both enantioselectivity and activity using 100 or 1000 moles of amine per mole of enzyme instead of 1 or 10 moles could arise from the formation of non-reactive enzyme-amine complexes, as was hypothezised by Garcia-Urdiales*et al.*, when studying the role of amines in the CaLB-catalyzed alcohol resolution (Garcia-Urdialeset al., 2001).

The effect of encapsulation prior to the acylation reaction of free CaLB, in the absence or presence of imprinting molecules, on enzyme activity and enantioselectivity toward pentan-2-ol was then studied.

Encapsulation of free CaLBleadsto an increase of *E* from 109.0 ± 2.7 for free enzyme (lipozyme) to 154 ± 1 for CaLBencapsulated in sol/gel matrix (Figure 4).The value

corresponding to the activity of the encapsulated CaLBis multiplied by a factor of 1.7 compared to free enzyme.A substantially improved enantioselectivityand activity of sol-gel entrapped CaLBcompared to non-immobilized lipase was also obtained by Ursoiu*et al.*in the acylation reaction of octan-2-ol(Ursoiu et al., 2012).

Enantioselectivityremainsalmost identical with or without addition of amines in the sol/gel matrix, at 1 or 1000 moles per mole of enzyme and with all aminestested.Enzymatic activity remained constant with or without amines added in sol/gel matrix for all the amines tested.

The enantioselectivity of CaLB toward pentan-2-ol varies substantially between the free form (E=109.0 ± 2.7),Novozym 435 (E=51.2 ± 1.0) and the encapsulated enzyme (E=154 ± 1).It has already been shown that Novozym 435, that corresponds to CaLB immobilizedon Lewatit VP OC 1600, a macroporous hydrophobic acrylic polymer resin, displays very different selectivity compared to free CaLB or CaLBimmobilized on other supports (Cabrera et al., 2009; Mateo et al., 2007). Recently, it was shown by Zisis*et al.* by combining experiment and simulation, that CalB is an interfacially activated enzyme and that the hydrophobicity of the support used for its immobilization influences both enzyme conformation and substrate specificity (Zisis et al., 2015).

For enzyme encapsulated in sol/gel matrix, neither the enantioselectivitynor the activity wereimproved by the addition of chiral aminesor by the primary amine.On the contrary, these parameters were significantly improved with the same amines when co-driedin hexane with the enzyme immobilized on acrylic resin.In a recent study, docking experiments into CaLBof various additives with imprinting effects, were performed, using the two different enzyme conformations(open and closed) observed by Stauch et al. in 2015 (Weiser et al., 2016). These studies revealed synergistic effects includingthe stabilization of the active conformation of CaLB and provision of extra space for the mobile active site-covering lids after theirremoval. These types of effects were caused by large additives only, whereas smaller additives were unable to significantly stabilizethe active conformation of the active site (Weiser et al., 2016).

On that basis, it is probable that the “imprinting effect” for the investigated amines is rather based on allosteric effects.Further studies are required to fully explain these data.

3.2. Enantioselective acylation of diols using CaLB and BC lipases

Trans-*(Z)-cyclooct-5-en-1,2-diol* ***(7)***was usedas a modelracemicsubstrate to study the ability of lipase to catalyze the resolution of these difunctionalized compounds through enantioselective acylation.The comparison of acyl donors and enzyme, together with the effects of immobilization of the enzyme, were carried out as outlined below.

Theracemicdiol***(7)***was synthesizedfromthe commercial cycloocta-1,2-diene in two steps : epoxidation followed by ring opening in acidic conditions(Figure 5), as previously described (Rouillard et al., 2014).

The preparation of an optically enriched monoester was first performed using free or immobilized CaLB(Novozym 435) to desymmetrize*meso*-symmetric diol ***(7)***usingvinyl ethanoate, vinyl propanoate and vinyl butanoate as the acylatingagents in THF solvent,at room temperature (Figure 6A and Table 1).

When vinyl ethanoate is used as the acyl donor, immobilized CaLBgives a higher conversion than free CaLB (16% compared to 8%) and also demonstrates a higher enantioselectivity for the (1*R*,2*R*) diol. It increased the enantiomeric excess (ee) of (1*R*,2*R*)-monoethanoate***(8a)***from 50%to 55% and the ee of (1*S*,2*S*)-diol ***(7b)***increasedfrom 4% to 9%. The immobilized CaLB was then chosen for reactions with three acyl donorscontaining different chain lengths.

The conversion and the enantiomeric excess of(1*R*,2*R*)-monoacetatewere enhanced when the chain length increased from C2 to C4.

Conversion increased from 16% using vinyl ethanoate to 33% using vinyl propanoate and 48%, using vinyl butanoate (Table 1).The enantiomeric excess of the monoester ***(8a)***increased

from 55%with a vinyl ethanoate donor to 73% or 96% using vinyl propanoate or vinyl butanoate, respectively. In all cases, the (1*R*,2*R*) diol was the preferred substrate for monoacetylation.The enantiomeric excess of the remaining (1*S*,2*S*)-diol ***(7b)***increased from 9% using a vinyl ethanoate donor to 55% using vinyl propanoateand more than95% using vinyl butanoate.

The same reaction wasthen performed using BC Lipase (Figure 6B and Table 2).

When vinyl ethanoate was used as an acyl donor, BC lipase resulted in a higher conversion than CaLB lipase, and interestingly,under the same conditionsshowed an inversion ofenantioselectivity, with a preference for (1*S*,2*S*)-diol, and the formation of monoester ***(8b)***. Enzyme immobilization slightly enhanced conversion (26% compared to 17%) but not selectivity (Table 2).No reaction was observedwhen an acyl donor with longer chainlength was used with immobilized BC Lipase.

Conclusion

In this study, it was shown that for pentan-2-ol resolution in a continuous solid/gas reactor enantioselectivityand temperature dependence of CaLB vary and depend on the conditions of immobilization of the enzyme. This indicates that the enzyme may take on different conformations induced by interactions with the immobilization supports. The acyl donor has a pronounced effect on CaLB-catalyzed resolution through acylation, due to the fact that the acyl and alcohol moieties are brought spatially close together during catalysis. A significant enhancement of resolution of the trans-*(Z)-cyclooct-5-en-1,2-diol*using CaLBNovozym 435 and vinyl butanoate,rather thanvinyl ethanoate, as acyl donor, was also noticed. The data presented here contribute to the researchintoimproving the stereochemical purity of

compounds obtained by biocatalysisas well asother possibilities such as the optimisation of solvent, temperature, water activity, choice of enzyme and enzyme immobilization methods.

Author Contributions

M.G. and V.T. designed the research. The experimental work was performed by N.F., B.B. and L.D. for the enantioselective acylation of pentan-2-ol using free and immobilized CaLB,by H.R. and R.D. for the enantioselective acylation of diols using CaLB and BC lipases and by A.B. for NMR spectra. M.G. wrote themanuscript with the cooperation of H.R. All authors discussed, edited and approved thesubmitted version.

Conflict of Interests

The authors declare that there is no conflict of interestregarding the publication of this paper.

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Table 1

Effect of immobilization and acyl donorfor *Candida antarctica* lipase B catalyzedenantioselective acylation of diol ***(7)***. Standard deviations were calculated with three values of ee and of yield.

Acyl donor	**Enzyme**	**Conversion (%)**	**Monoester *(8a*, *9a*** or***10a)*** **ee (%)**	**Diol *(7b)* ee (%)**
Vinyl ethanoate	free CaLB	8±2	50±2 (*1R,2R*)	4±2 (*1S,2S*)
Vinyl ethanoate	CaLB immobilized on acrylic resin	16±1	55±3 (*1R,2R*)	9±2 (*1S,2S*)
Vinylpropanoate	CaLB immobilized on acrylic resin	33±2	73±2(*1R,2R*)	55±2 (*1S,2S*)
Vinylbutanoate	CaLB immobilized on acrylic resin	48±1	96±1 (*1R,2R*)	>95 (*1S,2S*)

Table 2

Effect of immobilization and acyl donor for *Burkholderiacepacia*lipase catalyzed enantioselective acylation of diol ***(7)***.Standard deviations were calculated with three values of ee and of yield.

Acyl donor	**Enzyme**	**Conversion (%)**	**Monoester *(8b)* ee (%)**	**Diol *(7a)* ee (%)**
Vinyl ethanoate	free BC	17±2	60±2 (*1S,2S*)	12±2 (*1R,2R*)
Vinyl ethanoate	BC immobilized on diatomite	26±1	57±3 (*1S,2S*)	12±2 (*1R,2R*)
Vinylpropanoate	BC immobilized on diatomite	-NR	-	-
Vinylbutanoate	BC immobilized on diatomite	- NR	-	-