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Influence of the nature of the support in the catalytic performance of CALB: experimental and theoretical evidences

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Abstract

The immobilization of the lipase B of *Candida antarctica* (CALB) was carried out on various hydrophilic and hydrophobic supports through adsorption and covalent bond interactions. The catalytic performance of the biocatalysts was evaluated in the enantioselective esterification the *R/S*-ibuprofen with ethanol without co-solvents added. The present investigation demonstrates that the biocatalysts based on CALB immobilized by adsorption on hydrophobic supports (acrylic resin, polymethylmethacrylate, polystyrene, epoxy resin and polypropylene) possess higher conversion of ibuprofen and enantiomeric excess towards the *S*-enantiomer than CALB immobilized onto hydrophilic materials. Additionally, the enzyme loading is a key factor influencing the conversion of the substrate regardless the nature of the support material. High enzyme loading is detrimental for the specific activity of the biocatalysts. Experimental and molecular modeling investigations provided evidences of the influence of the water activity in the stereospecificity of the lipase.

Keywords: CALB, immobilized enzymes, hydrophobic support, hydrophilic support, *R/S*-ibuprofen, esterification.

List of abbreviations

APTS, 3-aminopropyl-triethoxisilane

a_w, water activity

BET, Brunauer-Emmett and Teller

CALB, *Candida antarctica* lipase B

CS, chitosan

E, enantiomeric ratio

eeS, enantiomeric excess

eeS_t, theoretical enantiomeric excess

EDS, energy dispersive X-ray microanalysis

EF, enantiomeric factor

EP, epoxy acrylic resin

GLU, glutaraldehyde

GLU-CS, glutaraldehyde-chitosan

ICP-AES, Inductively Coupled Plasma Atomic Emission Spectroscopy

MAG, magnetite

NPs, nanoparticles

NSAIDs, non-steroidal anti-inflammatory drugs

OA, oleic acid

PMMA, polymethylmethacrylate

PPL, polypropylene

PS, polystyrene

SA, specific activity

1. INTRODUCTION

Currently more than 50% of the active pharmaceutical ingredients used in the manufacture of drugs are chiral, and of these over 80% are marketed as racemates. However, the trend in the pharmaceutical industry is aimed at the use of the pure enantiomers as these have some advantages as reduction of metabolic burden, kidney or liver, minor drug interactions and less likely side effects. Therefore, the production of the pharmacologically active isomer is an aspect of increasing interest in the areas of drug development and manufacturing. The so called "chiral switch" that happens when a racemic drug is replaced with a purified single enantiomer version in the market is also a driving factor for the production of such substances.[1,2] The expiration of the patent of the synthesis of the racemate is the opportunity to introduce a new patent of the pure enantiomer. This strategy provides an extended profit for the pharmaceutical company that is producing the racemate, new opportunities to other manufactures and allows bridging studies that would lead to an easier pathway for approval. Ketoprofen and ibuprofen are examples of racemic non-steroidal anti-inflammatory drugs (NSAIDs) that underwent the chiral switch.

In this context, single isomers may be obtained by asymmetric synthesis or by kinetic resolution. The feasibility of those processes is directly related with the cost of the reagents involved, the cost-recyclability of resolving agent or catalyst, the quantity of waste generated and the catalyst/process productivity. In particular, the enzymatic kinetic resolution has aroused scientific interest in the last decades because is carried out at moderate temperatures (typically 45 °C) and the reaction mixtures are benign to the environment.[3,4] The commercial biocatalyst Novozym®435 composed by the lipase B of *Candida antarctica* (CALB) adsorbed

on a polymethylmethacrylate (PMMA) resin is the most widely used to catalyze the enantioselective esterification of the *R*-ibuprofen isomer within the racemic mixture. The commercial biocatalyst Lipozyme® IM composed of the *Rhizomucor miehei* lipase has also been used. In contrast, the catalytic performance of the *Candida rugosa* lipase has been investigated as the free enzyme and immobilized over agarose, SiO_2, Al_2O_3, cellulose acetate-titanium isopropoxide gel fiber, polypropylene and Accurel MP100 (hydrophobic polypropylene).[3]

Previously, some of us reported the esterification of racemic ibuprofen using just ethanol as reactive and solvent without the addition of a co-solvent catalyzed with Novozym® 435.[5,6] A 62 % of conversion with 54 % of enantiomeric excess towards *S*-ibuprofen was obtained under such conditions. Further investigations demonstrated that short chain alcohols cause a series of drawbacks in the commercial biocatalyst such as the disaggregation of the polymeric support, the desorption and modification of the secondary structure of CALB, strong adsorption of the alcohols on the enzyme catalytic triad and its consequent inhibition, and the modification of the inner and outer texture of the beads of the biocatalyst.[7-9] The decrease of the volume of ethanol at the expense of using an organic co-solvent[10] and the replacement of ethanol by another short-chain alcohol were investigated as alternatives to overcome those drawbacks.[11] However, both strategies are less efficient in terms of the progress of the reaction and the enantiomeric resolution compared to use ethanol as substrate and solvent.

The investigations clearly demonstrate that the nature of the material that composes the commercial biocatalyst Novozym®435 is a key factor in the stability of the biocatalyst. However, the literature has not addressed the influence of other materials as supports of CALB in the esterification of ibuprofen with alcohols acting simultaneously as acyl acceptors and solvents. In this regard, the suitable carriers for enzyme immobilization should possess certain characteristics such as high

affinity for proteins, availability of reactive functional groups or chemical modification, mechanical stability and rigidity, feasibility to be recovered and reused, high surface area, high biodegradability, low cost and, depending on the application, should be non-toxic (biocompatible). Therefore, considering those requirements, the present investigation evaluates hydrophilic and hydrophobic, organic and inorganic materials as supports for the immobilization of the lipase B of *Candida antarctica* through physical adsorption and covalent coupling. Moreover, the influence of the water activity is addressed through an experimental and theoretical point of view.

This research is a start point for development of novel biocatalysts active in the kinetic resolution of ibuprofen that overcome the drawbacks of Novozym® 435 discussed above.

2. EXPERIMENTAL SECTION

2.1. Materials

The *Candida antarctica* B lipase (CALB L, batches LCN02102 and LCN02103) was supplied by Novozymes Brasil (Paraná, Brazil) and pure *Candida antarctica* B lipase (35,500 g/mol) was purchased from Sigma Aldrich Argentina (10.9 U/mg). The commercial biocatalyst Novozym® 435 (batch LC200217) was obtained as a gift from Novozymes Brasil (Paraná, Brazil). The commercial biocatalysts known as SPRIN liposorb CALB®, SPRIN actisorb CALB®, SPRIN epobond CALB® were kindly donated by Piedmont S.A. (Pittsboro, USA).

The organic supports used for the immobilization of CALB were chitosan and polypropylene called CS and PPL within the text. The CS powder from prawns shell

(Primex Ingredients ASA Norwegian batch TM 369) possess 3-5 m^2g^{-1} of surface area, 60-90 mesh; 70,000-80,000 g/mol^{-1} and the degree of deacetylation is 85.2 %. The low molecular weight polypropylene powder (PPL) was obtained through polymerization using metallocenes and possesses 23 m^2g^{-1} of surface area.

A series of commercial oxides such as ZrO_2 (Evonik, 39 m^2g^{-1}, 20 nm), fumed SiO_2 (Cab-O-Sil, 300 m^2g^{-1}, 20 nm) and TiO_2 (anatasa P-25 of Degussa, 45.7 m^2g^{-1}, 20 nm) were assayed. Additionally, nanosized magnetite Fe_3O_4 (called MAG within the text) was synthesized according to the method described in the literature.[12,13]

Other reagents used in this study were glutaraldehyde (GLUT, Fluka 25 % w/w), 3-aminopropyl-triethoxisilane (APTS, Avocado Research Chemicals U.K.), oleic acid (OA, Anedra, 96.3 %), *R/S*-ibuprofen (Parafarm, 99.23 %), ethanol (Carlo Erba, 99.8 %) and potassium hydroxide in ethanol 1M (Riedel-de Haen).

2.2. Quantification of proteins

2.2.1. Q*uantification of proteins in the commercial crude extract*

The concentration of the lipase B of *Candida antarctica B* in the crude extract was determined through the precipitation of the protein with ammonium sulfate according to procedure published before.[14] This procedure allowed determined that the crude extract possesses 68 mg of precipitable material (using ammonium sulfate) per mL.

2.2.2. *Quantification of immobilized lipase through high resolution Inductively Coupled Plasma Atomic Emission Spectroscopy ICP-AES*

The protein concentration in the biocatalyst was determined by measuring the sulfur content through high resolution ICP-AES (Shimadzu ICPE 9000) in combination with a modified Bradford method.[15] The amount of lipase was calculated considering that a molecule of CALB (molecular weight equals to 33 kDa) possesses ten amino acids with sulfur in the structure.[16]

The enzyme was removed out of the commercial biocatalysts using aqua regia and further quantified through ICP-AES. Additionally, sulfur was quantified in the remaining solids (after treatment with aqua regia) through environmental scanning electron microscopy (ESEM FEI Quanta 200) with energy dispersive X-ray microanalysis EDS (EDAX SDD Apollo 40) in order to assure the complete removal of the protein. The samples were covered with a thin layer of carbon and further analyzed under high vacuum (P < 10^{-6} torr), 1000X magnification, 100 sec of acquisition and 19.99 kV.

2.3. Synthesis of the biocatalysts

2.3.1. *Lipase immobilization through adsorption*

The immobilization of CALB over PPL and CS was performed through the adsorption of the crude extract in a phosphate buffer at pH 7 at room temperature. The adsorption of CALB onto the oxides' NPs was performed in distilled water (pH 5) without the addition of a buffer to avoid the co-adsorption of ions onto the supports. The details of the immobilization were reported previously.[17-19] Polypropylene was pretreated with absolute ethanol for 3 min at 500 rpm in order to decrease the hydrophobicity of the microenvironment. This treatment favors the loading and distribution of the enzyme over the support.[20]

The materials obtained were called CALB/PPL, CALB/CS, CALB/TiO$_2$, CALB/SiO$_2$ and CALB/ZrO$_2$.

2.3.2. *Lipase immobilization through covalent bond*

The immobilization of the lipase B of *Candida antarctica* over functionalized chitosan and magnetite was performed through covalent bond. The surface of chitosan was modified by suspending 375 mg of the powder in 50 mL of a solution of glutaraldehyde 0.25 % v/v in phosphate buffer at pH 7. The immobilization was performed by contacting a solution of the lipase (pH 7, 0.014 M of ionic strength) with glutaraldehyde-chitosan at room temperature for 7 hours under stirring at 350 rpm. Then, the solid was recovered by filtration, washed with distilled water and dried at 50 ºC for 12 h.[17] This material was called CALB/GLUT-CS.

Magnetite Fe$_3$O$_4$ was coated with chitosan in a 2: 1 magnetite-chitosan weight ratio and further treated with glutaraldehyde in order to avoid the segregation of chitosan moieties from the magnetic NPs. The surface was functionalized with APTS and glutaraldehyde prior to the immobilization of CALB. Briefly, the MAG/CS/GLU/APTS/GLUT support was contacted with a solution of CALB at pH 5.8 under stirring for 7 hours and then washed three times with distilled water. The details of the immobilization have been published before.[21]

2.3.3. Specific surface area and pore size

The specific surface area and pore diameter of the biocatalysts were determined through the physical adsorption of nitrogen at -195.8 ºC using the Brunauer-Emmett and Teller (BET) method. The assay was performed in an equipment Micromeritics ASAP 2020.

2.4. Esterification of the *R*/*S*-ibuprofen with ethanol. Calculation of the enantiomeric excess **eeS**, enantiomeric ratio **E**, enantiomeric factor **EF** and specific activity **SA**.

The esterification of 0.5000 g (2.42 mmol) of *R/S*-ibuprofen was performed in ethanol as reactant and solvent with 4.76 % v/v of water added. The esterification was carried out at 45 °C under stirring (200 rpm) in a water bath (Julabo SW22, Germany) for 48 h. The reaction begins with the addition of 160 mg of biocatalyst per mL of alcohol. The reaction conditions correspond to those previously found to be optimal when using ethanol as nucleophilic agent and solvent.[5,6] Additionally, reactions were tested doubling the mass of the catalyst of CALB/PPL and CALB/MAG.

Additional experiments of esterification of *R/S*-ibuprofen with ethanol catalyzed with CALB in homogeneous fashion were performed in order to compare the catalytic activity of the free and the immobilized lipase. In this context, a certain volume of the crude extract containing 2.53 mg, 9.57 mg and 15.51 mg of the lipase was used in these experiments. Those quantities are similar to the amounts of the lipase CALB provided by 160 mg of CALB/TiO$_2$; SPRIN epobond CALB® and Novozym® 435, respectively. The assays were performed as described above.

Blank tests were also performed in order to determine the degree of progress and enantioselectivity of the non-catalyzed reaction.

A volume of 50 µL of the reaction media was taken for analysis and further diluted with 20.00 mL of MeOH (Carlo Erba 99.8 %) and tetraethylammonium acetate buffer TEAA 0.1 % v/v (Fluka) in a 60:40 ratio at pH = 4. The *R* and *S* enantiomers of ibuprofen were assessed through high performance liquid chromatography (HPLC) using a chiral column Nucleodex Beta-PM (Macherey-Nagel, Germany) with an UV detector at 230 nm.

The enantiomeric excess (*eeS* %) referred to the form *S*-ibuprofen was calculated according to the equation (1) where [S] and [R] account for the concentrations of the *S*-ibuprofen and *R*-ibuprofen respectively.[22,23]

$$eeS \% = \frac{[S] - [R]}{[S] + [R]} * 100 \qquad (1)$$

Additionally, the enantiomeric ratio (E) was calculated according to equation (2) being *X* the conversion of racemic ibuprofen towards the ethyl esters.[22,23]

$$E = \frac{[\ln(1-X)(1-eeS)]}{[\ln(1-X)(1+eeS)]} \qquad (2)$$

This parameter was defined by Chen *et al.* for the irreversible reactions in which the single substrate is converted into a product following the first or pseudo-first order, in the homogenous system and in the absence the secondary reactions. In 1987, the authors extended the definitions to reversible reactions.

It is worth noticing that Straathof and Jongejan studied methods of calculation of this parameter and discussed the influence of factors such as parallel reactions, kinetic and thermodynamic of the main reaction, enzymatic inhibition, phase homogeneity, chemical equilibrium, diffusion limitation, incomplete mixing and reactor type.[24] This report shows that the equation (2) is not of general application. Heinsman *et al.* reported that the diffusion of the substrates through the biocatalyst influence the validity of the E parameter when using immobilized enzymes.[25] Lopez-Belmonte *et al.* proposed a new parameter called Enantiomeric Factor (EF) that is independent of the reaction kinetics. The EF is defined as the relationship between

the experimental enantiomeric excess *eeS* and the theoretical enantiomeric excess *eeS$_t$* (equation 3).

$$EF = \frac{eeS}{eeS_t} = 100\,X\,(100 - X) \qquad (3)$$

The *eeS$_t$* corresponds to the enantiomeric excess towards the enantiomer that is preferentially catalyzed by the enzyme (*S*-ibuprofen in the case of CALB) as the only substrate of the reaction under time and conversion similar to the experimental assay.[26] Thus, an EF value of 1 indicates a perfect enantioselectivity and a value of zero indicates that no resolution occurs.[27] A conversion equals to 50% or above that value indicates that the enzyme converts both enantiomers. Therefore, for X% ≥ 50% the EF value must be calculated considering a theoretical *eeS$_t$* of 100%.

The ibuprofen remaining after the reaction was determined through titration with a standardized solution of potassium hydroxide in ethanol. The specific activity (SA) was calculated as the amount of profen converted to ethyl esters (in μmol) per amount of enzyme (in mg) and time (h). Similarly, the productivity was calculated as the amount of profen converted to ethyl esters (in μmol) per amount of biocatalyst (in mg) and time (h). The standard deviations of the specific activity and the enantiomeric excess are (+/- 0.01) and (+/- 0.5), respectively.[5,7,8,10]

2.5. Effect of the water activity in the kinetic resolution

The effect of the water activity a$_w$ on the activity and enantioselectivity of the biocatalysts was also studied. Three distinctive biocatalysts such as, Novozym® 435 (hydrophobic support, adsorbed enzyme), CALB/MAG (hydrophilic support, covalent coupling of the enzyme) and CALB/CS (hydrophilic surface, adsorbed

enzyme) were selected. The control of the water activity during the esterification of ibuprofen was performed with saturated solutions of lithium chloride (a_w = 0.11), potassium acetate (a_w = 0.23) and sodium bromide (a_w = 0.57).[28] An open eppendorf vial containing 0.60 mL of the saturated solution was placed inside the flask containing the profen and ethanol solution (with 4.76% v/v of water added), purged with nitrogen and allowed to equilibrate until the expected water activity was reached. The water activity was measured before and after the reaction with a thermo hygrometer HANNA HI 9564 inside a dry box, being verified the constant values of this parameter.

2.6. Molecular modeling

To explore the importance of steric interactions at the level of the active site and neighborhoods of the catalytic triad from CALB, a simple molecular mechanics study was carried out, using the same model and structures than previously reported in other manuscripts from some of us.[29]. The active site of CALB is a catalytic triad composed of serine (S) 105, aspartic acid (D) 187 and histidine (H) 224, and an oxyanion hole formed by threonine (T) 40 and glutamine (Q) 106 is found. The mechanism of action of this enzyme has been described as Ping Pong Bi Bi with the formation of two tetrahedral intermediates and an acyl–enzyme complex.[29] The CALB structure was obtained from the Protein Data Bank (1tcb; DOI:10.2210/pdb1tcb/pdb).

R/S-ibuprofen was modeled near the catalytic triad of CALB. The goal of this simple modeling approach was to compare the steric energy at an energetic minimum for R-ibuprofen or S-ibuprofen at two different situations such as the adsorption of the profen at the serine molecule (only the location near the serine as a first step) and the adsorption of ethanol to the acyl enzyme. Both situations were studied with and

without including two water molecules near the catalytic triad and the ibuprofen/ethanol species. The steric energy was calculated using the ChemBio3D Ultra 11.0 Software from Cambridge Soft.

The theoretical study does not take into account the role of the support in the access of the substrates and products to the catalytic triad of CALB. It is supposed that no diffusional problems take place.

3. RESULTS AND DISCUSSION

3.1. From commercial to synthesized biocatalysts: characteristics of the various support materials

The Table 1 summarizes the organic and inorganic materials used as supports, their hydrophilic and hydrophobic properties, the nature of the protein-support interaction, the specific surface area (of the support mainly), the protein loading, pore size and enzyme density.

The commercial biocatalysts such as, SPRIN liposorb CALB®, Novozym®435 and SPRIN actisorb CALB® and the material called CALB/PPL (prepared in this investigation) possesses the lipase B of *Candida antarctica* adsorbed onto a polymeric support material with hydrophobic nature. Similarly, the biocatalysts called, CALB/CS, CALB/TiO$_2$, CALB/ZrO$_2$ and CALB/SiO$_2$ have been prepared through adsorption onto hydrophilic materials such as chitosan (organic nature) and the transition metal oxides (inorganic supports). The interaction of the enzyme in the commercial SPRIN epobond CALB® and the biocatalysts called CALB/GLU-CS and CALB/MAG possesses covalent nature. The support of the former presents hydrophobic nature while the covalent materials prepared in this investigation possess hydrophilic nature.

The highest values of enzymatic loading (above 50 mg of protein per gram of biocatalyst) were obtained with the hydrophobic supports, such as polymethylmethacrylate (PMMA), epoxy acrylic resin (EP), polystyrene (PS) and polypropylene (PPL). An exception, within the materials of inorganic nature, is the zirconium oxide ZrO_2 that supports up to 100 mg of protein per gram even though possesses hydrophilic nature (see Table 1).

The enzymatic densities have no relationship with the pore dimension (ranged from 100 Å to 300 Å) of the assayed biocatalyst. It is worth noting that the procedure of BET area determination involves a pretreatment with high vacuum and/or thermal treatment. The determination of the BET area under these conditions has not relation whatsoever to the reaction media. Therefore, the specific surface areas of the transition metal oxides, chitosan (an essentially non-porous material) and PPL are maximum values for the final biocatalysts. In the case of CALB/MAG, the surface area was calculated taking into account oleic acid adsorption. Moreover, the dimensions of CALB are 30 Å × 40 Å × 50 Å suggesting that the enzyme is able to penetrate inside the particles without constrains. Therefore, the enzyme density depends on the surface nature and density of reactive groups of the various supports. Hydrophobic supports possess a series of accessible (exposed) groups such as, ester groups CH_3COOCH_3 in PMMA and other acrylic resins; epoxy groups (EP resin); methylene groups $-CH_2$ (polystyrene) and methyl groups $-CH_3$ in isotactic polypropylene.[8,30] In the present investigation, polypropylene shows the highest enzyme density (4.3 mg.m^{-2}). This phenomenon might be related with the exposure of the support to alcohol prior to the immobilization of CALB.[31,32] The polypropylene powder with controlled particle size (590-1100 μm) was contacted with ethanol under stirring for 3 min in order to decrease its hydrophobic nature and improve the capacity of the material for the adsorption of the enzyme. Furthermore, the pretreated polypropylene stabilizes the non-polar side chains inside the protein

and exposes the polar side chain residues.[31] In contrast the commercial SPRIN actisorb CALB® based on polystyrene, shows the lowest amount of CALB per unit surface area (0.4 mg.m^{-2}).

The bare transition metal oxides such as TiO_2, ZrO_2 and SiO_2 possess surface hydroxyls (Brønsted species) and Lewis cation species providing the anchoring sites for the immobilization of the proteins.[33] Previous investigations demonstrated that silica possess a lower density and reactivity of the surface hydroxyls than titania and zirconia resulting in a low surface coverage with metal oxide species.[33,34] This observation explains the low enzyme density of the SiO_2 support (0.2 mg.m^{-2}) even though possesses the highest surface area within the assayed materials (see Table 1).

Bare and functionalized chitosan show the highest enzyme densities between the hydrophilic supports (5.0 mg.m^{-2}). Chitosan possesses surface hydroxyls and a fraction of NH_2/NH_3^+ groups. Previous studies reported by Ferreira *et al.* suggested that the enzyme anchors to the surface through a twofold interaction.[35] For one side, the charged side chains of the protein are attracted by the NH_3^+ groups through an ionic interaction. Additionally, Van der Waals forces and hydrogen bridges between the enzyme and the hydroxyls and amino groups NH_2 of chitosan have been proposed.

The covalent bonding of CALB with functionalized chitosan is due to the reaction of the amino groups of the side chains of the protein with the aldehyde groups of glutaraldehyde. Ferreira *et al.*, among others, suggested that the lateral groups of lysine residues are directly involved in the reaction of lipases with glutaraldehyde.[35]

The magnetic support used in this contribution was composed by chitosan crosslinked to nanosized Fe_3O_4 with glutaraldehyde and further functionalized with 3-aminopropyl-triethoxisilane APTS.[21] In this particular case, the lipase is immobilized to the support through adsorption (on the APTS, the surface of

magnetite and oligomeric glutaraldehyde) and is covalently bonded due to the reaction with glutaraldehyde as discussed before.

3.2. Influence of the enzymatic loading and the nature of the supports in the biocatalytic performance

The Figures 1A and 1B show the conversion of ibuprofen and the specific activity (SA) as a function of the enzymatic loading of the biocatalysts based on the lipase B of *Candida antarctica* immobilized onto hydrophobic and hydrophilic supports, respectively. The open squares and triangles indicate the conversion and specific activities of those biocatalysts with covalent nature. The conversion of ibuprofen towards the ethyl esters does not show a correlation with the enzymatic loading in the particular case of the supports with hydrophobic nature (see Figure 1A). In general, the conversion reaches 60 % with the exception of CALB/PPL. However, the conversion increases with the enzyme's loading onto the hydrophilic materials until levels off at 50 mg.g^{-1} of immobilized enzyme as observed in the Figure 1B. The lowest conversion of ibuprofen (10 and 17 %) is obtained with CALB/CS and CALB/GLU-CS presenting the highest enzyme density (amount of enzyme per unit surface area equals to 5.0 mg.m^{-2}). This observation could be ascribed to the modification of the protein structure associated to the rearrangement of the molecules upon increasing the loading of enzyme onto the support. A high density of CALB may require a certain modification of the tertiary structure of the protein to be able to accommodate on the available surface of the support that also causes the aggregation of the immobilized lipase.[17]

The comparison between the Figures 1A and 1B demonstrates that the highest values of conversion of ibuprofen (~60 %) are achieved with the hydrophobic supports. The exception of CALB/PPL is related again with its high surface enzyme

density. Additionally, it comes straight forward from the figures that the immobilization through covalent coupling diminishes the specific activity compared with the adsorption of the enzyme regardless of the nature of the support. The specific activity normalizes the conversion per amount of protein therefore that behavior indicates that the covalent bonding of the enzyme to the support decreases the biocatalytic activity of the active sites. Additionally, the specific activity is between 1.2 and 4.0 $\mu mol.mg^{-1}.h^{-1}$ regardless of the hydrophobicity or hydrophilicity of the supports. The observation that the specific activity diminishes upon the increase in the enzyme loading is an evidence of the modification of the structure of the protein as will be further discussed in the following sections.

Further information about the effect of the immobilization on the catalytic activity of the lipase B of *Candida antarctica* is obtained by comparing the free and immobilized lipase. The Figure 2 compares the specific activity of free and immobilized CALB through adsorption and covalent bonding over hydrophobic and hydrophilic supports. The lipase adsorbed on a hydrophobic material such as acrylic resin AR (SPRIN liposorb CALB®) shows a similar activity as the free CALB. Meanwhile the enzyme immobilized over polymethylmethacrilate PMMA (Novozym® 435) and polystyrene PS (SPRIN actisorb CALB®) retain 70% of the activity of the free lipase.

The adsorption on hydrophilic materials and the covalent bonding greatly diminishes the activity of the enzyme, with the exception of CALB/TiO_2 that retains 73 % of the activity of the free lipase.

The Figures 3A and 3B show the enantiomeric excess ee*S* % as a function of the enzymatic loading on hydrophobic and hydrophilic supports, respectively. Again, the results evidenced that the covalent immobilization diminishes the enzymatic enantioselectivity, independently of the nature of the support. The negative ee*S*

value observed within the hydrophilic supports indicates an inversion of the enantiomeric excess that will be discussed later in this section and further addressed with a theoretical approach.

The enantiomeric excess towards the *S*-ibuprofen obtained with CALB immobilized through adsorption onto hydrophobic supports (ee*S* ≥ 30 %) is higher than the ones obtained with hydrophilic supports (ee*S* < 10 %). There is no particular correlation between the ee*S* and the enzymatic loading.

The enantiomeric excess towards *S*-ibuprofen is 11 %, -2.4 % and 3.1 % for 2.53 mg, 9.57 mg and 15.51 mg of the free CALB. In this regard, the immobilization on hydrophobic materials enhances the enantio-preference of the lipase towards the esterification of the *R*-enantiomer.

The Figure 4 compares the productivity, specific activity (SA) and enzymatic loading of the biocatalysts as a function of the nature of the various supports used in this investigation. The lipase CALB immobilized onto hydrophobic supports such as AR, PMMA and PS show the highest productivity in accordance with the observations discussed above. The high productivity of the commercial biocatalyst SPRIN liposorb CALB® (possessing an acrylic resin as support) correlates with the highest specific activity between the assayed materials. The high SA and the enzyme surface density (1.4 mg.m^{-2}) somehow evidence that the enzyme is well dispersed (without strong aggregation) onto the support and the active sites are not affected due to the immobilization process. In contrast, the productivity of Novozym®435 and SPRIN actisorb CALB® (supports materials of polymethylmethacrylate and epoxy acrylic resin, respectively) correlates with their enzymatic loading instead of the activity of the lipase. This observation evidences that the immobilized lipase is less active therefore, a higher loading than in the case of SPRIN liposorb CALB® is required to achieved a similar productivity.

Additionally, the figure shows the deleterious effect of the covalent immobilization on the biocatalytic performance as was discussed previously. In this context, the lowest values of productivity belong to those biocatalysts with covalent enzyme-support interaction (CALB/MAG, CALB/GLUT-CS and SPRIN epobond CALB®). The decrease in the specific activity from 2.52 to 1.47 $\mu mol.h^{-1}.mg^{-1}$ and the productivity from 0.054 to 0.031 $\mu mol.h^{-1}.mg^{-1}$ observed for CALB/CS and CALB/GLU-CS respectively is a clear evidence of the deleterious effect of glutaraldehyde on the esterification of ibuprofen. It is known that a negative aspect of covalent immobilization is the decrease of enzymatic activity either by involving amino acids of the active site in the binding to the support, by steric blocking of the active site and/or an increase in the stiffness of the enzyme and the active site. A covalent immobilization is preferred when enzymatic leaching problems should be avoided.

The Figure 4 also compares the results obtained with those assays performed by doubling the mass of biocatalyst. Particularly, CALB/magnetite was tested in the esterification of ibuprofen using a ratio of biocatalyst: ethanol of 160 $mg.mL^{-1}$ (MAG in Figure 4) and 320 $mg.mL^{-1}$ (MAG* in Figure 3), respectively. The productivity and the specific activity diminish upon increasing amount of biocatalyst due to the aggregation of the magnetic nanoparticles.[17]

Finally, the enantiomeric excess ee*S* %, ratio E and factor EF of the various supports are compared in the Figure 5. It is also worth notice that those systems prepared in this investigation and the commercial ones possess similar values of E and EF. The values of enantiomeric ratio E are in the range going from 0.5 to 4 regardless of the conversion of ibuprofen. Interestingly, CALB/PPL shows a negative value equals to -34.2 that corresponds to the highest EF (1.07) of the

assayed biocatalysts. An EF above unity indicates that the experimental enantiomeric excess is higher than the theoretical value.[36,37]

Additionally, the CALB/GLU-CS shows a negative enantiomeric excess towards *S*-ibuprofen (-4 %) indicating a certain inversion of the enantio-preference of the lipase.

3.3. Water activity, nature of the support and biocatalytic behavior relationships

Novozym® 435, CALB/CS and CALB/MAG were chosen specifically to investigate the influence of the water activity on the catalytic behavior as function of the nature of the supports and the enzyme-support interactions addressed in this investigation. As it was discussed before, the commercial Novozym® 435 possesses the lipase adsorbed onto a hydrophobic support. Additionally, the enzyme is adsorbed (ionic interaction and hydrogen bond might also be present) onto a hydrophilic support in CALB/CS. Moreover, CALB/MAG presents the enzyme bonded onto a hydrophilic support through a covalent interaction (see Table 1). The figures 6 and 7 show the specific activity and enantiomeric excess of Novozym® 435, CALB/CS and CALB/MAG versus the water activity of the reaction medium. The specific activity and the enantiomeric excess of the esterification carried under non-controlled water activity are also shown. Under these circumstances, the water activity varied from 0.392 at time zero to 0.683 in 24 h of reaction.

The control of the water activity of the reaction media possesses a clear impact in the specific activity of Novozym® 435. This commercial biocatalyst shows an increase in the specific activity at low a_w values (from 0.11 to 0.45) compared with the non-controlled reaction media (3.8 μmol.mg^{-1}.h^{-1} versus 2.4 μmol.mg^{-1}.h^{-1}). The enantiomeric excess towards *S*-ibuprofen is negligible at low a_w and reaches its highest value (55 %) at a_w 0.34.

The specific activity of the hydrophilic biocatalysts CALB/CS and CALB/MAG is clearly influenced by the water activity as can be observed in the Figure 6. In fact, the highest specific activity of the chitosan based biocatalyst is obtained in the non-controlled reaction media (maximum a_w). This observation indicates that water generated during the reaction promotes the activity of CALB/CS. Interestingly, the water activity influences the enantio-preference of the lipase immobilized on chitosan. CALB preferentially catalyzes the esterification of R-ibuprofen at low water activity ($a_w = 0.11$) and inverts towards the esterification of S-ibuprofen at a_w 0.34 onwards.

Similar to Novozym® 435, CALB immobilized onto magnetite reached the highest ee$S$ at a_w 0.34 evidencing a dependence of the enantiomeric excess with water activity. Previously Marszałł *et al.* reported that the catalytic behavior of *Candida rugosa* lipase immobilized onto magnetite cross-linked with glutaraldehyde was influenced by the water activity.[38] The authors determined that the enantiomeric excess towards R-ibuprofen increased with the addition of a salt hydrate pair and molecular sieves directly to the reaction media in order to remove the water generated during the esterification.

3.3.1. Influence of water activity in the stereo-selectivity of CALB: a theoretical explanation

Depending on the hydrophillicity/hydrophobicity of the support, the distribution/partition of water in support/lipase is different. The differences found in the enantiomeric excess towards S-ibuprofen at low (0.1) and higher (0.3 and beyond) water activity may be assigned to the different partition of water near the catalytic triad of CALB.

Some of us published some years ago a theoretical manuscript about the impact of a water molecule near the catalytic triad in ethyl oleate synthesis from oleic acid and

ethanol.[39] Even when docking methods and simulations may add further information about potential locations of the profen near the catalytic triad of the CALB, the number of combinations and the multiple situations considered: R/S profen and 1-2-3-4 water molecules, would not necessarily consider the actual structure of CALB under reaction or the hydration of CALB. In this sense, a docking procedure not necessarily provides the most probable conformations but the ones that the boundary conditions and restrictions correlated with the parameters are related. In this sense, we selected a simpler molecular modeling with the potential to give us some tools to explain sterically why a change in enantioselectivity takes place with the increase of the water activity. This does not imply that we have a high quality evidence, but we have not results against a potential sterically related explanation of the impact of water activity in CALB enantioselectivity. A different theoretical study was included in this contribution, considering the additional complexity of the ibuprofen molecule and the *R*/*S* enantiomers. The first question was if the coordination of *R* and *S*-ibuprofen enantiomers (the simple adsorption) was different when more than one water molecule were around the catalytic triad. The Figures 8A to 8D depicts a 3D representation of the profen interacting with the catalytic triad with one and four molecules of water. The second question was if the coordination of ethanol to the *R*-acyl enzyme or the *S*-acyl enzyme were different if more than one water molecule were present around the acyl enzyme (see Figures 9A and 9B). The results in terms of steric energies are presented in the Table 2. Even when the *R*-acyl enzyme shows a more negative steric energy for coordination, the difference is not more than a few kcal/mol. Besides this is only the first step of the mechanism. The difference is almost similar when the acyl enzyme is formed and ethanol is coordinated through acyl carbon at different positions with water far away from the active site (near 4 kcal/mol).

When two water molecules are included near the acyl enzyme and the ethanol, with a clear hydrogen bonding length, the difference favoring the *S*-acyl enzyme versus the *R*-acyl enzyme is now near 9.5 kcal/mol (see Figure 9B). When the two water molecules are placed far away the catalytic triad of the lipase, the difference again is near 2.5 kcal/mol. These results correlate well with the low preference for *S*-enantiomer at low water activity and the high preference for *S*-enantiomer at high water activity.

The coordination of ethanol to carbon of the acyl enzyme is important to take into account. First, the coordination of the hydrogen of the H-O group of ethanol to the serine oxygen of the acyl enzyme demonstrated to have a strong difference in steric energy favoring the *R*-enantiomer. It was clear from this results that this step is not present in the mechanism. Second, the coordination of the oxygen of the ethanol to the carbon of the acyl enzyme with the generation of tri-coordinated oxygen clearly demonstrated to generate less repulsion and an extensive H-bonding when two water molecules were present near the acyl enzyme.

The interaction of the alpha hydrogen to the carbonyl group is different in the case of the *S*-ibuprofen and in the case of the *R*-ibuprofen. Even when the alpha hydrogen is not enough acidic to generate an enolate anion, the interaction with the oxygen from the alcohol is not the same that the interaction with the oxygen from the serine. The difference among *S* and *R*-ibuprofen when ethanol is coordinated to carbon in a transition state can be assigned to the interactions of the alpha hydrogen in the alpha carbon to the carboxylate, that is also alpha carbon to the phenyl group; and the interaction with the water molecule placed laterally to the ibuprofen and coordinated to the oxygen from ethanol.

3.5. Discussion

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It is well known that adsorption and covalent binding are the most frequently used techniques to immobilize enzymes onto solid supports. The former ensures good recovery of activity but there is leaching in aqueous medium, while immobilization by covalent bond can cause major changes in the enzyme structure, which usually decreases the activity. In general, the hypothesis behind this strategy of immobilization consists of contacting lipases with hydrophobic materials that are similar to their natural substrates, and thus achieve immobilization of these enzymes in an open conformation.[40,41] The present investigation demonstrates that CALB adsorbed on hydrophobic supports (acrylic resin, PMMA, polystyrene, epoxy resin and polypropylene) is more active (in terms of conversion and enantiomeric excess) in the esterification of ibuprofen compared to the immobilization onto hydrophilic materials. This observation is ascribed to the modification of the enzymatic conformation due to the strong interaction between CALB and hydrophobic surfaces according to the experimental investigations and molecular dynamics simulations performed by Zisis *et al.*[42] The lipase B of *Candida antarctica* possesses a Ser-Asp-His catalytic triad with two mobile α-helices (α5 and α10) in the vicinity of the active site. In fact, the authors demonstrated that the α5 helix adopts various conformations depending on the hydrophobicity of the surroundings. Moreover, the conformation of this α-helix has a key role in regulating the size of the substrate that is accessible to the active site.

The biocatalysts prepared through adsorption on hydrophilic supports (ZrO_2, TiO_2, SiO_2, Fe_3O_4 and chitosan) show a similar specific activity as the ones prepared with hydrophobic supports with half the conversion of ibuprofen. However, is worth noting that the enzyme density is higher over the hydrophilic materials than over hydrophobic ones. Previous investigations by Al-Duri and Yong also reported a similar effect when adsorbing lipases over polypropylene (hydrophobic) and polypropylene/silica composite (hydrophobic-hydrophilic) materials.[43] In this context,

the enzyme loading is also a key factor influencing the conversion of substrate regardless the nature of the support material. The present investigation demonstrates that the higher the enzyme loading the lower is the specific activity of the biocatalysts prepared through adsorption. Those observations are in agreement with recent publications by Fernandez-Lopez *et al.* and Zaak *et al.*[44,45] High loadings of *Thermomyces lanuginosus* and the lipase B of *Candida antarctica* over the hydrophobic octyl-agarose prepared with solutions containing high concentration of the enzymes favors a closed packing of the molecules. The proximity also conducts to protein-protein interactions that somehow affects negatively on the thermal stability of the lipases and the residual activity. Previously, Fair and Jamieson proposed a model for the enzymatic adsorption over polystyrene latex that depends directly on the amount of adsorbed protein.[46] The explanation of this behavior is the aggregation of enzymes in the solution (promoted by high enzyme concentration) and at the hydrophillic surfaces (due to multiple hydrogen bonding and polarization). At low loading, the isolated enzyme molecules are rigidly and irreversible adsorbed onto the surface. At higher protein loading, the molecules form a partly ordered fluid structure with low interfacial free energy. This fluid possesses the freedom to adjust their configuration, lateral mobility and/or cooperative intermolecular interaction to form a monolayer. Finally, an ordered close-packed two-dimensional monolayer surface with a dense phase of protein occurs. The authors demonstrated that the surface coverage of bovine serum albumin increases when the pH of the medium was closed to the isoelectric point of the enzyme. This observation indicates that the surface enzyme "fluid" increases its configurational flexibility to form the monolayer.

The adsorption of CALB over the transition metal oxides TiO_2, SiO_2 and ZrO_2 is related with the surface species distribution at the pH of the immobilization. The lipase B of *Candida antarctica* possess an isoelectric point of 6, therefore at pH 5 (starting pH of the immobilization) it is expected that the positive charge is higher in

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average than the negative charge. Previously, some of us demonstrated that the adsorption of CALB over TiO_2 proceeds through the interaction of the lipase with surface >Ti-OH species.[19] These species predominate on the surface of anatase (α fraction ~ 1) with minor contribution of >TiO_2^- species ($\alpha = 0.1$).[47] According to the studies of Sato et al., water molecules form a hydrated multilayer on a ZrO_2 surface consisting of terminating H_2O adsorbates and hydrogen bonded H_2O layers.[48] A portion of the water molecules chemisorbed on zirconium atoms ($Zr–OH_2$) dissociates into H^+ and OH^-, forming polydentate and monodentate hydroxyls (>OH^+ and $Zr–OH^-$). According to the calculations of the authors, the amounts of $Zr–OH^-$ and $Zr–OH_2$ species (0.5 site fraction for each one) are comparable at pH 5. The higher contribution of negatively charged species of ZrO_2 compared with TiO_2 increases the available sites for the adsorption of the positively charged lipase. Moreover, this observation correlates with the high surface enzyme density of the ZrO_2 (2.6 mg.m^{-2} vs 0.4 mg.m^{-2} of TiO_2).

The adsorption of CALB over chitosan was performed at pH 7 in a phosphate buffered media and an ionic strength of 0.014 M. Chitosan is a linear copolymer of 2-amino-2-deoxy-D-glucopyranose and 2-acetamido-2-deoxy-glucopyranose. In addition to the available OH groups of the chitosan structure, the primary amine on the glucosamine residues can be protonated to $–NH_3^+$ in a wide pH and ionic strength range therefore, chitosan behaves in an aqueous solution as a cationic polyelectrolyte. The investigation of Cataldo et al. demonstrated that the protonation of chitosan is directly influenced by the ionic strength of the aqueous media. In fact, at the ionic strength of the adsorption of CALB, chitosan has a protonation equilibrium constant $logK^H = 6.6$ at 25 °C.[49] The high density of protonated surface sites of chitosan also correlates with the fact that CALB/CS possesses the highest density of adsorbed enzyme (5 mg.m^{-2}) among the hydrophilic supports.

It is well known that water is essential for the catalytic activity of enzymes in organic media however water molecules in excess reverse the direction of the esterification reaction. The water activity a_w is a relevant factor in the catalytic behavior of the assayed biocatalyst even though the reaction media possesses 4.76 % v/v of added water. An a_w below 0.7 enhances the specific activity in the particular case of Novozym® 435 (low enzyme density, hydrophobic support). However, the opposite effect is observed for CALB/CS (high enzyme density, hydrophilic support).

Many years ago, Valivety *et al.* reported an increase in the catalytic activity of *Rhizomucor miehei* lipase adsorbed at low loading on hydrophobic supports (polypropylene, anion-exchange silica, celite and anion-exchange resin) in the synthesis of dodecyl decanoate.[50] The authors attributed this observation to the competition for the available water between the enzyme molecules and the support. In fact, many investigations recognized two populations of enzyme-bound water. The first one is composed by tightly bound water where the water molecules do not exchange with other water molecules on the enzyme. The second population is formed by loosely bound water which exchanges freely with the surrounding solvent, and has properties that are indistinguishable from those of free water. Thus the enzyme's catalytically active conformation is possibly maintained by the tightly bound water whereas the loosely bound water induces an increase in enzyme activity by increasing enzyme flexibility and active-site polarity.[51] However, *Rhizomucor miehei* lipase is active at very low water activity.

In this context, the fact that the reaction medium possesses added water ensures the presence of the essential first population described above. The lipase adsorbed onto a hydrophobic support in its open conformation might contain an excess of water molecules of the second population since the support is repelling them towards the enzymatic layer. Previous investigations by some of us demonstrated that water and ethanol actually competes with the active site inhibiting the formation

of the acyl-enzyme intermediate.[6] Therefore, a low a_w somehow equilibrates the right amount of water in the microenvironment of the active sites.

An hydrophilic material such as chitosan and a polar organic solvent (such as ethanol) cause the stripping of water molecules from the enzyme. Therefore, a high a_w should be beneficial for the catalytic activity as observed in this investigation.

Depending on the conformation of the ibuprofen, the interaction in the case of *S*-ibuprofen is favored with water present and is not as favored without water. It seems that water changes the stereospecificity in the case of CALB, from *R* (the preferred one in general) to *S* (when water is present with the alcohol). In the case of no water near the catalytic triad, the *R*-ibuprofen shows a hydrogen near the oxygen of ethanol. This conformer seems to be slightly more stable than the *S*-ibuprofen where the alpha hydrogen interacts with the oxygen from the carbonyl group of the acyl enzyme. The methyl group bonded to the alpha carbon introduces an additional steric hindrance to the coordination of the oxygen from ethanol to the carbon of the acyl enzyme.

4. CONCLUSION

In this investigation, CALB was immobilized on supports of different nature and the resulting biocatalysts were applied to the esterification of rac-ibuprofen with ethanol without added co-solvents. Key evidences regarding the influence of the nature of the support and the immobilization on the catalytic features of a lipase were obtained. In this context, the following conclusions can be drawn:

• CALB adsorbed on hydrophobic supports is more active (in terms of conversion and enantiomeric excess) in the esterification of ibuprofen compared to the immobilization onto hydrophilic materials.

• The negative effect of the high enzymatic density on the conversion of the substrate was evidenced.

• Immobilization by covalent binding causes a decrease in the specific activity regardless of the physicochemical characteristics of the support

• The hydrophobic/hydrophilic nature of the support showed influence on enantioselectivity but not on the specific enzymatic activity.

• The influence of a_w on biocatalytic performance is different depending on the hydrophobic/hydrophilic nature of the support.

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FIGURES

FIGURE 1 Conversion (■) and specific activity SA (▲) versus enzymatic loading of biocatalysts with hydrophobic (1A) and hydrophilic supports (1B). The open squares (□) and open triangles (Δ) correspond to the conversion and specific activity of those biocatalysts with covalent nature, respectively.

FIGURE 2 Comparison of the specific activity SA of the free lipase (●) and immobilized through covalent bonding (●) and adsorption over hydrophobic (●) and hydrophilic (●) supports.

FIGURE 3 Enantiomeric excess ee$S$ % (●) vs. enzymatic loading of biocatalysts with hydrophobic (3A) and hydrophilic supports (3B). The open circles (○) correspond to those biocatalysts with covalent nature.

FIGURE 4 Enzymatic loading (gray circles ●), specific activity of the enzyme (black squares ■) and productivity (open squares □) of the biocatalysts as a function of the supports such as: AR, acrylic resin; PMMA, polymethylmethacrylate; PMMA PB, Novozym®435 provided by Piedmont Biofuels; PS, polystyrene; ZrO_2, zirconium oxide nanoparticles; PPL and PPL*, CALB/polypropylene tested in the esterification of ibuprofen using a ratio of biocatalyst: ethanol of 160 mg.mL^{-1} and 240 mg.mL^{-1}, respectively; TiO_2, titanium dioxide nanoparticles; SiO_2, silica nanoparticles; MAG and MAG*, CALB/magnetite tested in the esterification of ibuprofen using a ratio of biocatalyst: ethanol of 160 mg.mL^{-1} and 320 mg.mL^{-1}, respectively; CS and GLU/CS, bare chitosan and chitosan functionalized with glutaraldehyde.

FIGURE 5 Enantiomeric excess ee*S* % (open circles), enantiomeric ratio E (open triangles Δ) and enantiomeric factor EF (grey triangles ▲) of the biocatalysts as a function of the supports. Captions as presented in the figure 4.

FIGURE 6 Influence of the water activity on the specific activity of the esterification of ibuprofen with ethanol catalyzed with Novozym® 435 (▲), CALB/MAG (▲) and CALB/CS (▲). The open symbols corresponds to the specific activity of the non-controlled reaction media ($a_w = 0.7$).

FIGURE 7 Influence of the water activity on the enantiomeric excess towards *S*-ibuprofen in the esterification of ibuprofen with ethanol catalyzed with Novozym®435 (●) CALB/MAG (●) and CALB/CS (●).The open symbols corresponds to the enantiomeric excess of the non-controlled reaction media ($a_w = 0.7$).

FIGURE 8 3D molecular models of ibuprofen in the catalytic triad of the lipase (8A); lateral view of the profen with serine and histidine of the catalytic triad (8B) and lateral view with one (8C) and four water molecules (8D) shown in "stick " mode in the rectangle.

FIGURE 9 Molecular models of ethanol coordination to the acyl enzyme of *R*/*S*-ibuprofen with (A) and without (B) water near ethanol or carbonyl from acyl enzyme. These models were obtained with Chem3D Cambridge soft.

TABLE 1 Description of the nature and surface properties of the materials used as supports, protein-support interaction and enzymatic loading of the biocatalysts.

| BIOCATALALYST | SUPPORT MATERIAL | | Protein-support interaction | S_{BET} (m^2/g) | Pore size (Å) | ENZYME | |
	NATURE	SURFACE PROPERTY				Loading (mg/g) [*1]	Density (mg/m^2)
SPRIN liposorb CALB®	acrylic resin (AR)	Hydrophobic	Adsorption- Hydrophobic	36.4	298.7	50	1.4
Novozym®435	polymethylmethacrylate (PMMA)	Hydrophobic	Adsorption- Hydrophobic	72.0	273.9	100	1.4
Novozym®435 provided by Piedmont Biofuels SA	polymethylmethacrylate (PMMA PB)	Hydrophobic	Adsorption- Hydrophobic	72.0	273.9	70	1.0
SPRIN actisorb CALB®	polystyrene (PS)	Hydrophobic	Adsorption- Hydrophobic	179.8	115.7	70	0.4
SPRIN epobond CALB®	epoxy acrylic resin (EP)	Hydrophobic	Covalent bond	50.7	272.9	60	1.2
CALB/PPL	metallocenic polypropylene (PPL)	Hydrophobic	Adsorption- Hydrophobic	23.0[*2]	----	100	4.3
CALB/CS	chitosan	Hydrophilic	Adsorption-ionic interaction and hydrogen bond	4.0[*2]	----	20	5.0
CALB/GLU-CS	Functionalized chitosan	Hydrophilic	Covalent bond	4.0[*2]	----	20	5.0
CALB/TiO$_2$	TiO$_2$ anatase	Hydrophilic	Adsorption- ionic interaction and hydrogen bond	45.7	118.6	20	0.4
CALB/ZrO$_2$	ZrO$_2$	Hydrophilic	Adsorption- ionic interaction and hydrogen bond	39.0	168.0	100	2.6
CALB/SiO$_2$	SiO$_2$	Hydrophilic	Adsorption- ionic interaction and hydrogen bond	300.0	104.9	50	0.2
CALB/MAG	Functionalized Fe$_3$O$_4$	Hydrophilic	Covalent bond	79.5 [*2]	----	30	0.4

[*1] The enzyme loading corresponds to the amount of enzyme in milligrams obtained with ICP-AES analysis (with an uncertainty of +/- 1 mg) per gram of biocatalyst. [*2] This value is the BET area of the support without CALB. They are maximum values for the BET area of the

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biocatalyst. In the case of CALB/MAG, the surface area was calculated taking into account oleic acid adsorption (The pretreatment to measure BET with high vacuum and/or thermal treatment generates aggregation of MAG particles, obtaining mesoporosity).

TABLE 2 Steric energies of different conformations of *R*- and *S*-ibuprofen with and without water near the catalytic triad of CALB.

	R-Ibuprofen (kcal/mol)	*S*-Ibuprofen (kcal/mol)
Steric Energy- Ethanol far away	-241.6	-236.35
Ethanol acyl enzyme Coordinated to O Serine	-173.42	-138.53
Ethanol coordinated through Acyl Carbon-2 Water far away-Conf. 1	-181.1*	-177.6*
Ethanol Coordinated through acyl Carbon-2 Water far away-Conf. 2	-191.1*	-187.2*
Ethanol Coordinated through acyl enzyme with 2 water in H Bonding	**-201.137**	**-210.65**
Ethanol Coordinated through acyl enzyme without 2 water in H Bonding – Conformer 2	**-168.2**	**-166.5**

Conf. 1 is a conformer different than Conf. 2. The different name is related to different distances to Serine and different locations of ethanol methyl groups.

* The main point is that the difference in steric energy between R and S ibuprofen is similar (near 4 kcal /mol favoring R) whatever the conformer at this step, with water far away.

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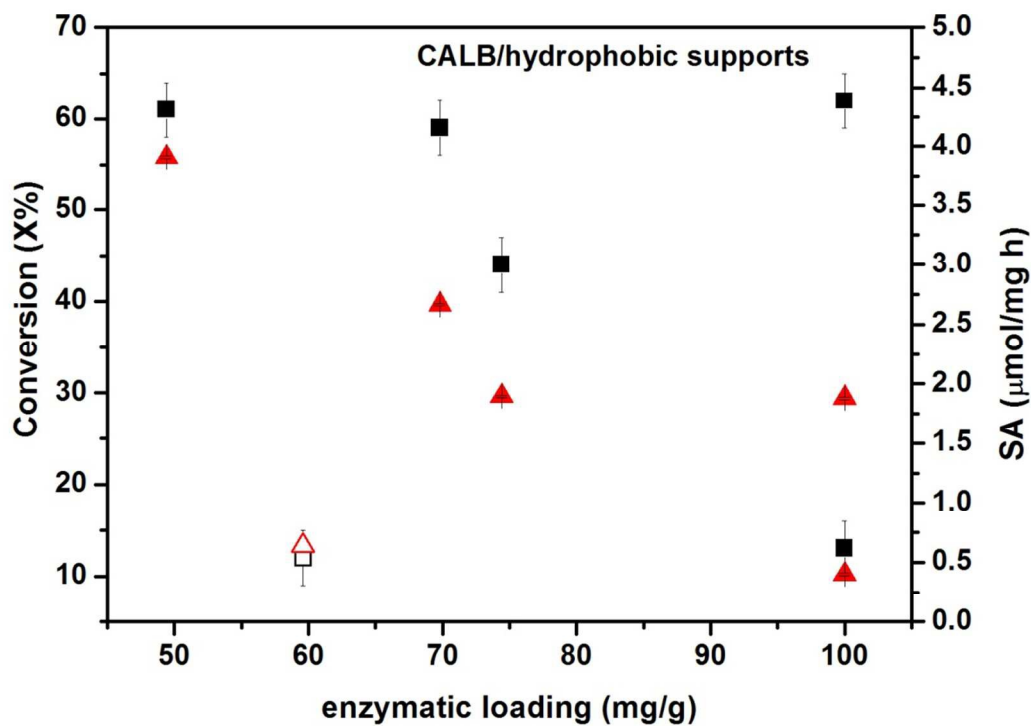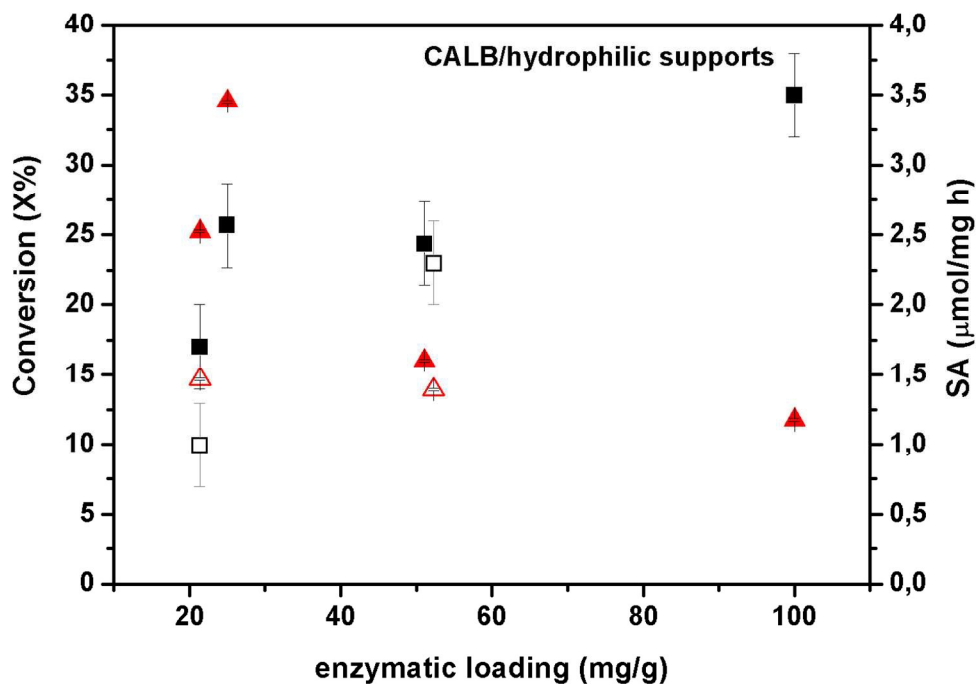
FIGURE 1A

FIGURE 1B

FIGURE 2
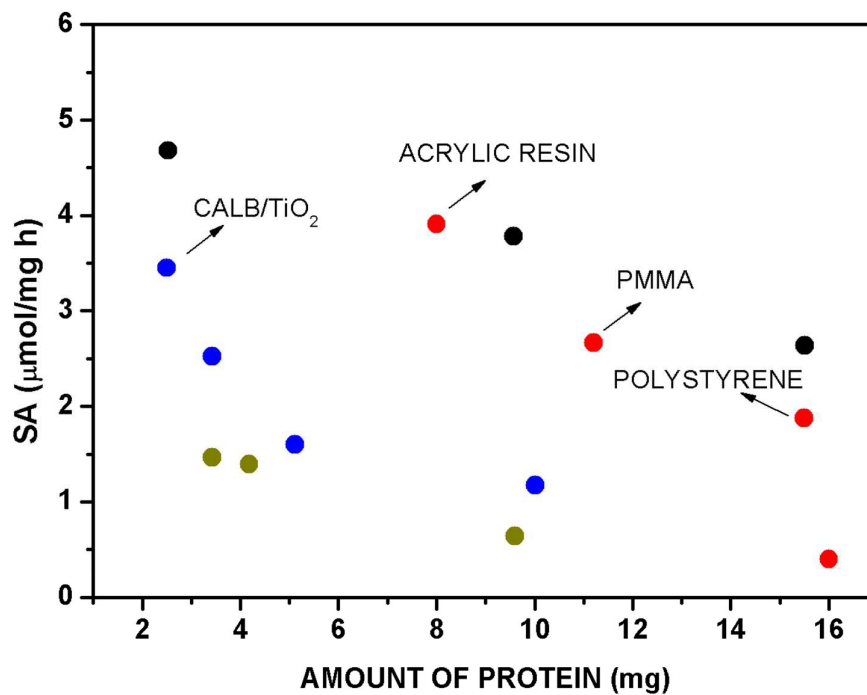
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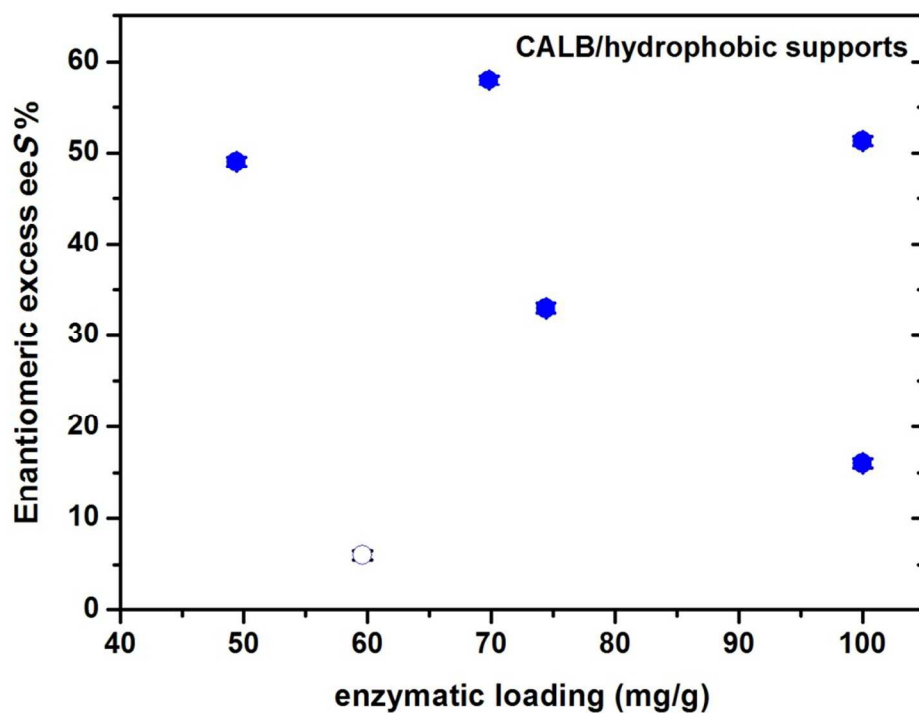
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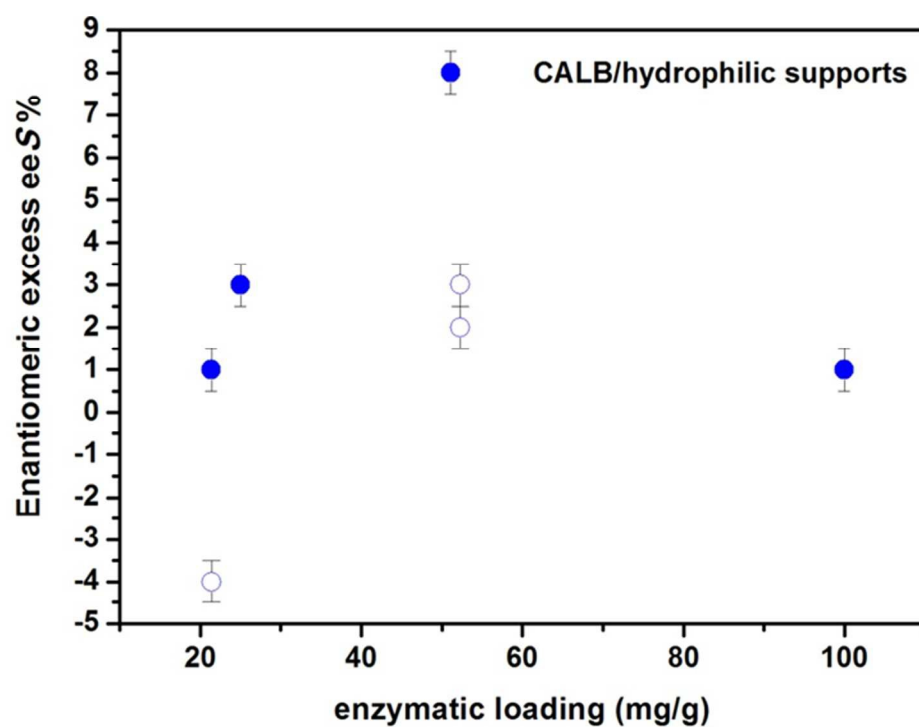
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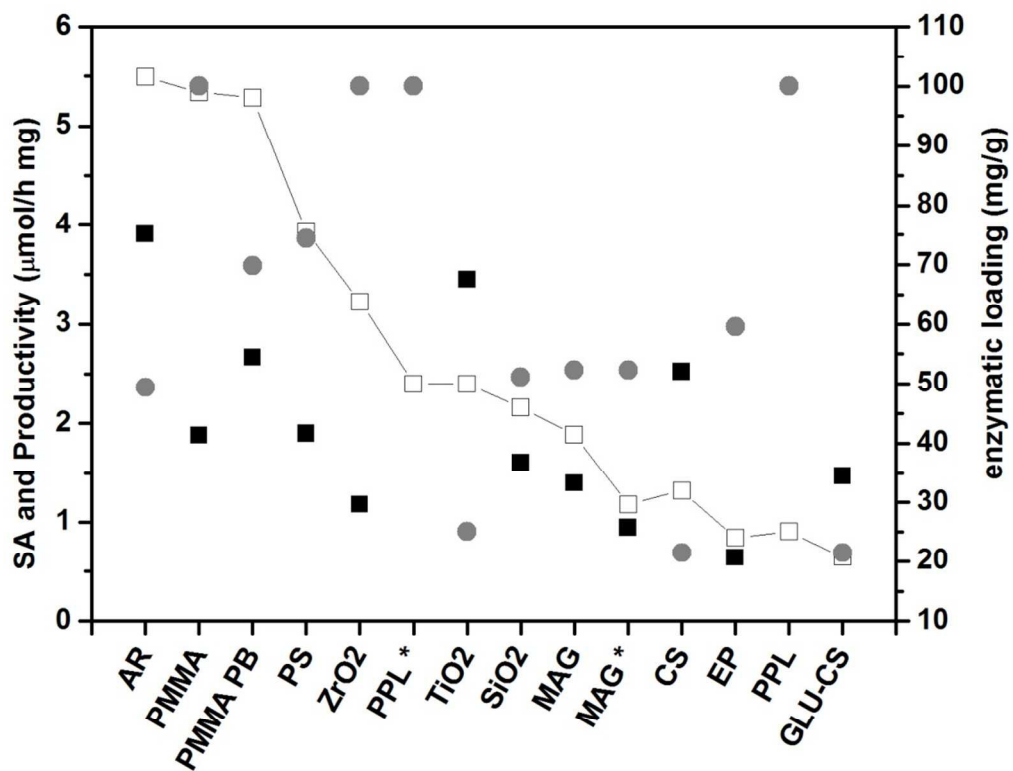
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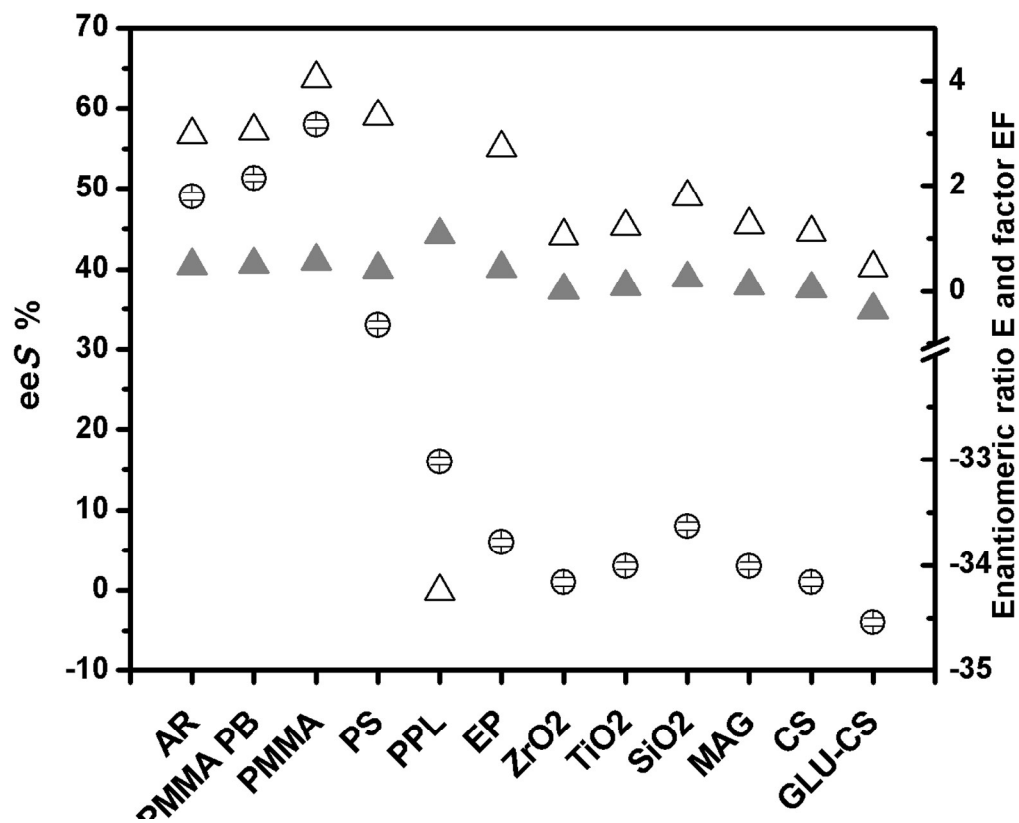
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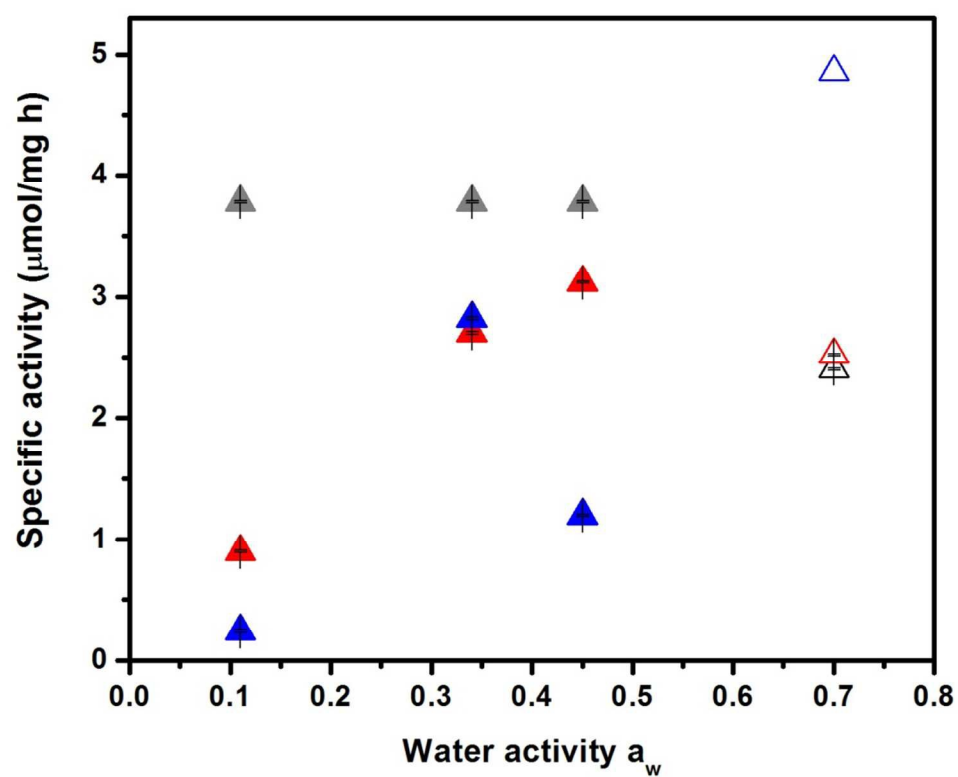
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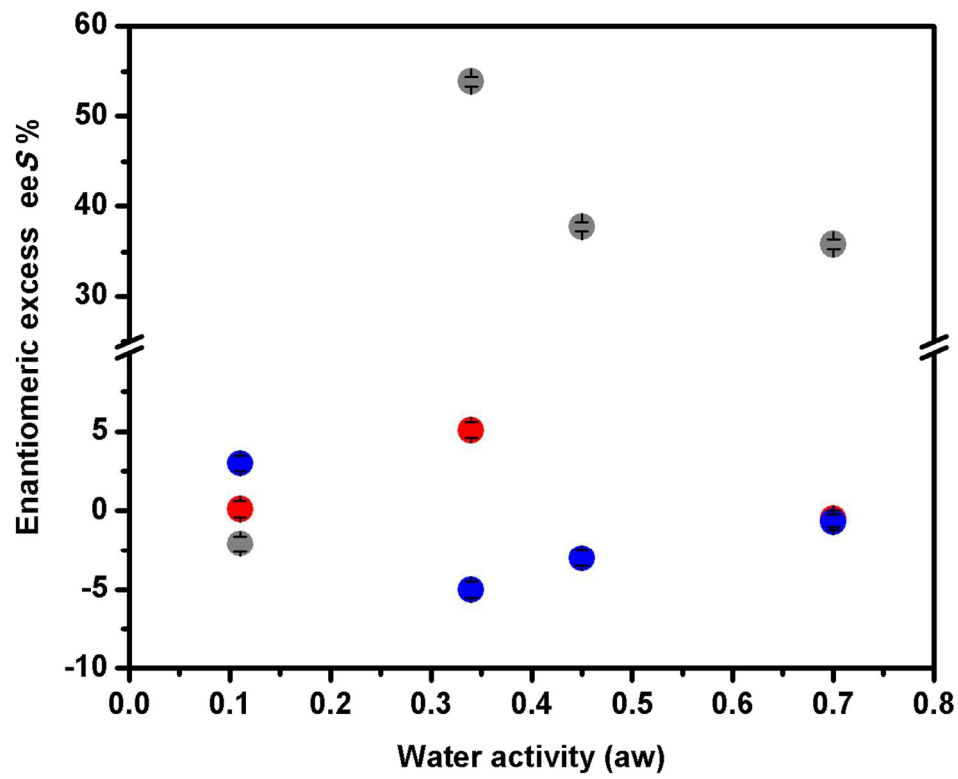
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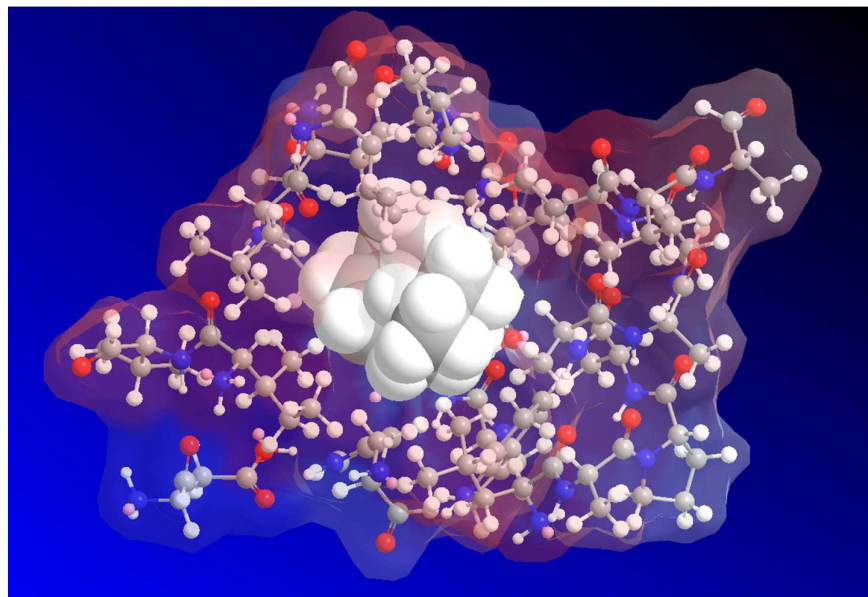
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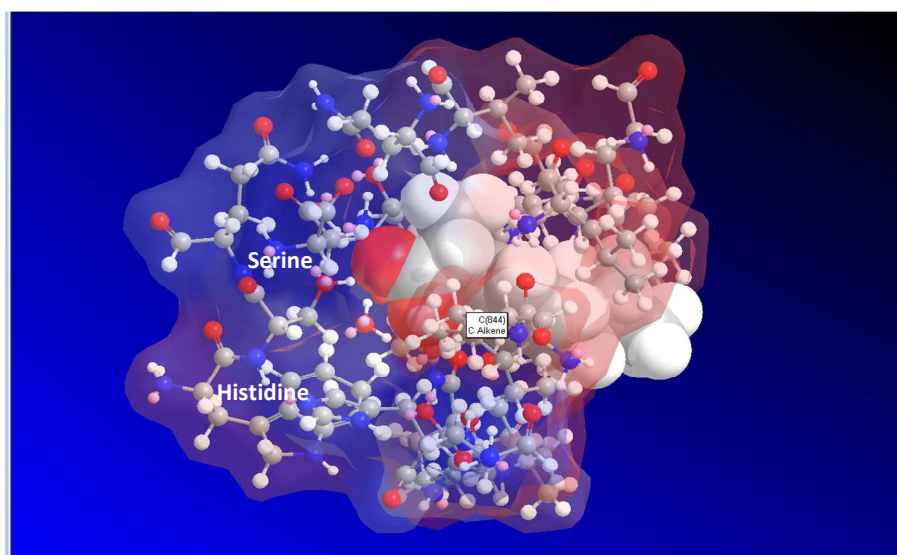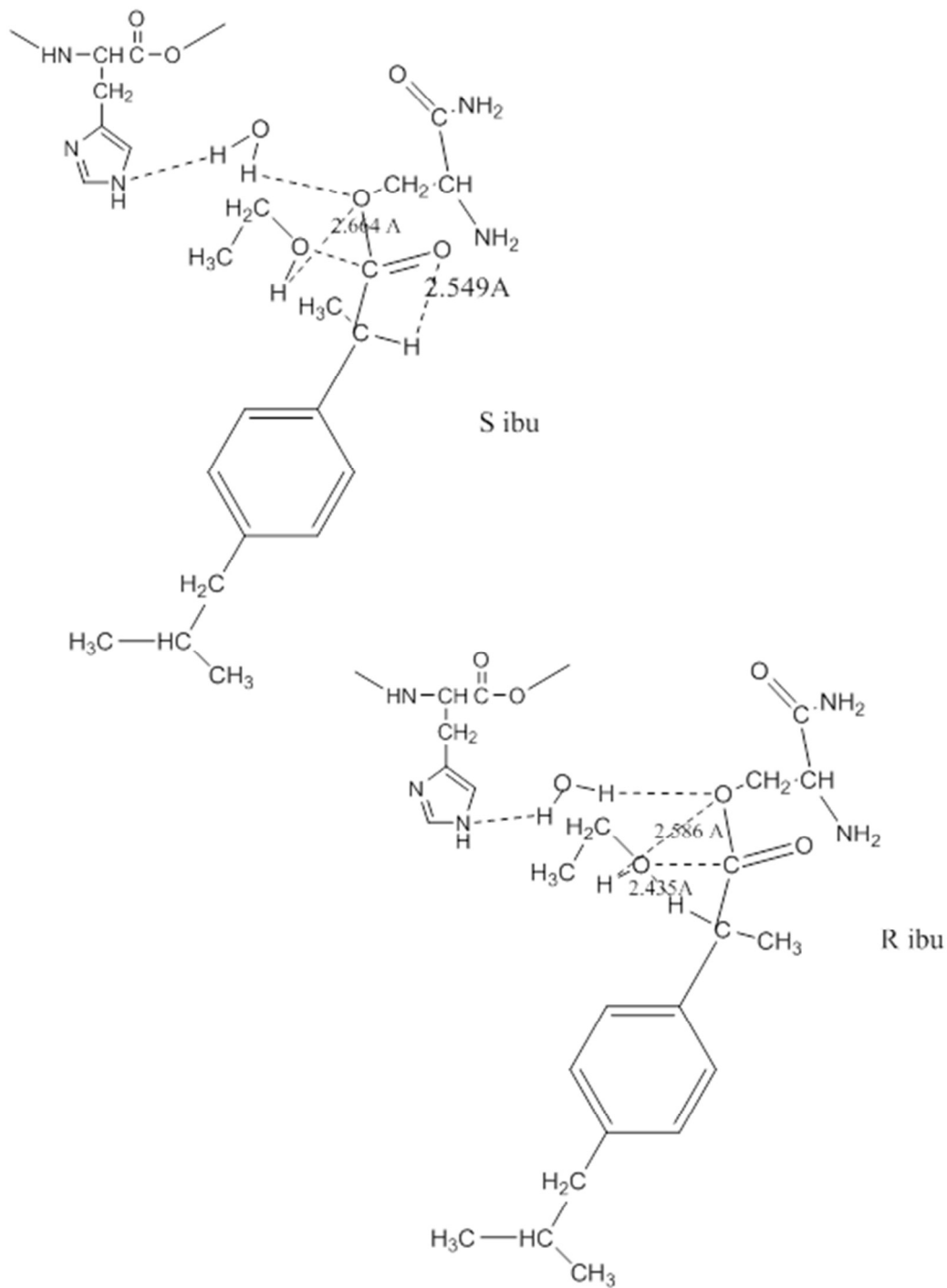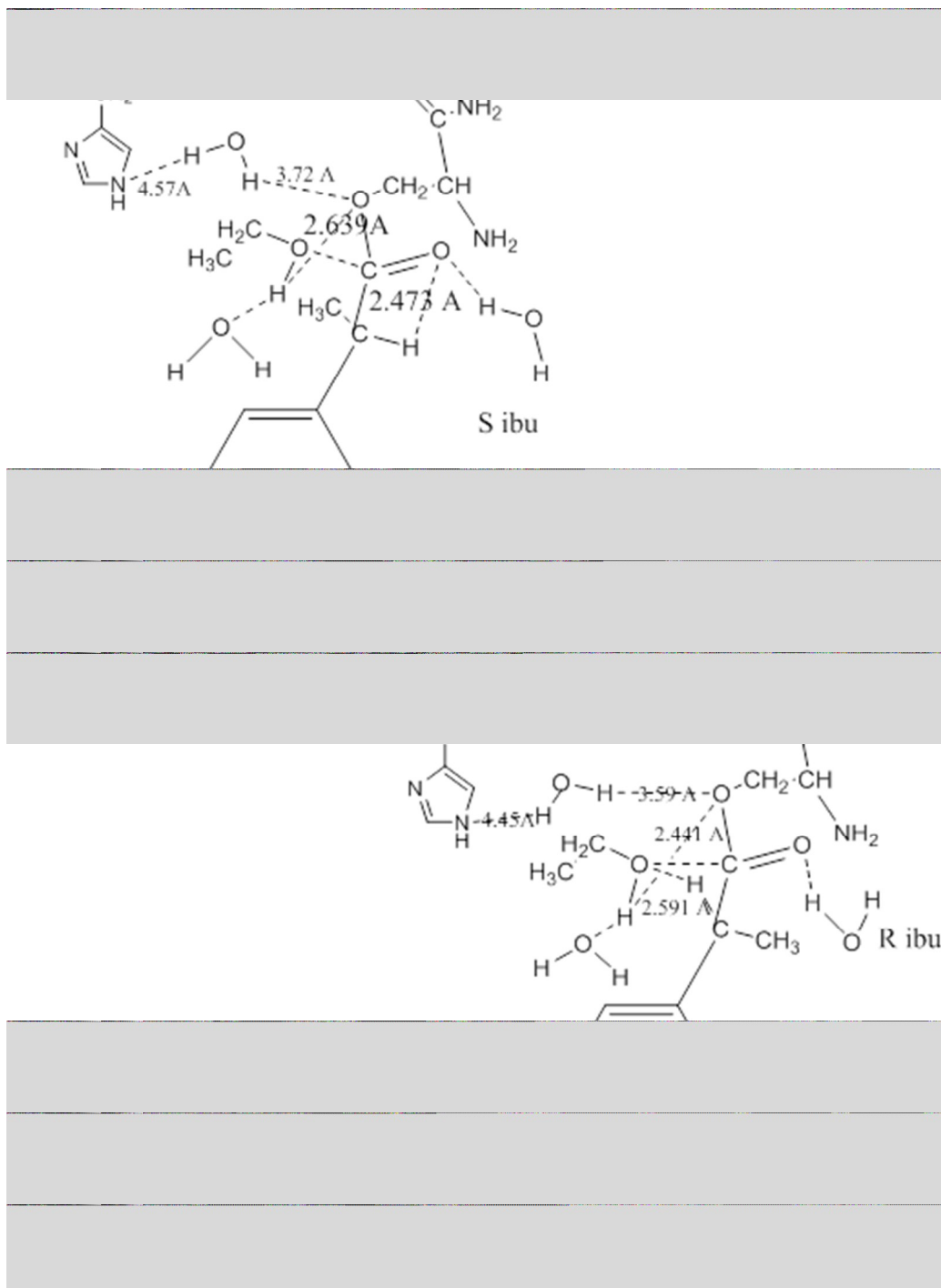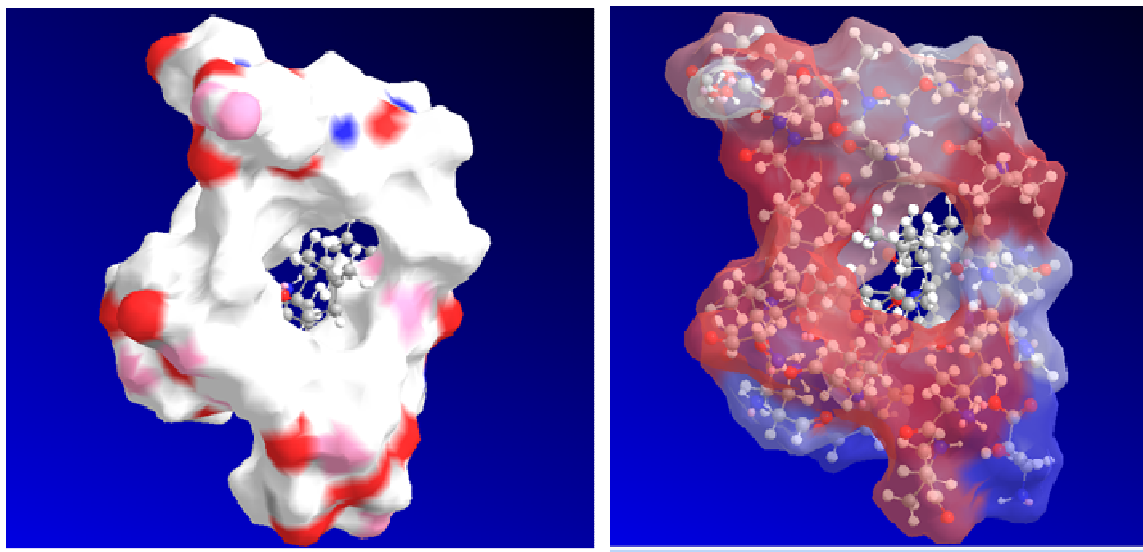
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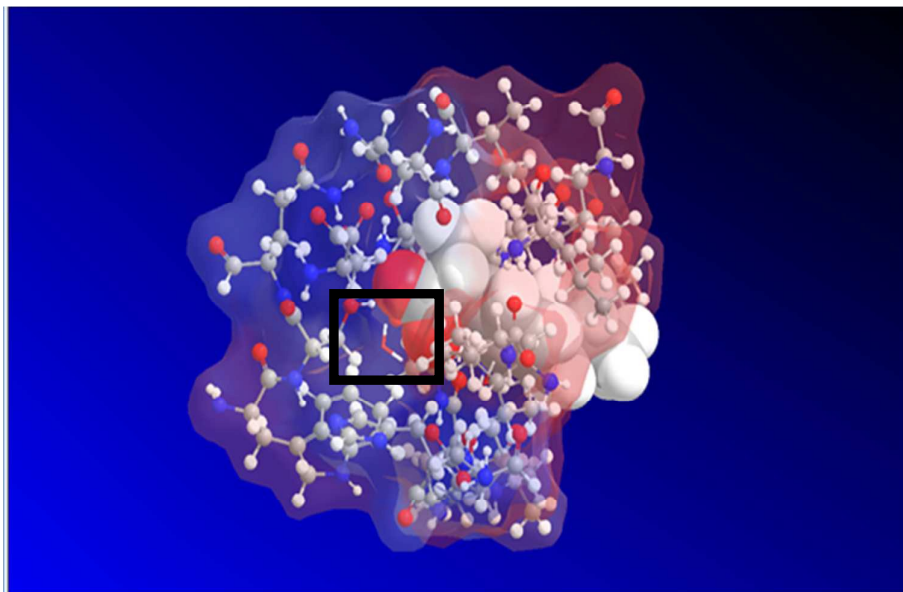
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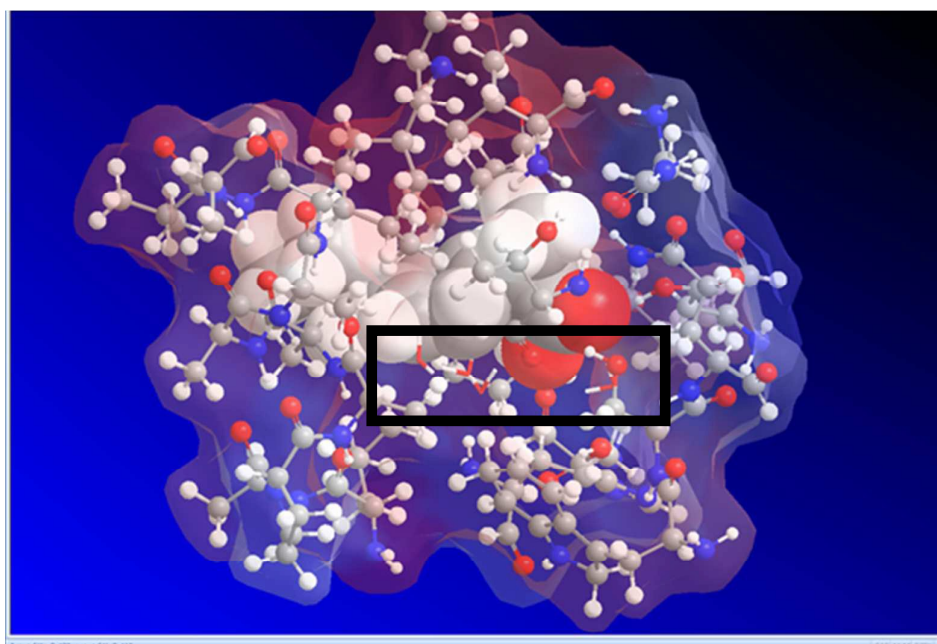
FIGURE 9A

FIGURE 9B

CALB immobilized on hydrophobic supports possesses higher conversion of ibuprofen and enantiomeric excess towards the *S*-enantiomer.