Research Article

Received: 20 December 2008 Revised: 11 March 2009 Accepted: 20 March 2009 Published online in Wiley Interscience: 13 May 2009

(www.interscience.wiley.com) DOI 10.1002/jctb.2200

Enantioselective esterification of ibuprofen with ethanol as reactant and solvent catalyzed by immobilized lipase: experimental and molecular modeling aspects

María L. Foresti,[a,b]* Marianela Galle,[a] María L. Ferreira[b] and Laura E. Briand[a]

Abstract

BACKGROUND: In recent years enantioselective esterification of racemic ibuprofen performed in organic co-solvent media such as isooctane and cyclohexane and catalyzed by lipases, has been proposed as an effective way to increase the concentration of S-ibuprofen in the racemic mixture. In this contribution, the enantioselective enzymatic esterification of (R,S)-ibuprofen with ethanol catalyzed by commercial Novozym 435 without the addition of a co-solvent is thoroughly investigated. Experimental data are further analyzed considering the results of extensive molecular modeling calculations.

RESULTS: The conversion of ibuprofen towards the ethyl esters and the enantiomeric excess towards S-ibuprofen are greatly affected by the ethanol and water contents of the reaction media. The optimum conditions for the esterification of racemic ibuprofen in a batch-type reactor were as follows: molar ratio of ethanol to ibuprofen = 7, 4.8% v/v of water, 160 mg of Novozym 435, 45 °C and 200 rpm. Under these conditions an enantiomeric excess of 54% and 63% of ibuprofen conversion were reached.

CONCLUSIONS: Results showed that the reaction in excess of the esterifying alcohol in a system free of additional organic solvents is possible if the proper conditions are set. Molecular modeling calculations demonstrated that the formation of dead-end compounds between the enzyme and ethanol/water may account for lipase inhibition at high concentrations of those compounds.
© 2009 Society of Chemical Industry

Keywords: ibuprofen; lipase; enantioselective; esterification; molecular modeling

INTRODUCTION

Ibuprofen ((R,S)-2-(p-isobutylphenyl)-propionic acid) is a nonsteroidal anti-inflammatory drug (NSAID) that was introduced in the late 1960s for the treatment of symptoms caused by arthritis such as swelling, pain and stiffness. Ibuprofen, as other nonsteroidal drugs like ketoprofen and flurbiprofen, is also known for its antipyretic and analgesic effects. Arylpropionic acids such as ibuprofen exist in two forms, the (R) and (S) enantiomers. However, it is well documented that the therapeutic activity of ibuprofen is mainly attributed to the (S) enantiomer, which is 160 times more effective than the (R) enantiomer.[1] Although the (R) enantiomer is known to undergo a unidirectional metabolic inversion of configuration to form the active (S) enantiomer,[1,2] it has been reported that this inversion occurs in competition with an acyl exchange reaction with endogenous triacylglycerols, which may result in ibuprofen accumulation in fatty tissue.[3] Besides the still unknown effects of accumulation of ibuprofen residues in fatty tissue, and just considering the total amount of NSAID ingested by patients, if the pure (S) enantiomer could be administered instead of racemic ibuprofen, the amount of total drug prescripted to produce the desired therapeutic effect could be significantly reduced.

Methods for enantiomer separation include mainly high performance liquid chromatography (HPLC, the most common method to date for the resolution of ibuprofen), physical separation methods such as diastereoisomeric crystallization (mainly by salt formation using L-lysine), and in the last decades, enzymatic kinetic resolution.[4] In particular lipase-catalyzed enantiomeric resolution of ibuprofen has been studied by a number of authors in the last two decades.[5–7] Enzymatic resolution is based on the difference in the rates of the reactions catalyzed by an enzyme on the two isomers of the molecule of interest. It can be performed either through hydrolysis, synthesis, oxidation or group transfer, depending on the functions present on the molecule. Due to

* *Correspondence to: María L. Foresti, Planta Piloto de Ingeniería Química -PLAPIQUI-CONICET-UNS, Camino la Carrindanga Km 7, 8000, Bahía Blanca, Argentina. E-mail: lforesti@plapiqui.edu.ar*

a *Centro de Investigación y Desarrollo en Ciencias Aplicadas- Dr. Jorge Ronco, Calle 47 n° 257, B1900AJK, UNLP, Centro Científico Tecnológico CONICET La Plata, Argentina*

b *Planta Piloto de Ingeniería Química -PLAPIQUI-CONICET-UNS, Camino la Carrindanga Km 7, 8000, Bahía Blanca, Argentina*

 © 2009 Society of Chemical Industry

the high selectivity of lipases this family of enzymes is able to efficiently distinguish between both enantiomers and selectively esterify one of them at a higher reaction rate. Depending on the lipase chosen as biocatalyst (R) or (S) stereopreference is obtained. The lipases most widely used for profen esterification are lipases from *Candida rugosa* and *Rhizomucor miehei*, which preferentially catalyze the esterification of S-ibuprofen, and lipase from *Candida antarctica B*, which preferentially esterifies the (R) enantiomer. The stereopreference of lipase from *Candida antarctica B* is advantageous since it allows the direct production of the (S) enantiomer as unreacted substrate of the enzymatic esterification.

The advantages of using enzymes for the enantioselective resolution of racemic drugs include the inherent enantioselectivity of lipases, the mild conditions in which these biocatalysts operate, and, if the enzyme is immobilized, the possibility of reusing the biocatalyst, and the avoidance of complex approaches for its separation from reaction products. On the other hand, a main disadvantage of this route is that enzyme's activity and enantioselectivity are highly dependent on the reaction conditions chosen (i.e. water content, reaction temperature, molar ratio of reactants, alcohol concentration, presence of inhibitors, mass of catalyst, type of solvent, etc.). Published works on lipase-catalyzed esterification of racemic ibuprofen generally analyze the effect on conversion to ester and enantiomeric excess of some of the mentioned variables.[5–8]

With regard to the medium chosen to carry out the reaction, lipase-catalyzed esterification of profens has generally been performed in organic medium. In particular, cyclohexane and isooctane have been the solvents most frequently chosen for (R,S)-ibuprofen resolution. In this contribution no organic solvent was added to the reaction medium and the reaction proceeded in a system composed only of substrates. However, since at the reaction temperature ibuprofen is solid, the ethanol used as the esterifying reagent was added in excess in order to solubilize ibuprofen. Considering the application of ibuprofen, performing the racemic resolution in a medium composed only of substrates appears to be an attractive alternative approach if high conversion to ester and high selectivity are achieved. Previous work on solventless esterification of ibuprofen (to the authors' knowledge there are only a few of them and from the same laboratory) used molar ratios alcohol/ibuprofen close to the stoichiometric values.[9–12] However, in that case solubilization of ibuprofen required the heating of the reaction mixture to high temperatures (70/80 °C) which, depending on the biocatalyst chosen, may be detrimental for lipase activity. On the other hand, it is herein proposed to work in excess of alcohol, which both solubilizes and esterifies ibuprofen, with no need for high temperatures that may be too high for lipase action. Although it is well-documented that molar ratios higher than unity may be highly detrimental to lipase activity,[13] the aim of this contribution was to demonstrate that it is possible to enantioselectively esterify (R,S)-ibuprofen successfully in excess of ethanol under several conditions. The biocatalyst chosen for this purpose was the commercial immobilized form of lipase from *Candida antarctica* B (Novozym 435). The effect on conversion and enantiomeric excess of the initial water content of the medium in which reaction takes place, the volume of ethanol added, the temperature level set, and the enzyme loading used, have all been assayed in order to optimize the conditions for ibuprofen enantioselective esterification in a medium free of organic solvent.

In the last part of the manuscript lipase-catalyzed esterification of ibuprofen is studied through molecular modeling methods. Molecular modeling appears to be an attractive method for predicting the ability of enzymes to discriminate between enantiomers of a substrate.[14] In this contribution computational calculations results are valuable tools for the analysis of experimental observations such as the R-enantiopreference of *Candida antarctica* lipase B and the lower conversions to ester observed at high water/alcohol concentrations. The complete mechanism of (R,S)-ibuprofen esterification with the corresponding steric energies involved in each step, as well as the formation of dead-end complexes between lipase and ethanol/water that may account for lipase inhibition at high concentration of those compounds are specially considered.

EXPERIMENTAL

Materials

The commercial immobilized biocatalysts Novozym 435 (*Candida antarctica* B lipase (CALB), immobilized on an acrylic resin) and Lipozyme RM IM (*Rhizomucor miehei* lipase (RM) immobilized on a macroporous ion-exchange resin) were provided by Novo Nordisk AS (Denmark). (R,S)-2-(p-isobutylphenyl)-propionic acid (racemic ibuprofen, 99.23%) was purchased from Parafarm (Argentina). Absolute ethanol (Carlo Erba, 99.8%, Italy), potassium hydroxide (Carlo Erba, 85.0%) and other chemicals used were all analytical grade.

Esterification reaction

Reactions were performed in closed 100 mL vials, which were kept at constant temperature and stirring (200 rpm) in a shaker bath (Julabo SW22, Germany). In all cases 0.5 g of ibuprofen (2.42 mmol) were dissolved in definite amounts of ethanol with no heating. For the standard reaction the amount of ethanol used was 20 mL (molar ratio of ethanol to ibuprofen = 141), temperature was set at 45 °C, and the mass of catalyst used was 100 mg. In order to determine the effect of water on the conversion of ibuprofen and the enantiomeric excess of the esterification reaction, different quantities of water (W) were added to the initial mixture of substrates, namely 0, 1, 4.8, 9.1 and 20% v/v. When the effect of the volume of ethanol used was assayed, the amount of alcohol was alternatively reduced to 10, 5, 3, 2, and 1 mL ethanol (molar ratios of ethanol to ibuprofen of 71, 35, 21, 14 and 7, respectively). Once the best initial water/ethanol amounts were determined the effect of reaction temperature was checked at 37 °C and 55 °C. Finally, the mass of catalyst was varied in the 25–200 mg range.

Analysis of samples

Chiral analysis of both enantiomers of ibuprofen was conducted by chiral HPLC analysis using a Nucleodex beta-PM (Macherey-Nagel, Germany) with a UV detector operated at 230 nm. The mobile phase (metanol/0.1% TEAA pH 4.0 (60/40 v/v)) was operated at a flow rate of 0.700 mL min^{-1}. All samples were run in triplicate with a 2% relative error. Enantiomeric excess (ee) referred to the form (S) of the remaining ibuprofen was calculated according to the following equation, where [S] and [R] account for the concentrations of the (S) and (R) enantiomers respectively.

$$ee = [([S] - [R])/([S] + [R])] \times 100 \quad (1)$$

Final ibuprofen conversion was also verified by titration of the final reaction mixture. Because ibuprofen is the only acid

 © 2009 Society of Chemical Industry

compound present in the reaction mixture, titration of the samples with a basic solution of known concentration proved to be an accurate and reliable method, possessing an average relative error of 3%. Conversion results obtained by this methodology were in agreement with results obtained by HPLC.

Both the relative errors involved in ibuprofen conversion (3%), and in the enantiomeric excess (2%) are indicated with error bars in the figures presented in this investigation.

Biocatalyst reuse

The operational stability of Novozym 435 was assayed by using 160 mg of the immobilized biocatalyst in successive reaction batches of (R,S)-ibuprofen esterification under optimum conditions determined in the parametric analysis. At the end of each batch the commercial biocatalyst was recovered from reaction medium and washed with ethanol to remove any substrate or product retained. After drying at room temperature the immobilized lipase was added to fresh reaction medium.

RESULTS AND DISCUSSION

Feasibility of lipase-catalyzed resolution of (R,S)-ibuprofen in excess of ethanol

Kinetic resolution of racemic mixtures catalyzed by lipases in organic medium has proved successful for a number of profens. Not only ibuprofen but also other non-steroidal anti-inflammatory drugs of the aryl propionic class (naxoprofen, surprofen, flurbiprofen and ketoprofen), have all been resolved by lipase-catalyzed enantioselective esterification carried out in organic media.[15,16] In most cases the organic solvents chosen have been hydrophobic ones with high log P (logarithm of the partition coefficient (P) of the solvent between 1-octanol and water), a parameter used to quantify the hydrophobicity/hydrophilicity of the compound. It is known that lipases develop higher activity in hydrophobic media since hydrophilic solvents remove the essential water from the biocatalyst leading to lower enzymatic activity.

In this contribution we hereby propose a different approach, in which no additional organic solvent is added to the reactants mixture. Instead, and due to the solid nature of ibuprofen in the temperature range chosen for reaction, the alcohol used for ibuprofen esterification is added in excess in order to guarantee profen solubilization. Previous reports on solvent-free esterifications of ibuprofen catalyzed with lipases were performed in stoichiometric ratios of reactants that required heating the substrate mixture to dissolve ibuprofen.[9–12] It is widely accepted that alcohols may inhibit lipases, the concentration of the former determining biocatalytic activity in lipase-catalyzed esterifications. In fact, in most reports dealing with esterifications catalyzed by lipases, reactant ratios close to stoichiometric values are usually determined as the best conditions for high activity and minimal lipase inhibition.[17,18] In spite of the previous comments, assay of the commercial catalyst Novozym 435 in the esterification of racemic ibuprofen in a medium with 20 mL of ethanol resulted in high activity with only partial deactivation even after 48 h of reaction.[19] On the other hand, under the same conditions of ethanol excess Lipozyme RM IM suffered a drastic reduction in activity with almost total deactivation in the first hours of reaction (data not shown). The excess ethanol used deactivated the *Rhizomucor miehei* lipase, which is known to be strongly deactivated by alcohols,[20] and in particular by ethanol.[21] The position of the active site of this lipase on the external surface of the enzyme promotes unhindered access of alcohols to the catalytic triad. In view of the previous results, Novozym 435 was the catalyst chosen for the parametric analysis described in the following sections.

Parametric analysis of lipase-catalyzed resolution of (R,S)-ibuprofen in excess of ethanol

Among other methods for separation of enantiomers, the enzymatic method is recognized as an attractive route because of the inherent enantioselectivity of enzymes. On the other hand, activity and stereoselectivity of biocatalysts are highly dependent upon a number of reaction parameters that must be optimized in order to make the process economically viable. In the following sections the effect on both esterification conversion and enantiomeric excess of the initial water content of the mixture, the alcohol volume used, the reaction temperature level achieved, and the mass of biocatalyst chosen are discussed in detail. In an attempt to find the best conditions for the reaction, the effect of each parameter is assayed keeping all other variables constant at the best values previously determined.

Effect of water content

Water content is an important factor affecting both the activity and enantioselectivity of lipases. Literature dealing with lipase-catalyzed esterifications performed in non-aqueous medium agrees on the fact that even if the reaction medium can be an organic solvent or a mixture of substrates, water is absolutely essential to keep the enzyme active. Moreover, it is now widely recognized that enzyme flexibility increases with increasing hydration levels, and it has been experimentally confirmed that the enhancement in enzyme activity usually observed upon addition of low amounts of extra water is related to an increase in enzyme flexibility.[22] Regarding the effect of water content on enzyme enantioselectivity the work of Broos *et al.*,[22] – a time-resolved fluorescence anisotropy study on the molecular flexibility of active-site labeled enzymes suspended in organic solvents – gave the first experimental evidence that enzyme flexibility and enzyme enantioselectivity are also correlated. However, published literature still shows different opinions on whether the increased flexibility of hydrated enzymes may increase or decrease enzyme enantioselectivity. The experimental work of Broos proved that enzyme flexibility in the order of (sub)-nanoseconds enables the rapid sampling of a large repertoire of enzyme conformations, thus enhancing the probability of reaching a conformational state capable of binding and converting an enantiomeric substrate.[22] Flexibility of the enzyme induced by protein hydration appears essential to maximize favorable interactions with the substrate. On the other hand, some reports agree on the fact that an enzyme with more conformational flexibility caused by a higher hydration level has less possibility to discriminate between the two enantiomers.[23]

In the case of (R,S)-ibuprofen resolution, the effect of water content on reactions performed in organic solvent media has been studied by a number of authors.[7,9,24,25] Among others Xie *et al.*[24] studied the effect of water added to enzyme activity and enantioselectivity for the esterification of racemic ibuprofen with *n*-butanol catalyzed by *Candida rugosa* lipase in several hydrophobic solvents. Authors found that lipase could be dramatically activated by adding a very small amount of water, and that activation was highly enantioselective for the esterification of (S) ibuprofen. In that contribution, for all solvents assayed profiles

 © 2009 Society of Chemical Industry

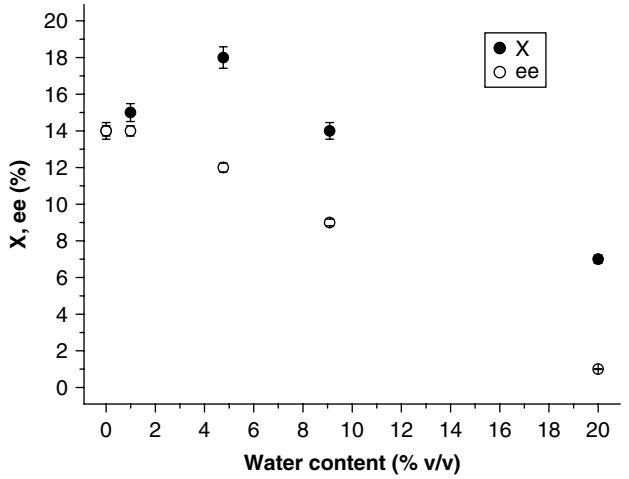


Figure 1. Effect of initial water content (*W*) on total (R,S)-ibuprofen conversion (*X*) and enantiomeric excess of substrate (*ee*). 0.5 g ibuprofen, 20 mL ethanol, 100 mg Novozym 435, $T = 45\,^{\circ}$C, 200 rpm, 72 h.

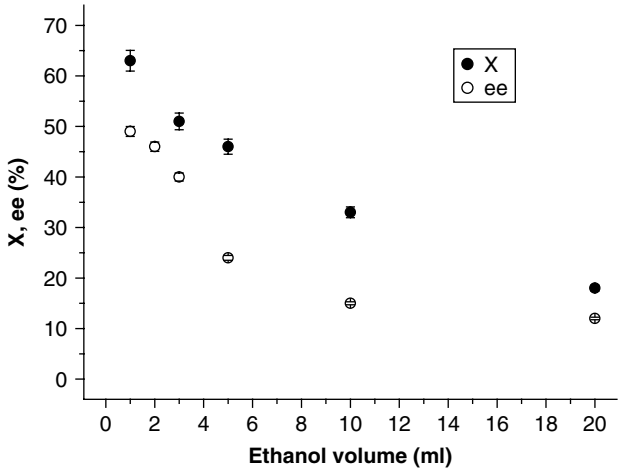


Figure 2. Effect of ethanol volume on total (R,S)-ibuprofen conversion (*X*) and enantiomeric excess of substrate (*ee*). 0.5 g ibuprofen, 4.8% v/v water, 100 mg Novozym 435, $T = 45\,^{\circ}$C, 200 rpm, 72 h.

of both racemate conversion and enantioselectivity with water content were notably similar with a marked optimum for added water contents of around 0.1%.[24]

Figure 1 shows the effect of water on conversion and enantiomeric excess achieved in the esterification of (R,S)-ibuprofen with ethanol in the absence of added organic solvent after 72 h of reaction. In terms of total acid conversion to ester, a maximum at a water content equal to 4.8% is observed. This behavior is typical of lipase-catalyzed esterifications in which a certain degree of lipase hydration has a positive effect on biocatalyst flexibility and therefore on its activity. On the other hand, since water is a by-product of the reaction, its addition promotes the reverse reaction: the hydrolysis of the ester formed. Then, the optimum aqueous content of lipase-catalyzed esterifications results from a compromise between the positive influence of water on lipase activity, and the reduction of the net rate of the synthesis by ester hydrolysis. Moreover, it has been proposed that water may form dead-end complexes with lipase, rendering it irreversibly deactivated.[26] As will be further illustrated by molecular modeling results, deactivation reactions may also account for the reduction of the esterification activity at high water contents.

In terms of enantiomeric excess data included in Fig. 1 reveals that for initial water contents in the 0–4.8% v/v range, water has no significant effect on selectivity. However, for increasing water contents the enantiomeric excess of substrate is severely reduced. These results are in line with data from Pepin and Lortie[9] and also with data from Trani *et al.*[10], who found that for resolution of (R,S)-ibuprofen in solventless medium catalyzed by Novozym 435 low water activities induced higher enantioselectivity. In a compromise between achieving high enantiomeric excess while keeping the biocatalyst active, for further experiments the initial water content was set at $W = 4.8\%$ v/v.

Effect of alcohol volume

In enzymatic stereoselective esterification reactions, alcohols act as a nucleophile and their concentration is known to affect reaction rate and enantioselectivity.[27] Thus, alcohol concentration is a key parameter to consider in lipase-catalyzed resolutions, and it has been an issue usually considered in parametric analysis of the enzymatic resolution of (R,S)-ibuprofen.[5,6,8] In the present contribution initial experiments in which the effect of water was assayed were performed in a medium containing 20 mL of ethanol. Such a high amount of ethanol (molar ratio of ethanol to ibuprofen = 141), was selected in order to compare with previous experiments made in isooctane medium, which required 20 mL of solvent in order to solubilize racemic ibuprofen (data not shown). However, the pharmaceutical was shown to be much more soluble in ethanol, which allowed operation in homogeneous medium for alcohol amounts of even 1 mL (molar ratio of ethanol to ibuprofen = 7). At a fixed amount of (R,S)-ibuprofen (0.5 g), data for enantioselective esterification reactions performed in systems with 1, 2, 3, 5, 10 and 20 mL of ethanol (which implies that the molar ratio of ethanol to ibuprofen was set at 7, 14, 21, 35, 71 and 141, respectively), are shown in Fig. 2.

Data included in Fig. 2 reveal that total conversion to ester is gradually increased when the ethanol volume is reduced from 20 mL to 1 mL. The well-known inhibitory effect of alcohols on lipase activity is reduced when lower volumes of ethanol are used, leading to higher reaction conversions. Formation of dead-end complexes between lipase and ethanol at high alcohol concentrations is proposed. The thermodynamic feasibility of competitive inhibition is analyzed for the different reactions proposed later on.

At this stage it must also be pointed out that, of course, increasing the volume/mass of a reactant (in this case ethanol), indirectly leads to additional effects caused by the concomitant modification of the concentration of the other compounds involved in the reaction medium (i.e. ibuprofen, biocatalyst). Even though the analysis of alcohol effects is focused on its inhibitory effect, lateral effects of ethanol addition must at least be pointed out. In detail: let's keep the idea – based on the well-known inhibitory effect of alcohols on lipase activity – that the main effect of ethanol is inhibitory (let's call this effect 1). Besides, upon increasing the amount of ethanol, dilution of ibuprofen concentration undoubtedly takes place (effect 2); and dilution of the biocatalyst (mass kept constant at 100 mg) also occurs (effect 3). These last two effects may be considered as 'negative effects' for the advance of the esterification reaction, which, together with lipase inhibition, contribute to a lower reaction rate (lower concentration of reactant and of the biocatalyst), and justify the lower conversions achieved at high ethanol volumes.

 © 2009 Society of Chemical Industry

On the other hand, there are also some other effects of the ethanol volume increment, which can be considered as 'positive' for promoting the esterification reaction; such as dilution of the inhibitory effect of the acidic moieties of ibuprofen on biocatalyst activity (effect 4), and the shift of equilibrium towards esterification products by addition of a reactant in high excess (effect 5). In the case of the amount of water added, the percentage of water was kept constant with respect to the total volume of the reaction mixture, so, in this case, added water is not diluted by the increasing volumes of ethanol used. However, the ratio of water volume to lipase weight is actually increased (the amount of water is increased to keep its percentage constant in relation with the total reaction volume, and the amount of catalyst is always the same), and therefore a potential and indirect additional effect of ethanol volume increment, related to the increase in the water/biocatalyst ratio may also be considered (effect 6).

Regarding the enantioselectivity of the process, in Fig. 2 the enantiomeric excess of substrate shows a profile similar to the one obtained for ibuprofen conversion, significantly increasing for the lower amounts of alcohol assayed. These results are in a good agreement with those reported in a number of published articles on (R,S)-ibuprofen esterification which present an enantiomeric excess profile with alcohol concentration that resembles conversion or initial rate profiles.[6,8,27]

Considering the analysis presented above, in a direct or indirect form, the increase of ethanol volume in the system increases the inhibitory or deleterious effects exerted by the alcohol. Additional effects reported by other authors such as the stripping of water from the active site by the ethanol,[28] and the dilution of an allosteric potential effect of ibuprofen using higher ethanol volumes,[29] may also contribute to the parallel decrease of enantioselectivity and activity experimentally observed for higher contents of ethanol. For further experiments alcohol volume was set at 1 mL.

Effect of reaction temperature

At the optimum conditions of ethanol and water contents discussed in the previous section, the reaction was performed at 37 °C and 55 °C. Higher reaction temperatures were not assayed since they caused too much ethanol evaporation making it difficult to keep the reaction volume constant. The results in terms of total conversion and enantiomeric excess are shown in Fig. 3. It can be observed that the highest activity of the biocatalyst is obtained at 45 °C with a total ester conversion of 63%. At 55 °C ibuprofen conversion towards the ester shows a slight reduction with respect to that at 45 °C, which illustrates the increase in the deactivation rate shown by enzymes at high temperatures. On the other hand, at 55 °C the enantiomeric excess shows a much more abrupt decrease, with a reduction in ee of almost 10 points. Enantioselectivity of lipases is known to be reduced by high temperatures, which promote deformation of the active site. Enhanced flexibility of the active site reduces the ability of the biocatalyst to distinguish between enantiomers leading to a decrease in the observed enantioselectivity. A quick look at the literature regarding lipase-catalyzed resolution of NSAIDs illustrates the effect of temperature on the enantioselectivity shown by lipases of different origin.[7,8,17] In the work by Ong *et al*.[17] enantioselective esterification of racemic ketoprofen catalyzed by free CALB enantioselectivity increased with increasing reaction temperature up to 40 °C. When the temperature was increased to 45 °C enantioselectivity showed an abrupt decrease. In the work by Won *et al*.[8] enantioselectivity of *Candida rugosa* lipase in (R,S)-ibuprofen esterification in isooctane also decreased significantly

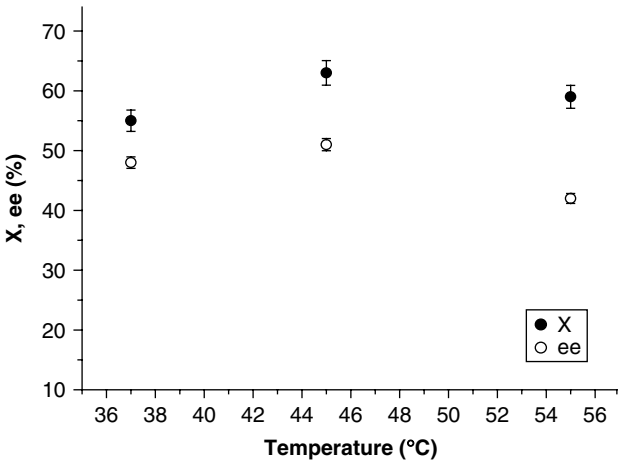


Figure 3. Effect of temperature on total (R,S)-ibuprofen conversion (*X*) and enantiomeric excess of substrate (*ee*). 0.5 g ibuprofen, 1 mL ethanol, 4.8% v/v water, 100 mg Novozym 435, 200 rpm, 72 h.

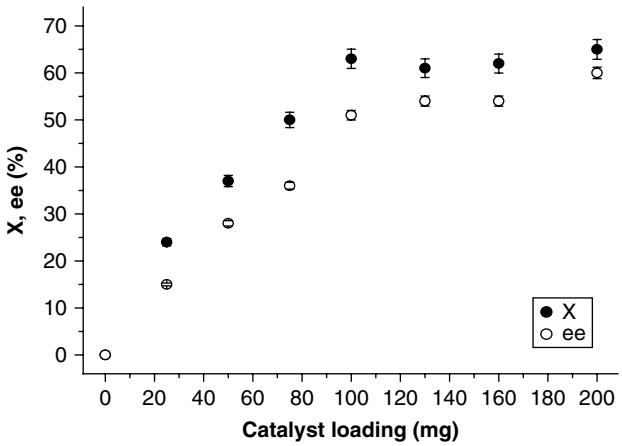


Figure 4. Effect of the mass of biocatalyst on total (R,S)-ibuprofen conversion (*X*) and enantiomeric excess of substrate (*ee*). 0.5 g ibuprofen, 1 mL ethanol, 4.8% v/v water, $T = 45$ °C, 200 rpm, 72 h.

for increasing temperatures, finding an optimum at 30 °C (the lowest temperature level assayed). Finally, for the reaction catalyzed by Lipozyme IM (containing the lipase form *Rhizomucor miehei*) Lopez-Belmonte *et al*.[7] report an inverse relationship between the enantioselectivity factor and temperature.

Effect of biocatalyst loading

The influence of the amount of Novozym 435 used on conversion and enantiomeric excess is shown in Fig. 4. As can be observed, both total conversion to esters and enantiomeric excess increase linearly with the mass of biocatalyst up to a mass of 100 mg. For higher amounts of biocatalyst no significant increase in conversion was observed, leading to lower productivity of the catalyst, measured as the specific activity (mmol ibuprofen esterified mg^{-1} biocatalyst h^{-1}). Regarding the enantiomeric excess of the process, ee continued increasing for biocatalyst masses higher than 100 mg, reaching a maximum value of 60% for the reaction catalyzed with 200 mg of Novozym 435. No ester formation was detected in the absence of enzyme. Due to the high catalyst loading used in the reaction, reuse of the Novozym 435 was attempted in order to reduce biocatalyst cost.

 © 2009 Society of Chemical Industry

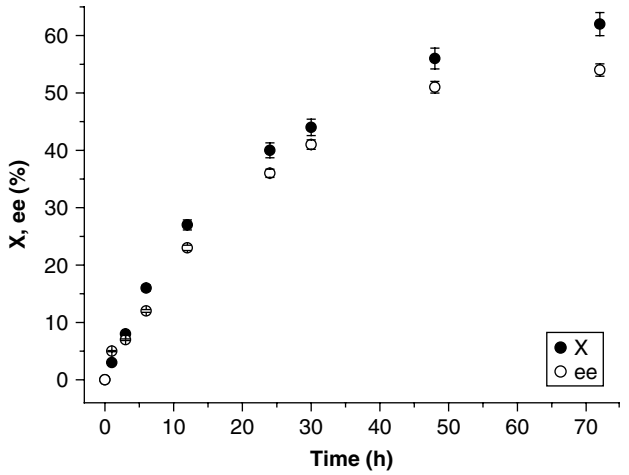


Figure 5. (R,S)-ibuprofen enantioselective esterification course at optimum conditions determined. 0.5 g ibuprofen, 1 mL ethanol, 4.8% v/v water, 160 mg Novozym 435, $T = 45\,^{\circ}C$, 200 rpm.

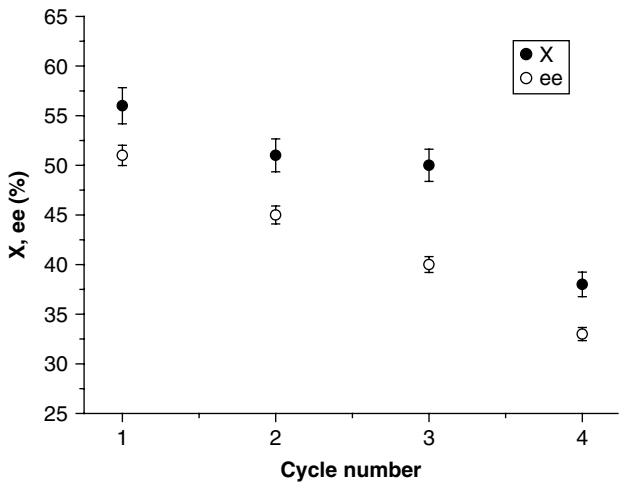


Figure 6. Biocatalyst reuse. 0.5 g ibuprofen, 1 mL ethanol, 4.8% v/v water, 160 mg Novozym 435, $T = 45\,^{\circ}C$, 200 rpm, 48 h.

Reaction kinetics

In the best conditions determined in the parametric analysis (0.5 g ibuprofen, 1 mL ethanol, 4.8% v/v water, 160 mg Novozym 435, $T = 45\,^{\circ}C$, 200 rpm) reaction conversion and enantiomeric excess was determined as a function of time. Kinetic data are included in Fig. 5.

As it is shown in Fig. 5, the enantioselective esterification of racemic ibuprofen performed in excess of ethanol without additional organic solvents, proceeds at a high rate in spite of the excess of alcohol employed. In fact, conversion to ester values are very close to those found for a similar process carried out in organic media.[18] In terms of the enantioselectivity of the reaction, the enantiomeric excess evidences a continuous increase with reaction time, achieving a value of 54% in 72 h of reaction. Comparison with reports of (R,S)-ibuprofen resolution catalyzed by Novozym 435 and performed in organic solvent medium shows that both higher[18] and lower[30] enantiomer excess/enantioselectivity values have been reported. Novozym 435 has also been used for (R,S)-ibuprofen resolution in solvent-free medium, which required temperature levels above $70\,^{\circ}C$.[9–12] In those conditions, applying vacuum in order to remove water formed during the reaction, authors report having achieved an enantiomeric excess of 95%,[12] and of 85% when the process was scaled-up to 100 g.[10]

Reuse experiments

Reuse of Novozym 435 was assayed in order to check if in the system with 1 mL of ethanol, alcohol impelled a drastic inhibition on the biocatalyst that might deteriorate its activity or enantioselectivity. Figure 6 shows reuse data after several 48 h uses of Novozym 435. Assay conditions were those determined as optimum (0.5 g ibuprofen, 1 mL ethanol, 4.8% v/v water, 160 mg Novozym 435, $T = 45\,^{\circ}C$, 200 rpm). Data included in Fig. 6 suggest that when 1 mL of ethanol is used (molar ratio of ethanol to ibuprofen = 7), the alcohol medium does not impose a drastic inhibition on Novozym 435, which after four uses still retains 68% of its initial activity. Regarding the enantioselectivity of the reused catalyst, data shows a gradual decrease with an enantiomeric excess of 65% of that attained in the first use. In terms of enantiomeric excess, the decrease registered might be due to the enhanced flexibility of the active site of lipase induced by water, temperature and ethanol contact during previous uses.

MOLECULAR MODELING OF (R,S)-IBUPROFEN ESTERIFICATION

As has been mentioned in the introduction, in this section experimental data from the enantioselective esterification of (R,S)-ibuprofen is discussed in terms of molecular modeling results. Structure–function relationship studies by means of molecular graphics and molecular modeling contribute to the understanding of coordination steps, reaction pathways and inhibition reactions in lipases. In the case of lipase-catalyzed enantioselective reactions, the study of the binding of the different enantiomers of chiral substrates has given several clues about the enantioselectivity preferences of lipases using esterifications or transesterifications as model reactions.[14]

In the following sections the enantiopreference of lipase from *Candida antarctica* B (CALB) towards the (R) enantiomer of ibuprofen is studied considering the different steps of the 'Ping Pong Bi Bi' mechanism of reaction, with special attention to the coordination and reaction of each enantiomer at the catalytic triad of CALB. Formation of dead-end complexes between (a) the catalytic triad and water/ethanol prior to ibuprofen coordination, and (b) the catalytic triad and ethanol after the acyl-enzyme formation step, are also considered. The dead-end complexes formed are analyzed considering published literature on competitive inhibition exerted by solvents, alcohols and water.[26,31–33]

Different aspects of the modeling will be discussed taking into account the experimental findings reported in the previous section, together with the most recent molecular modeling literature on the topic.

Methodology

Enzyme structure

The crystal structure of *Candida antarctica* lipase B was obtained from the Protein Data Bank.[34] Lipase from *Candida antarctica* B consists of 317 amino acid residues and has an α/β hydrolase fold. The catalytic triad of CALB is formed by Serine 105, Histidine 224 and Aspartic Acid 187.[34]

Reaction pathway calculation

The reaction pathway of the esterification of (R,S)-ibuprofen with ethanol was modeled following the Ping Pong Bi Bi mechanism.

 © 2009 Society of Chemical Industry

For clarification purposes the conformations for which steric energies were calculated – following the steps of the Ping Pong Bi Bi mechanism – have been labeled according to the following: (1) ibuprofen and ethanol far away from each other and from the enzyme; (2) tetrahedral intermediary I with coordination of ibuprofen: (3) acyl-enzyme and water near the active site; (4) tetrahedral intermediary II with coordination of ethanol and water near the active site; (5) resulting ester near the catalytic triad and water near the active site; (6) ester far away from water and from the enzyme. The tetrahedral intermediary I includes the coordination of the hydrogen of the Serine to the basic nitrogen of the Histidine and to the hydroxyl of the ibuprofen. Moreover, the tetrahedral intermediary I (conformation 2) may have the ethanol close to the intermediary or far away from the active site. The identification of one of the oxygens as an alkoxide, with interaction with the oxyanion hole and of the oxygen from the hydroxyl of the acid was included. The tetrahedral intermediary II includes the coordination of the hydrogen of ethanol to the basic nitrogen of Histidine, as well as the oxygen from ethanol to the tetrahedral carbon.

Following these steps and taking into account all the molecular aspects of the active site of CALB, Molecular Mechanics Version 2 (MM2) from Chem3D- Cambridge Soft was used to model the complete active site and tunnel of *Candida antarctica* lipase B and the complete molecules of ethanol and ibuprofen (with the different binding modes (I and II) of both enantiomers (R and S)). Residues included were those selected by Raza *et al*.[35] (in bold the residues of the catalytic triad): 38–42, 47, 73, 104–109 (**105**), 132–134, 138, 140, 141, 144, 150, 151, 153, 154, 157,**187**,188–190, **224,** 225, 278, 281, 282, 285. At this level, the role of ethanol presence at the coordination of ibuprofen was modeled to evaluate the importance of Van der Waals forces as well as H-bonding effects.

In the case of the modeling of inhibition reactions caused by ethanol or water the Parameterizer Model Revision 3 (PM3) was used to obtain the ΔH_f for the initial and final states of the reactions proposed. For this level of modeling the catalytic triad and the oxyanion hole from *Candida antarctica* lipase B were considered. Inhibition reactions with water or ethanol before the coordination of the ibuprofen, and after the acyl-enzyme formation were both analyzed.

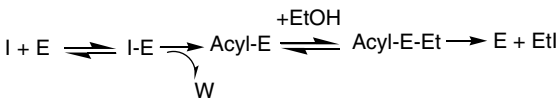
Reaction enantioselectivity calculation

Enantioselectivity is related to the difference in the free energy gaps between the ground states of the enantiomers and the corresponding transition states of the enantiomers after reaction with the enzyme's catalytic triad. Since the thermodynamic ground states of the enantiomers are the same, enantioselectivity is determined by the difference in free energy between the two transition states.[14] According to Burkert *et al*.[36] the translational and rotational terms of the Gibbs free energy cancel to a very good approximation when the energy difference between two isomers is computed. Moreover, as reported by Hæffner *et al*.[14] the force field energy (steric energy) gives a good approximation of the enthalpic part of the Gibbs free energy; and the difference in steric energy is close to the free energy difference in enzyme configurations with substrates where the entropy contribution is of minor importance to the enantioselectivity. With a base on the work of Hæffner, Raza *et al*.[35] presented different subsets of parameters to calculate potential energies: function-based; structure based and energy based; but in the context of a Molecular Dynamics study which is not what we present in this contribution. In the current approach complexes with the most likely substrate configurations were preselected for energy minimization considering the published literature on the topic. In view of the previous it must be pointed out that results included in the following sections give evidence of the relevance of the interactions of the enantiomers with ethanol, but are limited by the number of configurations in the substrate and intermediaries that were considered.[37]

The reaction mechanism

As described earlier, MM2 was applied to each step of the mechanism of the (R,S)-ibuprofen esterification catalyzed by immobilized CALB. Scheme 1 depicts the mechanism of the synthesis. In general terms – without considering inhibition by substrates or water- the mechanism involves the reaction of CALB with ibuprofen to yield a lipase–ibuprofen complex (the tetrahedral intermediary I), followed by the transformation of the tetrahedral intermediary I into the acyl-enzyme and water release. After the acyl-enzyme is formed the second substrate (ethanol) interacts with the acyl-enzyme to produce a ternary complex (the tetrahedral intermediary II) which finally yields ethyl-ibuprofen and the free lipase. The mechanism described is shown in Scheme 1 where I, E, W, Et, EtI account for ibuprofen, the enzyme, water, ethanol and the ethyl–ibuprofen ester, respectively.

Taking into account the racemic nature of ibuprofen the molecular modeling of the reaction mechanism needs to consider the different binding of the two enantiomers in the active site of lipase. Figure 7 shows the structures of both enantiomers of ibuprofen. Considering the spatial distribution of the ligands around the chiral carbon of ibuprofen, a small ligand S (in this case the hydrogen), a medium ligand M (the CH_3 group), and a large ligand (L) (the substituted phenyl group or Ph) can be identified, following the proposal of Haeffner *et al*.[14] for secondary alcohols. Considering this distribution of groups according to their relative size, Orrenius *et al*.[38] analyzed the binding of secondary alcohols in the active site of CALB during transesterification reactions. In the work mentioned, the authors postulated the existence of two different orientations for enantiomers binding in the active site of lipase. In mode I the large group of the enantiomer points out from the active site, whereas in mode II the large



Scheme 1. Ping Pong Bi Bi mechanism for (R,S)-ibuprofen esterification with ethanol.

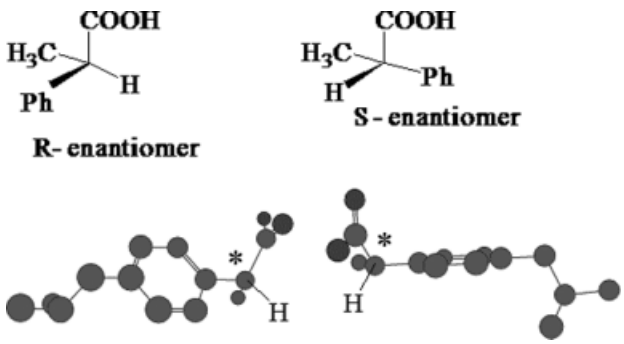


Figure 7. Structures of (R) and (S) enantiomer of ibuprofen.

 © 2009 Society of Chemical Industry

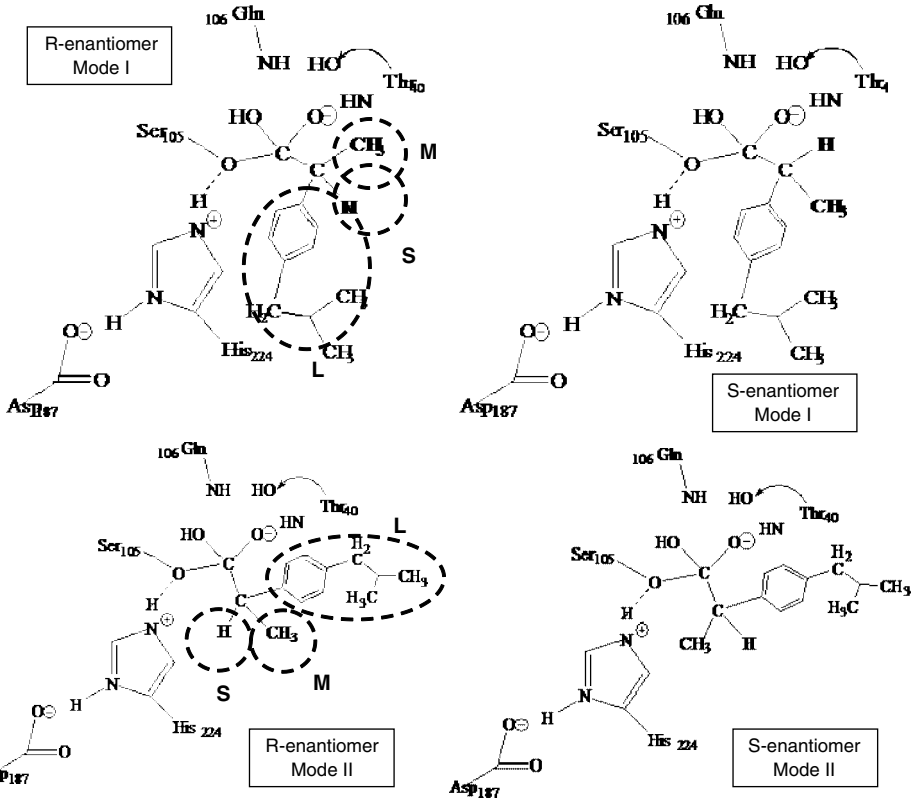


Figure 8. Binding modes for (R) and (S) enantiomer of ibuprofen at the active site of CALB.

group points into the interior of the active site. According to the authors, when the large group is larger than ethyl, the selectivity increases dramatically, justifying the high enantioselectivity of CALB for secondary alcohols. In line with the previous proposals, two possible orientation modes for the binding of ibuprofen enantiomers at the tetrahedral intermediary I are shown in Fig. 8.

Steric energies involved in the reaction mechanism have been be calculated considering the four configurations binding possibilities described (R and S for modes I and II) by steric energy minimization until a cutoff was reached (0.1 kcal mol^{-1}). Figure 9 shows the steric energies involved in each step of the mechanism, with ethanol present since step 1 in the neighborhood of the active site. Figure 9 shows that the pathway for reaction of the (R) enantiomer in mode I is the most favored in steric energy terms. Besides, molecular mechanics results show that the intermediary II formation step is the key for the reaction to take place. The differences in steric energy found between the tetrahedral intermediary II (step 4) and the acyl enzyme (step 3) were +19.4 and +54.9 kcal mol^{-1} for the (R) and (S) mode I, and +53.1 and +39.5 kcal mol^{-1} for the (R) and (S) mode II, respectively. Based on the previous considerations it appears that the sequence for the reaction to take place is: R mode I > S mode II > R mode II > S mode II. The difference between the steric energy of the (R) enantiomer and the steric energy of the (S) enantiomer at the intermediary II step (step 4) resulted in −64.6 kcal mol^{-1} for mode I and + 20.3 kcal mol^{-1} for mode II, explaining the enantiopreference for the (R) enantiomer in the case of the mode I. These results are in line with the conclusions of Orrenius *et al.*[38] who found that productive bindings of enantiomers of secondary alcohols were those including the mode I of the (R) enantiomer and the mode II of the (S) enantiomer.

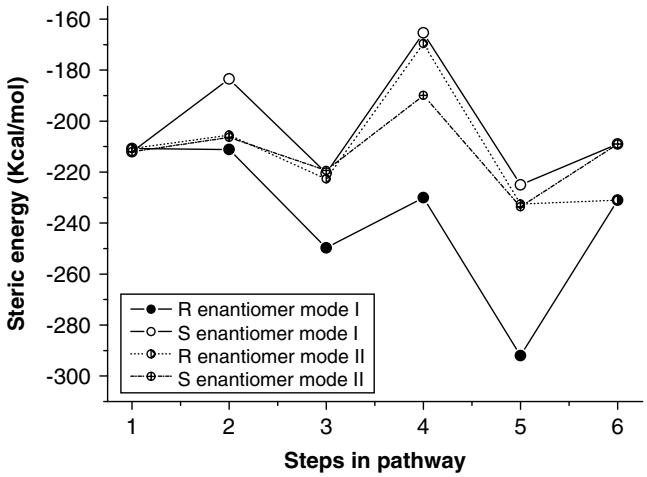


Figure 9. Evolution of the steric energy involved in the mechanistic steps of (R,S)-ibuprofen esterification with ethanol catalyzed by CALB.

When the presence of ethanol was assayed, molecular mechanics results showed that for the (R) enantiomer and for both binding modes, the presence of ethanol was favorable to step 2 in steric energy terms (data not shown). However, in the case of the (S) enantiomer, the presence of ethanol in the neighborhood of the catalytic triad increased the steric energy for mode II and decreased the steric energy for mode I tetrahedral intermediary I formation. Looking again at Fig. 8 it follows that for both modes the (R) enantiomer sterically interacts with ethanol through its hydrogen from the chiral carbon. In the case of the (S) enantiomer,

 © 2009 Society of Chemical Industry

for both coordination modes it is its methyl group from the chiral carbon which interacts with ethanol.

Competitive inhibition by ethanol/water

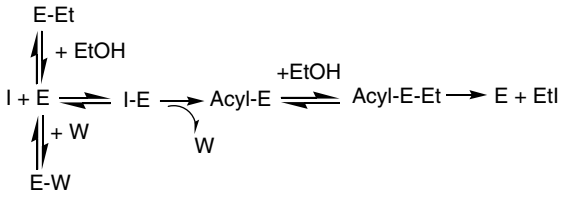
Competitive inhibition has been proposed, exerted by solvent molecules that bind into the active site as the normal enzyme substrate.[26] When competitive inhibition takes place, the substrate molecule cannot enter the active site while the inhibitor is there. This effect leads to a lower apparent affinity of the substrate to the binding site, which is evidenced by increased values of the dissociation constant (K_D) and the Michaelis–Menten constant (K_M).

In recent years several articles have discussed the role of alcohols and/or water as competitive inhibitors in lipase-catalyzed esterifications, alcoholysis and transesterifications.[26,31–33] Many of them studied the influence of water on kinetic parameters of a Ping Pong Bi Bi mechanism, and results showed that water raises K_M values for alcohol by acting as a competitive inhibitor of the nucleophile alcohol. In the alcoholysis of methyl propionate and n-propanol catalyzed by immobilized lipase B from *Candida antarctica* in a continuous solid/gas reactor, it was determined that alcoholysis kinetics fitted a Ping Pong Bi Bi mechanism with dead-end inhibition by the alcohol. Water was also found to act as a competitive inhibitor of methyl propionate with a higher inhibition constant than n-propanol.[31] More recent work by the same authors confirmed the formation of dead-end complexes between the alcohol and lipase and water and lipase, determining that the latter occurs after optimal hydration level for catalysis has been achieved ($a_w > 0.1$).[26] In the synthesis of dodecyl decanoate in hexane catalyzed by immobilized forms of lipases from *Rhizomucor miehei* and *Candida rugosa*, Valivety *et al*.[32,33] noticed that an increase in water activity led to an increase in the dissociation constant of the enzyme for the acyl substrate; concluding also that water acted as a competitive inhibitor.

Considering the previous references together with the experimental data obtained for the effect of water and alcohol concentration on (R,S)-ibuprofen esterification, some possibilities of competitive inhibition of lipase from *Candida antartcica* B exerted by ethanol and water have been proposed and analyzed using molecular modeling methods (PM3). Formation of lipase–water and lipase–ethanol dead-end complexes has been considered. As reported by Uppenberg *et al*.,[34] the region around the catalytic Serine (Ser-105) is polar in nature so the hydroxyl of the alcohol or of water may bind to this region to form the dead-end complex. Figures 10 to 12 present the different situations related to inhibition that will be discussed in the following sections.

Formation of dead-end complexes prior to ibuprofen coordination

Molecular modeling of competitive inhibition reactions implies considering that inhibitors (in this case ethanol and water) are present from the start in the neighborhood of the catalytic triad (instead of considering them far away from the catalytic triad until alcohol coordination). Given the highly polar nature of the environment of the catalytic triad the stabilization/desestabilization effects of an H-bonding network should be analyzed with care when water and other polar substrates such as short length alcohols at high concentrations (or activities) are used. The stabilizing effects of multiple H-bonding caused by water/ethanol at the substrate coordination step, which contribute to lowering the intermediates and the transition states energy, have previously been reported.[39] H-bonding networks have been used in enzymatic catalysis to understand several aspects.[40] In the references mentioned, the presence of only one water molecule between the Serine and Histidine was considered. In the current analysis the impact of a higher number of water molecules in the neighborhood of the active site, or coordinated as a network to the catalytic triad, is evaluated.



Scheme 2. Ping Pong Bi Bi mechanism for (R,S)-ibuprofen esterification with ethanol. Formation of dead-end complexes with water and ethanol prior to ibuprofen coordination.

Scheme 2 depicts the mechanism of the synthesis of ethyl-esters of ibuprofen catalyzed by CALB, considering inhibition by water and/or ethanol prior to the formation of the tetrahedral intermediate I. The terms E-Et and E-W account for the dead-end species.

Figure 10 illustrates the reactions proposed for ethanol with the Serine and Histidine residues of the catalytic triad of CALB prior to ibuprofen coordination. Formation of dead-end complexes might be promoted by high ethanol contents as described earlier. Results from PM3 molecular calculations without coordinated ibuprofen revealed that the reaction of ethanol with Serine (initially with a hydroxyl group) has an overall enthalpy change of +4.8 kcal mol^{-1}, whereas the reaction with Histidine to provide the NH-CHOEt- moiety has a reaction enthalpy of +0.2 kcal mol^{-1}. If the reaction is supposed to provide the N-OEt moiety as product, the reaction is strongly unfavored from the enthalpic point of view, with an overall enthalpy change of more than +40 kcal mol^{-1}.

In the case of the modeling of water inhibition, we hereby propose the presence of a network of H-bonding water pool near the active site reaction which may interact with the Histidine residue. This situation would operate at high water contents of the reaction media. In the modeling performed, only two water molecules at specific configurations were considered. As illustrated in Fig. 11, in the first place the proposed inhibiting reaction implies the disruption of the Serine–Histidine close interaction through the hydrogen from Serine. After that first step two reactions seem possible. The first one is the formation of N-H—COHH in the Histidine. The second one is the formation of N-OH-CH_2 in the Histidine. Both reactions take place by water addition to a double bond of the Histidine. PM3 calculations revealed that the formation of N-H–COHH moiety in the Histidine is favored by −8.4 kcal mol^{-1}. On the other hand, formation of N-OH-CH_2 is not sterically favored, with an overall enthalpy change of +39.6 kcal mol^{-1}. Reaction of water with the Serine residue is discarded because no products other than Serine and water are possible.

Formation of dead-end complexes after ibuprofen coordination and acyl-enzyme formation

The reactions shown in Figs 10 and 11 are the inhibition reactions that may take place before coordination of the ibuprofen. However, we hereby propose the existence of inhibition reactions that may take place after the formation of the acyl-enzyme which may also stop the esterification pathway (Scheme 3).

 © 2009 Society of Chemical Industry

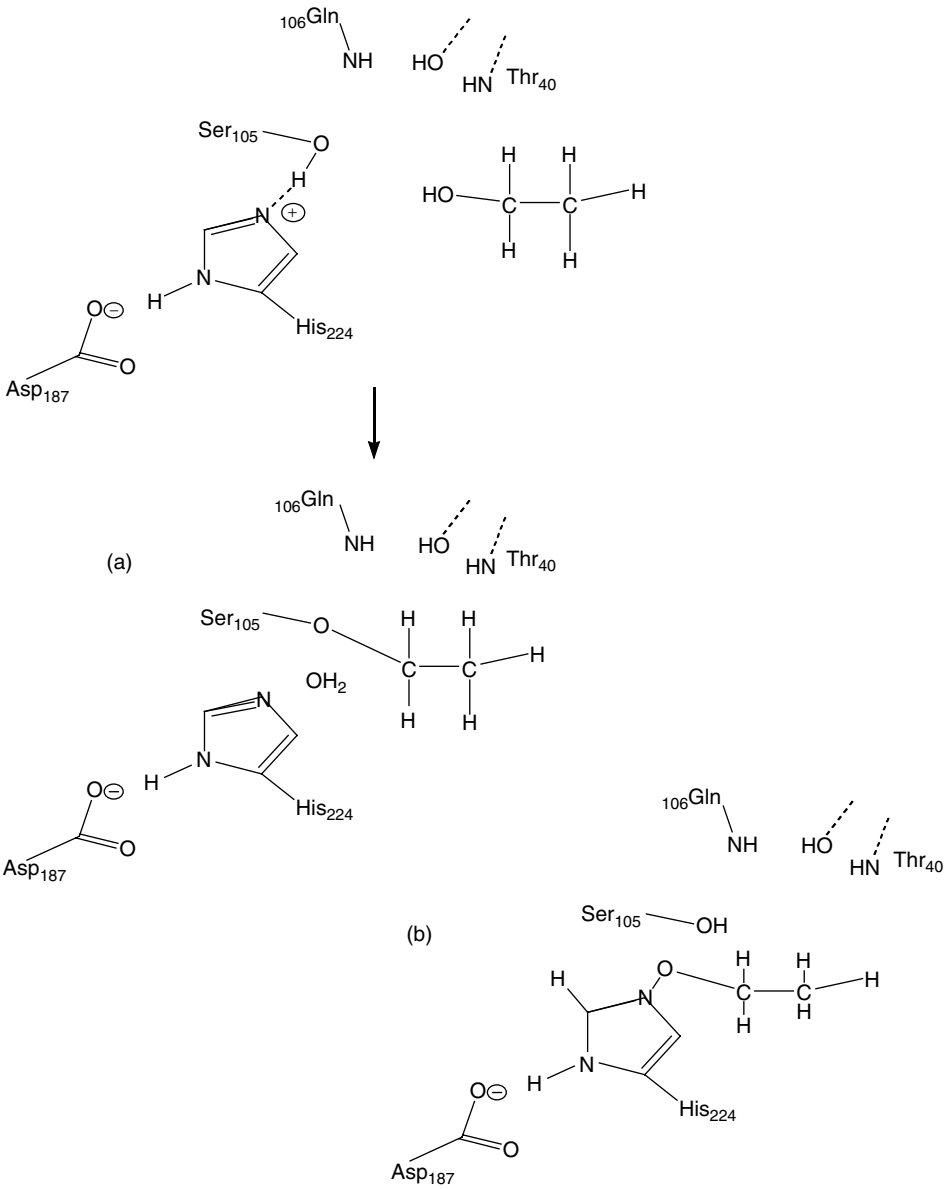
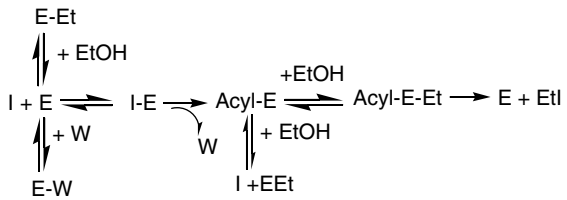


Figure 10. Inhibition by ethanol prior to ibuprofen coordination. Formation of dead-end complexes due to reaction of ethanol with aminoacids of the catalytic triad. (a) Reaction with Serine; (b) reaction with Histidine.



Scheme 3. Ping Pong Bi Bi mechanism for (R,S)-ibuprofen esterification with ethanol. Formation of dead-end complexes with water and ethanol.

In the case of water, once the acyl-enzyme has been formed the hydrophobic phenyl group severely hinders the access of water to the catalytic triad, reducing the probability of lipase inhibition. The results of Graber *et al.*[26] suggest that the impact of water on the formation of the AcylE-Et is not important. Different findings provided evidence for a 'shielding effect' protecting the acyl intermediate from its environment, where the organic solvent interferes very little with the enzyme for this particular intermediary.[26]

Regarding ethanol inhibition once the acyl-enzyme is formed, we propose the generation of addition reactions of ethanol to double bounds present in Histidine from the catalytic triad. PM3 results of the calculation including the active site and the oxyanion hole show that reaction would be favored for (R) and (S) enantiomers, which coordinate in mode II since in that case ethanol coordination is sterically unhindered (Fig. 12). In the case of the coordination of enantiomers in mode I the shielding effect exerted by the hydrophobic phenyl group would limit the access of ethanol to the Histidine residue, reducing the probability of the formation of a dead-end complex (Fig. 12). The approach of ethanol to the acyl enzyme to form the tetrahedral intermediary II would also be at first sight hindered for the mode I. However, the approach of ethanol by the less hindered side – away from the phenyl

 © 2009 Society of Chemical Industry

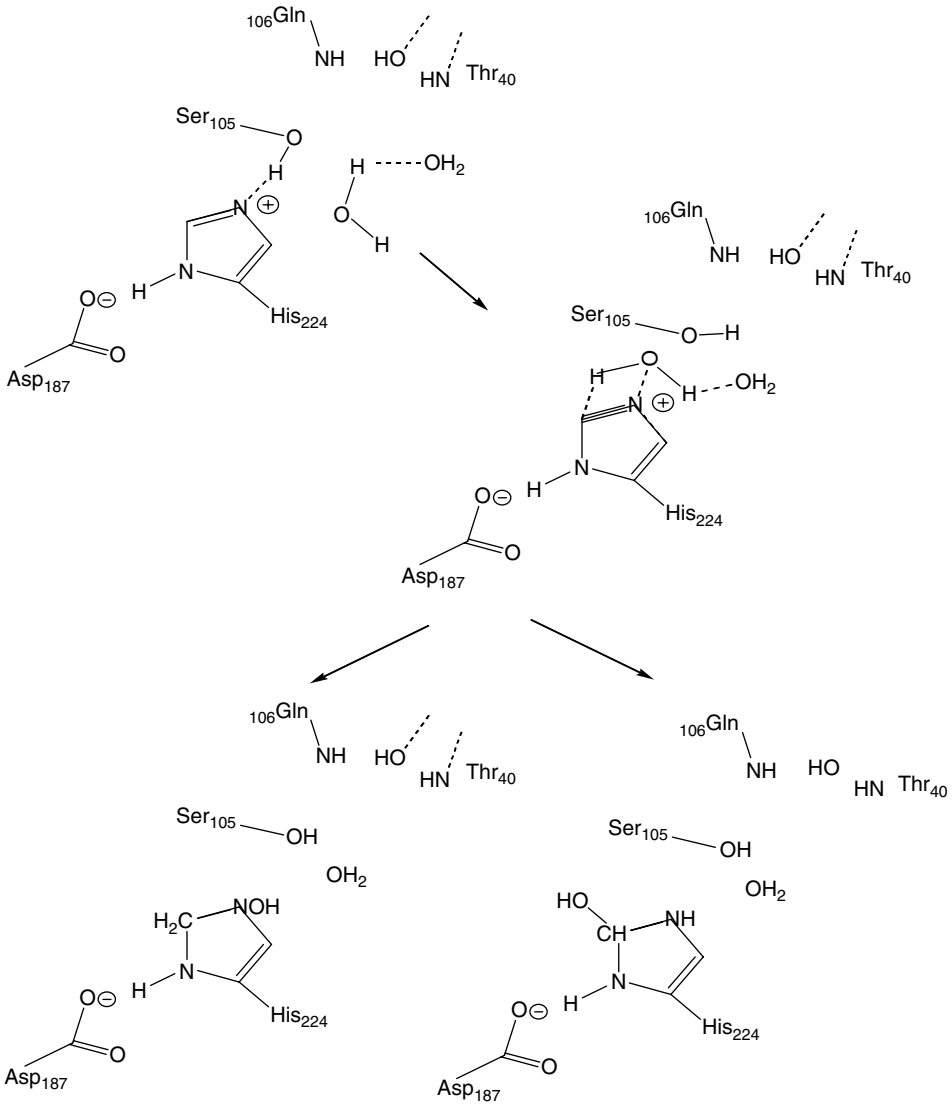


Figure 11. Inhibition by water prior to ibuprofen coordination. Formation of dead-end complexes due to reaction of water with Histidine from the catalytic triad.

group, going 'under' this group to the acyl enzyme by the 'alcohol pocket' seems possible. The alcohol pocket includes the following residues: Leu 144, ILe 189Ala 282 Leu 278 and V 286. These residues are situated away from Histidine and forming a 'bed' for the placement of the ethyl group of the alcohol. In this case, the hydroxyl of ethanol placed protruding into the acyl enzyme is not hindered.[37] In terms of the energy involved in the reactions, proposed PM3 results show that the formation enthalpy of the dead-end complex with a NH-CHOEt moeity present at the Histidine 224- when Serine is bonded to an acyl enzyme model (CO-CH3) is endothermic in near 31 kcal mol^{-1} (PM3) at this step, whereas it is 0.2 kcal mol^{-1} before ibuprofen coordination. The inhibition reaction with ethanol is not favored after the formation of the acyl enzyme (something that is intuitive for steric reasons). This value is in the same range as those reported here for the steric energy difference between the acyl enzyme and the tetrahedral intermediary II obtained by MM2 (from 19.5 to 55 kcal mol^{-1}). However, they cannot be compared straightforwardly to propose more or less impact of inhibition depending on ibuprofen mode coordination because they are obtained by different methods and models.

CONCLUSION

Enantioselective esterification of racemic ibuprofen catalyzed by Novozym 435 has been succesfully performed in the absence of additional organic solvents. Working at the lowest excess of ethanol possible (1 mL) it did not promote a drastic reduction in biocatalyst activity, with total conversion values comparable with those registered in organic solvent media.[17] The assay of different alcohol volumes, several initial water contents, reaction temperature levels and biocatalyst loadings, revealed that the esterification of 0.5 g of ibuprofen with 1 mL of ethanol in a system with 4.8% v/v water and 160 mg Novozym 435, operated at 45 °C resulted in the best conditions assayed in terms of both total conversion and enantiomeric excess. Even though for practical purposes the enantiomeric excesses registered still need to be enhanced, to the best of our knowledge, it has been proved for the first time that the lipase-catalyzed enantioselective esterification of racemic ibuprofen at moderate temperatures (45 °C) in a medium free of organic co-solvents is a feasible process. Reuse of the biocatalyst in a system with a molar ratio of ethanol to ibuprofen of 7 did not promote drastic deactivation of the lipase, which could

 © 2009 Society of Chemical Industry

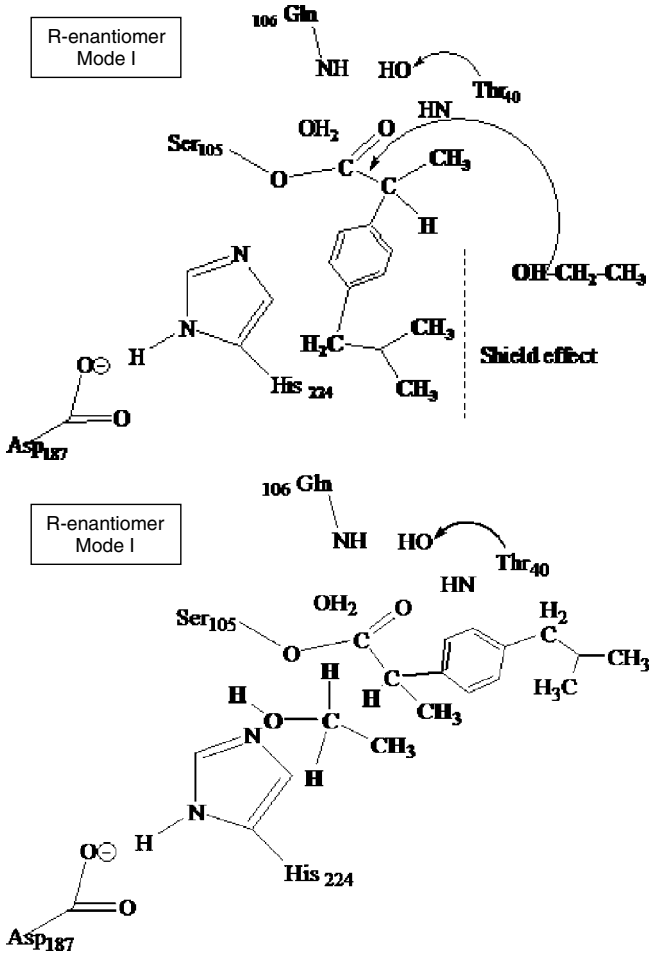


Figure 12. Accessibility of ethanol to acyl-enzyme from R-ibuprofen. Coordinations modes I and II.

be used up to four times, with activity/selectivity of around 65% of that measured in the first use.

Results for the effect of water and ethanol revealed that high concentrations of those compounds (20% v/v of water and 20 mL of ethanol, respectively) were highly detrimental to ester production. Inhibition reactions that may justify the behavior observed were therefore proposed and molecular mechanics modeling was used to determine their thermodynamic feasibility. Modeling results revealed that potentially irreversible reactions of ethanol and water with the Histidine moiety of the catalytic triad were enthalpically favored and could take place prior to ibuprofen coordination. Moreover, molecular mechanics calculations performed for different coordinations modes of the (R)/(S) enantiomers explained the enantioselectivity of CALB for the (R) enantiomer of ibuprofen. In this aspect, consideration of the energies involved in each step of the esterification mechanism demonstrated that the esterification of (R) enantiomer is favored, mainly if coordination in mode I takes place.

ACKNOWLEDGEMENTS

The authors thank CONICET for a postdoctoral fellowship, Agencia Nacional de Promoción Científica y Tecnológica (PICT RED 729/06) and Universidad Nacional de La Plata (project 11-X485) for financial support.

REFERENCES

1 Adams SS, Bresloff P and Manson GC, Pharmacological difference between the optical isomers of ibuprofen: evidence for metabolic inversion of the (−)isomer. *J Pharm Pharmacol* **28**:256–257 (1976).
2 Mills RFN, Adams SS, Cliffe EE, Dickinson W and Nicholson JS, The metabolism of ibuprofen. *Xenobiotica* **3**:589–598 (1973).
3 Carvalho PO, Cass QB, Calafatti SA, Contesisni FJ and Bizaco R, Alternatives for the separation of drug enantiomers: ibuprofen as a model compound. *Braz J Chem Eng* **23**:291–300 (2006).
4 Tung HH, Waterson S, Reynolds S and Paul E, Resolution of ibuprofen via stereospecific crystallization. *AIChE Symp Ser* **91**:64–68 (1995).
5 Sanchez A, Valero F, Lafuente J and Sola C, Highly enantioselective esterification of racemic ibuprofen in a packed bed reactor using immobilised *Rhizomucor miehei* lipase. *Enzyme Microb Technol* **27**:157–166 (2000).
6 Ikeda Y and Kurokawa Y, Enantioselective esterification of racemic ibuprofen in isooctane by immobilized lipase on cellulose acetate-titanium iso-propoxide gel fiber. *J Biosci Bioeng* **93**:98–100 (2002).
7 López Belmonte MT, Alcántara AR and Sinisterra JV, Enantioselective esterification of 2-arylpropionic acids catalyzed by immobilized Rhizomucor miehei lipase. *J Org Chem* **62**:1831–1840 (1997).
8 Won K, Hong J, Kim K and Moon S, Lipase-catalyzed enantioselective esterification of racemic ibuprofen coupled with pervaporation. *Process Biochem* **41**:264–269 (2006).
9 Pepin P and Lortie R, Influence of water activity on the enantioselective esterification of (R,S)-ibuprofen by *Candida antarctica* lipase B in solventless media. *Biotechnol Bioeng* **63**:502–505 (1999).
10 Trani M, Ducret A, Pepin P and Lortie R, Scale-up of the enantioselective reaction for the enzymatic resolution of (R,S)-ibuprofen. *Biotechnol Lett* **17**:1095–1098 (1995).
11 Ducret A, Trani M, Pepin P and Lortie R, Comparison of two HPLC techniques for monitoring enantioselective reactions for the resolution of (R,S)-ibuprofen: Chiral HPLC vs achiral HPLC linked to an optical rotation detector. *Biotechnol Tech* **9**:591–596 (1995).
12 Ergan F, Trani M and Lortie R, Selective esterification of racemic ibuprofen. *Ann NY Acad Sci* **750**:228–231 (1995).
13 Manjon A, Iborra JL and Arocas A, Short-chain flavor esters synthesis by immobilized lipase in organic media. *Biotechnol Lett* **3**:339–344 (1991).
14 Hæffner F, Norin T and Hult K, Molecular modelling of the enantioselectivity in lipase catalysed transesterification reactions. *Biophys J* **74**:1251–1262 (1998).
15 Bhandarkar SV and Neau SH, Lipase-catalyzed enantioselective esterification of flurbiprofen with n-butanol. *Electron J Biotechnol* **3**:195–201 (2000).
16 Park HJ, Choi WJ, Huh EC, Lee EY and Choi CY, Production of optically active ketoprofen by direct enzymatic esterification. *J Biosci Bioeng* **87**:545–547 (1999).
17 Ong AL, Kamaruddin AH, Bhatia S, Long WS, Lim ST and Kumari E, Performance of free *Candida antarctica* lipase B in the enantioselective esterification of (R)-ketoprofen. *Enzyme Microb Technol* **39**:924–929 (2006).
18 Carvalho P, Contesisni FJ and Ikegaki M, Enzymatic resolution of (R,S)-ibuprofen and (R,S)-ketoprofen by microbial lipases from native and commercial sources. *Braz J Microbiol* **37**:329–337 (2006).
19 Foresti ML, Martino R, Ferreira ML and Briand LE, Estudios exploratorios de la esterificación eco-compatible de ibuprofeno por vía enzimática tendientes a la resolución racémica de la droga. XV Congreso Argentino de Catálisis – IV Congreso Mercosur de Catálisis, La Plata, Argentina, ID-150 (2007).
20 Soumanou MM and Bornscheuer UT, Improvement in lipase-catalyzed synthesis of fatty acid methyl esters from sunflower oil. *Enzyme Microb Technol* **33**:97–103 (2003).
21 Piyatheerawong W, Iwasaki Y, Xu X and Yamane T, Dependency of water concentration on ethanolysis of trioleoylglycerol by lipase. *J Mol Catal B: Enzym* **28**:19–24 (2004).
22 Broos J, Visser AJ, Engbersen JF, Verboom W, van Hoek A and Reinhoudt DN, Flexibility of enzymes suspended in organic solvents probed by time-resolved fluorescence anisotropy. Evidence that enzyme activity and enantioselectivity are directly related to enzyme flexibility. *J Am Chem Soc* **117**:12657–12663 (1995).
23 Wehtje E, Costes D and Adlercreutz P, Enantioselectivity of lipases: effects of water activity. *J Mol Catal B: Enzym* **3**:221–230 (1997).
24 Xie Y and Liu H, Effect of water content on enzyme activity and enantioselectivity of lipase-catalyzed esterification of racemic

 © 2009 Society of Chemical Industry

ibuprofen in organic solvents. *J Chem Ann NY Acad Sci* **864**:570–575 (1998).
25 Arroyo M, Moreno JM and Sinisterra JV, Alteration of the activity and selectivity of immobilized lipases by the effect of the amount of water in the organic medium. *J Mol Catal A: Chem* **97**:195–201 (1995).
26 Graber M, Bousquet-Dubouch MP, Lamare S and Legoy MD, Alcoholysis catalyzed by *Candida antarctica* lipase B in a gas/solid system: effects of water on kinetic parameters. *Biochim Biophys Acta* **1648**:24–32 (2003).
27 Wu JY and Liu SW, Influence of alcohol concentration on lipase-catalyzed enantioselective esterification of racemic naproxen in isooctane: under controlled water activity. *Enzyme Microb Technol* **26**:124–130 (2000).
28 Trodler P and Pleiss J, Modeling structure and flexibility of *Candida antarctica* lipase B in organic solvents. *BMC Struct Biol* **8**:9–18 (2008).
29 Köhler J and Wünsch B, The allosteric modulation of lipases and its possible biological relevance. *Theor Biol Med Model* **4**:34–49 (2007).
30 Arroyo M and Sinisterra JV, High enantioselective esterification of 2-arylpropionic acids catalyzed by immobilized lipase from *Candida antarctica*: a mechanistic approach. *J Org Chem* **59**:4410–4417 (1994).
31 Bousquet-Dubouch MP, Graber M, Sousa N, Lamare S and Legoy MD, Alcoholysis catalyzed by *Candida antarctica* lipase B in a gas/solid system obeys a Ping Pong Bi Bi mechanism with competitive inhibition by the alcohol substrate and water. *Biochim Biophys* **1550**:90–99 (2001).
32 Valivety RH, Halling PJ, Peilow AD and Macrae AR, Relationship between water activity and catalytic activity of lipases in organic media. Effects of supports, loading and enzyme preparation. *Eur J Biochem* **222**:461–466 (1994).
33 Valivety RH, Halling PJ and Macrae AR, Water as a competitive inhibitor of lipase-catalysed esterification in organic media. *Biotechnol Lett* **15**:1133–1138 (1993).
34 Uppenberg J, Hansen M, Patkar S and Jones TA, The sequence, crystal structure determination and refinement of two crystal forms of lipase B from *Candida antarctica*. *Structure* **2**:293–308 (1994).
35 Raza S, Fransson L and Hult K, Enantioselectivity in *Candida antarctica* lipase B: a molecular dynamics study. *Protein Sci* **10**:329–338 (2001).
36 Burkert U and Allinger NL, *Molecular Mechanics*. *ACS Monograph* 177, ACS, Washington DC (1982).
37 Otto RT, Scheib H, Bornscheuer UT, Pleiss J, Slydatk C and Schmid RD, Substrate specificity of lipase B from *Candida antarctica* in the synthesis of arylaliphatic glycolipids. *J Mol Catal B: Enzym* **8**:201–211 (2000).
38 Orrenius C, Hæffner F, Rotticci D, Öhrner N, Norin T and Hult K, Chiral recognition of alcohol enantiomers in acyl transfer reactions catalysed by *Candida antarctica* lipase B. *Biocatal Biotransform* **16**:1–15 (1998).
39 Foresti ML and Ferreira ML, Molecular modeling of ethyl oleate synthesis catalyzed by lipases from *Candida rugosa* and *Candida antarctica* B. Effect of structural water, XXI Simposio Iberoamericano de Catálisis, Málaga, España, I-1029 (2008).
40 Word JM, Lovell SC, Richardson JS and Richardson DC, Asparagine and glutamine: using hydrogen atom contacts in the choice of side-chain amide orientation. *J Mol Biol* **285**:1735–1547 (1999).

 © 2009 Society of Chemical Industry