Research Article

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Lipase B of *Candida antarctica* co-adsorbed with polyols onto TiO_2 nanoparticles for improved biocatalytic performance

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Abstract

BACKGROUND: The immobilization of the lipase B of *Candida antarctica* CALB over TiO_2 nanoparticles was thoroughly investigated with the isotherms of adsorption at various temperatures with and without the addition of sorbitol and glycerol. The surface composition, secondary structure and the effect of the addition of the polyols was addressed.

RESULTS: The maximum dispersion limit of protein on TiO_2 nanoparticles (NPs) is 0.073 ± 0.007 μmol m^{-2}. Glycerol and sorbitol co-adsorb on the TiO_2 NPs reaching 45% of the surface composition of the biocatalyst. The optimized material was able to catalyze the esterification of 52% of R/S-ibuprofen with ethanol (0.31 ± 0.01 μmol min^{-1} mg^{-1}) with 41% of enantiomeric excess towards S(+)-ibuprofen in 24 h reaction. Under similar reaction conditions, the commercial counterpart Novozym® 435 showed 34% conversion (0.091 ± 0.003 μmol min^{-1} mg^{-1}) and 16% of enantiomeric excess.

CONCLUSIONS: The molecular association between the protein and the polyols exerts a positive cooperativism which prevents aggregation of the protein and protects its active conformation. The residual esterase activity of the immobilized CALB compared with the free lipase depends directly on the amount of co-adsorbed polyols. Moreover, polyols boost the catalytic performance in the kinetic resolution of racemic ibuprofen showing an optimum at the maximum coverage of polyols on the biocatalysts.

Supporting information may be found in the online version of this article.

Keywords: lipases; TiO_2 NPs; biocatalysis; immobilization; adsorption

INTRODUCTION

Immobilized enzymes are those physically confined or localized in a certain defined region of space with retention of their catalytic activities that can be used repeatedly and continuously, improving the economy of the process.[1,2] Immobilization is a strategy to improve enzyme stability in order to increase their applications, especially in those processes involved in the food and pharmaceutical industries, drug delivery technologies, bio-affinity chromatography and biosensors, among others. Immobilization strategies are basically of five types: simple adsorption, covalent binding, entrapment, crosslinking and affinity bonds.[3,4] Among them the easiest method for enzymatic immobilization is physical binding (adsorption). The adsorption mechanism involves interactions forces, such as van der Waals, hydrophobic and electrostatic. The main disadvantage of this technique is the desorption of the active surface layer due to changes in pH, temperature and ionic strength, among other factors, which leads to poor operational stability.[5] On the other hand, this could also be regarded as an advantage, because when the enzymatic activity of the biocatalyst decays, the support can be easily regenerated and re-loaded with fresh enzyme.[2]

The immobilization strategy is chosen according to the final application of the immobilized enzyme. When the biocatalysts produced are used in organic solvent media (in reactions such as enantiomeric resolution, biodiesel production and other condensation reactions), adsorption onto inert supports seems the strategy of choice. The decrease in the catalytic activity of the enzymes is related to their aggregation and denaturation due to loss of structural water. Immobilization, thus, spreads the enzyme on a relatively large surface area, enabling the mass transfer of substrates and products and increasing its catalytic activity.[6] The nature and

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morphology of the material used as support are of key importance as well as the enzyme loading since the best specific activity is often obtained when the enzyme forms a monolayer on the surface of the support.[7]

Metal oxides, silica and other minerals are inorganic supports highly resistant and stable to adverse effects that could be produced in the reaction media.[8] Nowadays iron oxides such as magnetite are among the most widely used, owing to their magnetic properties. In addition, oxide based supports are functionalized to allow a more efficient attachment of the enzyme.[5,9,10] SiO_2 based materials are also widely used for simple adsorption immobilization processes as mesoporous supports (allowing the regulation of pore diameter) or granulated silica.[6,11] Silica together with other organic and inorganic supports was also used to immobilize *Rhizomucor miehei* lipase either by covalent binding or by simple adsorption for ethanol esterification with oleic acid. Interestingly, the best esterification yields were obtained when the enzyme was immobilized through simple adsorption on silica.[12] Castro *et al.* reported the use of amorphous and crystalline niobium oxide as supports for immobilization of *Candida rugosa* lipase either by physical adsorption or by covalent. In this case, the niobium oxide beads were treated with γ-aminopropyltriethoxysilane and glutaraldehyde.[13] The biocatalysts were tested in the esterification of n-butanol with butyric acid in heptanes and the operational stability was measured. The investigation demonstrated that the biocatalysts obtained by simple physical adsorption had poor operational stability, whereas this parameter was significantly improved when the enzyme was attached by covalent binding to the support.

A summary of the literature concerning the use of titanium based materials as support of lipases, procedures of immobilization, applications and biocatalytic performance is presented in Annex 1 of the Supplementary information.[14–21] In this context, the co-adsorption (or entrapment) of the enzymes with cellulose acetate, poly(sodium acrylate)-g-methoxypoly(ethylene oxide) and polyacrylamide mixed with titanium dioxide have been investigated as suitable matrices for the preparation of active biocatalysts.[14,15,19–21] The application of TiO_2 as a single support for enzyme immobilization is much less reported in literature.[17,18] Previous work described the preparation of a biocatalyst through the adsorption of a plant peptidase (araujiain) onto TiO_2.[17] Similarly, a biocatalyst based on the immobilization of commercial CALB (Sigma Aldrich) onto titania was characterized in terms of structural changes of the adsorbed protein.[18] Other metallic oxides, such as Al_2O_3, SnO_2, ZrO_2, Fe_2O_3 and TiO_2 were used for coating WO_3 in order to obtain different oxide based supports for simple adsorption of *Psedomonas cepacia* lipase. The biocatalysts were successfully applied in the racemic resolution of methyl (±)-mandelate ((±)-1) in different ionic liquids.[16]

The most popular immobilized lipase-based biocatalysts are those obtained by contact of the enzyme with hydrophobic supports, showing interfacial activation.[1] There is no doubt that the commercial biocatalyst Novozym® 435 composed of CALB immobilized on beads of polymethylmethacrylate is by far the most widely investigated.

However, it has been demonstrated that short chain alcohols (methanol, ethanol, 1- and 2-propanol) dissolve the macroporous resin.[22–24] With this in mind, the use of alternative supports, such as metal oxides, represents an interesting alternative. The present investigation extends a previous report about the immobilization of CALB onto titania nanoparticles (TiO_2 NPs). This contribution addresses the immobilization of a commercial extract of CALB with the controlled addition of polyols at various temperatures. Structural changes in the adsorbed enzymes were investigated and correlated with their biological activity in terms of hydrolysis and esterification reactions. Finally a self-assembled model between the lipase and the polyols on the surface of the TiO_2 NPs is proposed.

MATERIALS AND METHODS

Chromatographic purification

The method used to obtain and purify the lipase B of *Candida antarctica* from the crude extract Lipozyme® was reported previously.[25] The purification through size-exclusion chromatography was performed in an Äkta purifier equipment using Sephacryl S-100 (GE Healthcare) packed in a XK 16/40 column (GE Healthcare). After removal of the nonsoluble fraction, a volume of 1.0 mL of the crude extract was loaded onto the column at a volumetric flow rate of 0.5 mL min^{-1} and eluted with 1.5 column volumes (CV) of the buffer 0.1 mol L^{-1} ammonium acetate-ammonia ($NH_4CH_3CO_2$-NH_3) at pH 8.4. The eluted fraction containing the lipase B of *Candida antarctica* was separated and lyophilized.

Immobilization onto TiO_2: isotherms of adsorption

The purified lipase and the crude extract (with and without the addition of sorbitol and glycerol) were immobilized on titanium dioxide through a conventional adsorption method. The nanoparticles of titanium dioxide of the anatase phase (Aeroxide® P-18 Evonik Ind., 43.1 ± 0.1 m^2 g^{-1}) were 20 nm on average and previously calcined at 500°C for 12 h. The oxide support was contacted with aqueous solutions (prepared with deionized distilled water) of protein ranging from 0.29 mg mL^{-1} to 1.10 mg mL^{-1} from purified CALB and the commercial crude extract (CE) in order to assess the isotherm of adsorption at various temperatures. Further experiments were performed with a fixed volume of crude extract (1.20 mL) with the addition of variable volumes (0–5.0 mL) of an aqueous solution containing 25 w/v% of sorbitol and 25 w/v% of glycerol.

The preparations were performed by contacting 20.0 mL of the solution containing the lipase with 100.0 mg of TiO_2 in duplicate. Each system was stirred either in a shaker (200 rpm) or by magnetic stirring in order to investigate the effect of stirring on the preparation.

After incubation, the mixtures were centrifuged at 9600xg for 20 min in 50 mL falcon tubes. The supernatants were separated for further analysis and the solids were re-suspended in distilled water and centrifuged under similar conditions. The supernatants were separated to analyze. The solids were freeze dried for 48 h.

The amount of lipase adsorbed in the course of the immobilization was determined through the Bradford method. The analysis was performed over the starting solutions and the series of samples (0.50 mL) of the supernatant solution that were taken during the preparation. The supernatant (50 μL) was centrifuged and mixed with 2.50 mL of the Bradford´s reagent and the absorbance was measured at 595 nm in an Agilent 8453 E spectrophotometer. The concentration of protein was determined through a calibration curve performed with CALB Sigma Aldrich as described previously.[25] Each assay was performed four times to establish the error associated with the measurement.

In general, the biocatalysts are named CALB/TiO_2 preceded by the amount of immobilized protein in terms of micromols of protein (the molecular weight of the protein is 33 478 Da) per unit surface area of titanium dioxide.[26] In addition, the word CALB has

a subscript such as: $CALB_{P30}$, $CALB_{CE30}$ and $CALB_{GS30}$ indicating that the material was prepared either with purified lipase (P), the crude extract (CE) or the addition of glycerol and sorbitol (GS) and the temperature of the adsorption is also indicated (in this example, 30°C).

Mechanism of adsorption

The fitting of the adsorption equilibrium data (isotherm of adsorption) to the Langmuir, Freundlich and Hill models was investigated.[27–29] The inverse of the amount of protein adsorbed (C_{ads}, μmol m^{-2}) versus the inverse of the concentration of protein in contact with the TiO_2 NPs at equilibrium (C_{eq}, mg mL^{-1}) was represented according to the linear form of the Langmuir model. The Langmuir model corresponds to Equation (1) and the linear form to the Equation (2):

$$C_{ads} = Q_{MAX}\frac{K_L\, C_{eq}}{1 + K_L C_{eq}} \tag{1}$$

$$\frac{1}{C_{ads}} = \frac{1}{Q_{MAX}} + \frac{1}{K_L Q_{MAX}}\frac{1}{C_{eq}} \tag{2}$$

where Q_{MAX} is the maximum amount of protein adsorbed to form a monolayer and K_L is the Langmuir adsorption constant that indicates the affinity of the binding sites.

The Freundlich model was investigated through the linear fit of the logarithm of the amount of protein adsorbed (C_{ads}, μmol m^{-2}) as a function of the logarithm of the concentration of protein at the equilibrium (C_{eq}, mg mL^{-1}) with their corresponding errors. The Freundlich model corresponds to Equation (3) and the linear form to Equation (4),

$$C_{ads} = K_F C_{eq}^{\frac{1}{nF}} \tag{3}$$

$$\log C_{ads} = \log\ K_F + \frac{1}{n_F}\ \log C_{eq} \tag{4}$$

where K_F and n_F indicate the adsorption capacity and adsorption intensity, respectively.

In addition, Equation (5) which corresponds to the Hill model was applied to the data of the isotherm of adsorption,

$$C_{ads} = \ Q_{MAX}\ \frac{C_{eq}^{n_H}}{\left(\ K_H + C_{eq}^{n_H}\right)} \tag{5}$$

where Q_{MAX} is the maximum capacity of adsorption of the support; K_H is the Hill constant and n_H is the cooperativity coefficient of the binding interaction.

The fitting to a linear (Langmuir and Freundlich models) and sigmoidal (Hill model) function was determined with OriginPro 8.0 software. The best fitting was estimated by maximizing the determination coefficient R^2 and minimizing the residual sum of squares Σ^2.

Quantification of the immobilized protein with electron microprobe analysis

The amount of immobilized protein on TiO_2 was calculated as the difference between the initial concentration of protein of the solution in contact with the oxide support and that in the supernatant solution at the end of the adsorption. In addition, X-ray microanalysis EDS was applied to quantify the amount of carbon, oxygen and titanium in order to calculate the amount of adsorbed protein. The analysis was performed on highly pure CALB, TiO_2 and the immobilized biocatalysts compressed in thin wafers that were fixed on an aluminum sample holder using silver based paint to avoid any contamination with carbon. The analyses were carried at 0.3 and 0.6 Torr, 12.5 kV, spot 7 and 80 s count with an equipment EDAX SS Apollo 40 coupled to an ESEM microscope FEI Quanta 200.

Determination of the secondary structure of the protein through isotopic exchange and in situ infrared spectroscopy

The secondary structure of the immobilized protein was determined through isotopic exchange with D_2O coupled with diffuse reflectance infrared spectroscopy DRIFTS. The spectra were recorded using a Harrick module with praying mantis mirrors set-up (Harrick Scientific Co). A Nicolet 8700 FTIR spectrometer with a MCT-A cryogenic detector was used to acquire the spectra (4 cm^{-1} resolution, 100–250 scans). The spectrometer and the mirrors that direct the radiation towards the cell are continuously purged with dry air (from a Parker Balston generator) in order to eliminate the contribution of CO_2 and water vapor from the spectra.

This isotopic exchange with deuterium oxide coupled with infrared analysis allows investigation of the Amide I signal (1700–1600 cm^{-1}) without the interference of the bending vibration of O–H species that typically appears at 1640 cm^{-1}. The percentage contribution of each structure was obtained through the deconvolution, integration and further normalization of the corresponding signals involved in the Amide I. The details of the methodology were published before.[23,24]

Determination of the esterase activity

The esterase activity of free and immobilized CALB was determined using *p*-nitrophenyl dodecanoate (Sigma Aldrich) as substrate.[25,30] The enzymatic release of *p*-nitrophenol was monitored by increasing absorbance at 405 nm (molar absorptivity ε: 17983 $M^{-1}cm^{-1}$) in an Agilent 8453 E spectrophotometric system.[25] The biocatalytic activity was calculated using the initial rate of reaction in saturating substrate condition. The enzymatic activity was expressed in International Units (IU), one unit being the amount of enzyme that releases 1 μmol of *p*-nitrophenol/min under the assayed conditions.

The esterase activity of the free lipase (i.e. in a homogeneous system) was assayed with 100 μL of the sample and 2.70 mL of buffer Tris–HCl buffer (0.1 mol L^{-1}, pH 8.0) and Triton X-100 (0.0075 % v/v in the reaction medium). This system was maintained at 37°C in the thermostatized cell of the spectrophotometer. The substrate (200 μL of a 2 mmol L^{-1} solution with a final concentration of 0.148 mmol L^{-1} in the reaction media) was added after reaching the desired temperature and the absorbance was measured every 5 s for 90 s in order to obtain the initial rates.

The hydrolytic activity of the immobilized lipase was assayed with 2.5 mg of the biocatalyst and 17.1 mL of the previously mentioned buffer, incubated in thermostatized glass flasks at 37°C and kept with magnetic stirring for 30 min before addition of the substrate (900 μL of a solution 2 mmol L^{-1} with a final concentration of 0.105 mmol L^{-1} in the reaction media). Samples (800 μL) were withdrawn until the end of the reaction (about 10 min). The enzymatic reaction was stopped in an ice bath, centrifuged in the cold to separate the supernatants, and the absorbance measured. The fact that the auto-hydrolysis of the substrate is negligible was demonstrated through similar experiments to those described without enzyme (blank assays).

The desorption of protein out of the oxide support was assayed through determination of the esterase activity of the supernatant solution in contact with the biocatalyst exposed to a buffer solution under similar conditions as described before.

Esterification of racemic ibuprofen with ethanol

The esterification of racemic ibuprofen with ethanol was assayed in order to investigate the catalytic activity of the immobilized CALB. In addition, the activity of the commercial biocatalyst Novozym® 435 was determined for comparison purposes.

Esterification of the profen was performed with and without co-solvent added. In the former situation, 10 mL of a solution of R/S-ibuprofen 0.12 mol L^{-1} (Parafarm 99%) and ethanol 0.12 mol L^{-1} (Carlo Erba pro-analysis) in isooctane (Merck pro-analysis) was assayed with 20 mg of biocatalyst. The solubility of the profen in isooctane allowed an equimolar (1:1) ratio of ibuprofen and alcohol to be obtained. Additional experiments varying the molar ratios profen:ethanol (1:3, 1:5 and 1:7) were also performed.

The alcohol was used as acyl-acceptor and solvent in the second set of experiments. In this context, a volume of 1.45 mL of R/S-ibuprofen 2.4 mol L^{-1} in absolute ethanol containing 4.8% of added water (alcohol:profen molar ratio equal to 7:1) was assayed with 20 mg of biocatalyst.

The reaction was performed at 45°C under orbital shaking (200 rpm) in glass flasks. The temperature of reaction corresponds to the optimum for the kinetic resolution of profens with the commercial Novozym® 435 as described in previous investigations.[23,24,31] The conversion of ibuprofen and the enantiomeric excess towards the S(+)-enantiomer were determined through titration with potassium hydroxide and chiral HPLC analysis, respectively. Details of the analysis are published elsewhere.[23,24,31] The specific activity was calculated as the micromols of ibuprofen converted to esters per milligram of protein per time of reaction in minutes. Novozym® 435 contains 15.5 mg of protein per 100 mg according to our previous investigations.[23,24]

In addition, the enantiomeric ratio *E* was calculated with the conversion of ibuprofen (*c*) and the enantiomeric excess of the substrate S(+)-enantiomer (ee_s) according to Equation (6) reported by Chen *et al.*[32]

$$E = \frac{\left[\ln\left(1-c\right)\left(1-ee_s\right)\right]}{\left[\ln\left(1-c\right)\left(1+ee_s\right)\right]} \quad (6)$$

Stability of the biocatalyst

The stability of the biocatalysts was investigated in terms of desorption of the active enzyme towards the reaction media (composed mainly of isooctane-ethanol or solely the alcohol) used for the esterification of ibuprofen. A certain amount (20 mg) of the biocatalyst 0.06 $CALB_{GS30}/TiO_2$ was contacted with 10 mL of a mixture containing 0.12 mol L^{-1} of ethanol in isooctane mimicking the reaction media used for the esterification of the profen. Alternatively, a similar amount of biocatalyst was contacted with 1 mL of ethanol containing 4.8% of added water. The systems were maintained at 45°C for 24 h at 200 rpm in a shaker bath. The biocatalyst and the supernatant solution were separated by centrifugation. Then, the corresponding 'fresh' reaction mixture (similar to those described earlier) was added to the solid and the reaction carried out for 24 h.

On the other hand, a certain amount of ibuprofen was added to the supernatant solution in order to achieve 2.4 mol L^{-1} and 0.12 mol L^{-1} of the profen in ethanol and ethanol-isooctane, respectively. These mixtures were maintained at 45°C, 200 rpm for 24 h allowing reaction in a homogeneous fashion if the lipase was in solution. The assays were performed in triplicate.

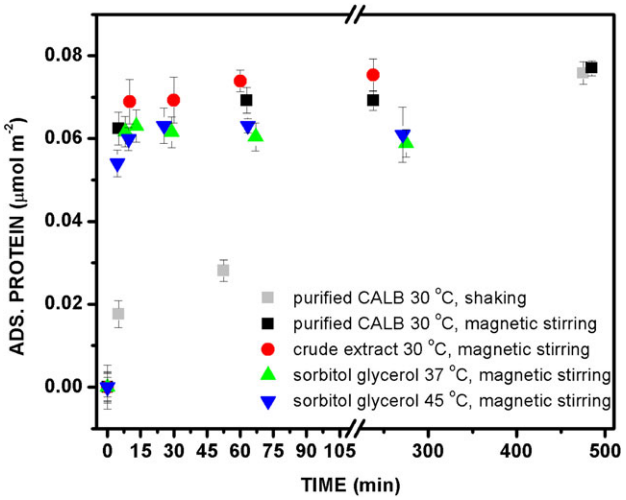


Figure 1. Protein adsorbed as a function of the time of contact between an aqueous solution (0.8–1.0 mg mL^{-1} of protein) of the lipase B of *Candida antarctica* (CALB) with TiO_2 NPs at various temperatures. Magnetic stirring and orbital shaking conditions are compared.

RESULTS AND DISCUSSION

Insights to the immobilization of CALB over an oxide support: maximum dispersion limit of protein on the TiO_2 NPs

Figure 1 shows the amount of protein adsorbed in μmol of protein per unit surface area as a function of time when the TiO_2 NPs were contacted with 0.8 to 1.0 mg mL^{-1} of the purified CALB (under shaking and magnetic stirring) at 30°C. Additional experiments were performed at 30°C, 37°C and 45°C under magnetic stirring using the crude extract (with and without sorbitol and glycerol) as the source of the enzyme. In particular, only the data containing 0.3 mol L^{-1} and 0.6 mol L^{-1} of sorbitol and glycerol, respectively is shown for brevity.

The figure shows that the adsorption of protein is almost complete in the first 25 min contact of the TiO_2 NPs with the enzyme when the preparation is performed under magnetic stirring regardless of the temperature. The process is clearly slower under shaking than magnetic stirring, indicating mass transfer constraints. The results demonstrate that 23% of the total protein was adsorbed in the first 5 min of contacting the oxide support with the protein solution under shaking at 30°C. However, the amount of protein adsorbed increased to 81% of purified CALB and 91% of protein of the crude extract when magnetic stirring was used at the same temperature.

In this context, the adsorption isotherms were performed under magnetic stirring in order to achieve an optimum contact (low mass transfer) between the protein and the NPs. Figure 2 shows the isotherms of adsorption, i.e. the amount of protein adsorbed per unit surface area, as a function of the concentration of protein remaining in contact with the support when the adsorption reached equilibrium. Purified protein, crude extract, the addition up to 0.3 mol L^{-1} of sorbitol and 0.6 mol L^{-1} of glycerol and various temperatures (30°C, 37°C and 45°C) were assayed. The amount of adsorbed protein increased upon contacting the oxide support with an initial solution containing from 0.35 mg mL^{-1} to 0.68 mg

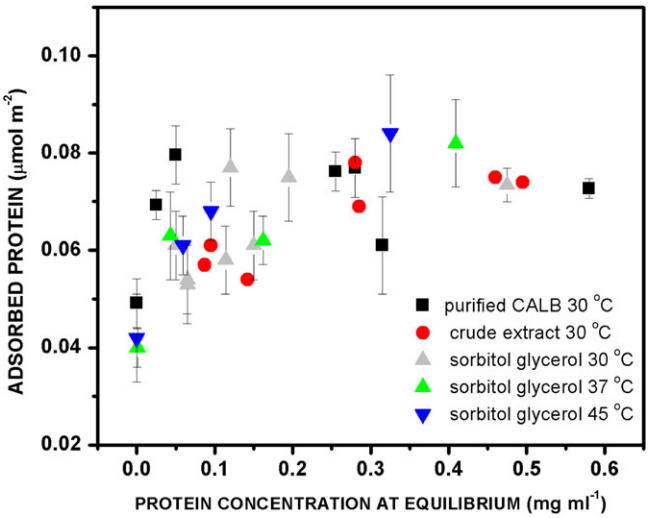


Figure 2. Isotherms of adsorption of protein over TiO_2 NPs in contact with aqueous solutions of purified CALB, crude extract and with the addition of sorbitol and glycerol at various temperatures.

mL^{-1} (0.2 mg mL^{-1} of protein at the equilibrium) regardless of the source of enzyme or the presence of additives. Above this last concentration the amount of adsorbed protein leveled off evidencing that the maximum dispersion limit was reached.

The Langmuir, Freundlich and Hill models were applied in order to assess the mechanism of adsorption.[27–29] Table 1 shows the experimental maximum dispersion limit of protein according to the isotherms of adsorption and the fitting of the experimental data to the adsorption models. In addition, the theoretical maximum dispersion limit (Q_{MAX} and K_F) according to the three adsorption models, the coefficient of determination R^2, the residual sum of squares Σ^2; and the various constants derived from the models are presented.

Experimentally, the maximum dispersion limit of protein onto the TiO_2 NPs is on average 0.073 μmol m^{-2} (with standard deviation 0.007), equivalent to 10.54 mg of protein per 100 mg of support. Moreover, the maximum dispersion limit obtained in this investigation differs only by 29% from the value reported previously.[18]

A statistical analysis of the errors (R^2 and Σ^2) shows that the equation of the Hill model (Equation (5)) provides the best fit to the adsorption of protein (purified, crude extract and with added polyols) at 30°C. This model is applied to non-ideal competitive adsorption of various species on a homogeneous substrate. The Hill model assumes that the adsorption is a cooperative phenomenon since the binding of an adsorbate on an adsorption site might influence the binding ability of other sites. Considering the assumptions of this model it is not surprising that the material 0.06 $CALB_{GS30}/TiO_2$ that was prepared with protein and polyols shows the best fit to this model.

Table 1 shows that the experimental maximum dispersion limit of the materials prepared at 30°C is in agreement with the theoretical value Q_{MAX} provided by the Hill model. There is also a fairly good agreement with the values obtained with the Langmuir model even though the fitting of the data to that model is not the best. In addition, the values of n_H and K_L of 0.08 $CALB_{CE30}/TiO_2$ and 0.06 $CALB_{GS30}/TiO_2$ indicate high affinity for the protein and the polyols to the adsorption sites of the titanium dioxide.

In contrast, the adsorption data of the materials prepared at 37°C and 45°C (0.06 $CALB_{GS37}/TiO_2$ and 0.08 $CALB_{GS45}/TiO_2$) fit the Freundlich model. Again this model refers to a non-ideal adsorption and is not restricted to a monolayer. The theoretical adsorption capacity K_F obtained with the Freundlich model is indicative of the amount of protein required for a multilayer kind of adsorption. Interestingly, the experimental maximum dispersion limits of the materials prepared at 37°C and 45°C (0.067 ± 0.007 μmol m^{-2} and 0.071 ± 0.009 μmol m^{-2}, respectively) are below the values of K_F (0.081 ± 0.007 μmol m^{-2} and 0.10 ± 0.02 μmol m^{-2}, respectively).

The observation that the mechanism of adsorption changes upon increasing temperature might be related with modification of the secondary structure of the protein as discussed in the following sections.

Co-adsorption of protein and other carbon based molecules on TiO_2 NPs: addition of glycerol and sorbitol

Table 2 shows the mass of carbon and titanium obtained through EDS analysis, the amounts of protein, TiO_2 and other carbon species of CALB (purified and the commercial crude extract) immobilized over TiO_2 NPs (see the Supplementary information: Annex II

Table 1. Experimental maximum dispersion limit and application of Langmuir, Freundlich and Hill models to the adsorption equilibrium of the lipase B of *Candida antarctica* on TiO_2 NPs using various sources of protein, addition of polyols and temperatures

	Experimental maximum dispersion limit	Langmuir				Freundlich				Hill				
Biocatalyst	(μmol m^{-2})	Q_{MAX} (μmol m^{-2})	K_L (mg mL^{-1})	R^2	Σ^2	K_F (μmol m^{-2})	n_F	R^2	Σ^2	Q_{MAX} (μmol m^{-2})	K_H	n_H	R^2	Σ^2
0.08 $CALB_{P30}/TiO_2$	0.074 ± 0.006	----	----	NA	NA	----	----	0	4.28	0.074 ± 0.005	0.017	3.8	0.83	0.93
0.08 $CALB_{CE30}/TiO_2$	0.075 ± 0.009	0.082 ± 0.006	24.4 ± 0.6	0.66	2.02	----	----	0.32	7.89	0.075 ± 0.002	0.19	87.6	0.80	0.89
0.06 $CALB_{GS30}/TiO_2$	0.076 ± 0.007	0.079 ± 0.005	31.6 ± 0.4	0.83	1.36	0.082 ± 0.006	6.8	0.79	1.87	0.074 ± 0.001	0.15	69.6	0.94	0.29
0.06 $CALB_{GS37}/TiO_2$	0.067 ± 0.007	0.069 ± 0.004	1507.2 ± 0.5	0.66	4.72	0.081 ± 0.007	10.1	0.89	1.87	----	----	----	NA	NA
0.08 $CALB_{GS45}/TiO_2$	0.071 ± 0.009	----	----	0.37	151	0.10 ± 0.02	5.3	0.98	0.02	----	----	----	NA	NA

NA not applicable
Q_{MAX} theoretical maximum dispersion limit according to the Langmuir and Hill models
K_L binding affinity constant
K_F adsorption capacity
nF adsorption intensity
K_H Hill constant
n_H cooperativity coefficient
Σ^2, sum of residual square

Table 2. Weight percentage of carbon and titanium obtained through EDS analysis, amounts and weight percentage of protein, oxide support and other carbon species obtained through EDS and the Bradford method for CALB immobilized over TiO_2

	0.05 $CALB_{P30}/TiO_2$	0.08 $CALB_{P30}/TiO_2$	0.08 $CALB_{CE30}/TiO_2$	0.06 $CALB_{GS30}/TiO_2$	0.06 $CALB_{GS37}/TiO_2$	0.08 $CALB_{GS45}/TiO_2$
EDS weight %						
C	3.61	4.56	6.13	6.59	5.80	6.07
Ti	56.52	54.90	54.93	57.49	58.78	58.53
Calculated mass (mg)						
Protein	6.75	8.52	8.50	7.66	7.48	9.46
Other carbon species	0.00	0.00	4.00 [a]	6.31 [a]	4.54 [a]	2.54 [a]
TiO_2	94.28	91.58	91.63	91.48	95.19	94.79
Protein weight % [b]						
EDS	7.16	9.30	9.28 [c]	8.37 [c]	7.86 [c]	9.98 [c]
Bradford	7.08	10.94	10.90	9.35	8.49	12.10
Carbon species weight %	0.00	0.00	4.38	6.90	4.77	2.68

[a] The amount of other carbon based species was calculated subtracting the mass of carbon that undoubtedly belongs to the protein from the mass obtained through the EDS analysis.
The result was converted to mass of glycerol and sorbitol that possess 39.5 % of carbon (see Supplementary information for details).
[b] The weight % of protein was calculated per 100 mg of TiO_2 NPs.
[c] Calculated with the equation: EDS weight % = 0.5544 Bradford weight % + 3.2348, obtained with the data of 0.05 $CALB_{P30}/TiO_2$ and 0.08 $CALB_{P30}/TiO_2$ (see Supplementary information for details).

for details of the analysis and the calculations). The data obtained through the Bradford method is also presented for comparison. The data demonstrate that the material obtained through the adsorption of protein of the crude extract over TiO_2 NPs named 0.08 $CALB_{CE30}/TiO_2$ contains 9.28 mg of protein and 4.38 mg of other carbon based species (per 100 mg of support) that can be ascribed to polyols such as glycerol and sorbitol that compose the crude extract.[33] The amount of those compounds represents 32% of the surface composition of the biocatalyst.

The materials named 0.06 $CALB_{GS30}/TiO_2$, 0.06 $CALB_{GS37}/TiO_2$ and 0.08 $CALB_{GS45}/TiO_2$ were prepared with a similar amount of sorbitol–glycerol mixture (3.60 mL of a mixture containing 25% w/v of sorbitol and 25% w/v of glycerol plus the amount of those polyols in the crude extract corresponding to 0.3 mol L^{-1} and 0.6 mol L^{-1} of sorbitol and glycerol in the preparation, respectively). Those biocatalysts contain 45%, 38% and 21% of adsorbed polyols, respectively. It is clear from comparison of the percentages of carbon based species 0.08 $CALB_{CE30}/TiO_2$ and 0.08 $CALB_{GS45}/TiO_2$, that the amount of adsorbed polyols diminishes with increase of temperature of the preparation.

Secondary structure of the adsorbed protein: influence of the temperature and addition of polyols

The percentage contribution of aggregates, α-helix, β-sheet, β-turns and random to the secondary structure of the lipase B of *Candida antarctica* CALB, the commercial Novozym® 435 and CALB immobilized over TiO_2 NPs at various coverages (expressed in micromols of protein per unit surface area of the oxide support) and temperatures, is shown in Table 3.

The percentage contribution of each structure was obtained through the deconvolution, integration and further normalization of the corresponding signals of Amide I as indicated earlier. The contributions of α-helix and random structures correspond to the area of the signals at 1654 cm^{-1} and 1643 cm^{-1}, respectively. The contribution of the β-sheet structure was obtained by adding the area of the signals appearing at wavenumbers 1631 cm^{-1}, 1637 cm^{-1} and 1686 cm^{-1}. Similarly, the contribution of the β-turn structure corresponds to the addition of the areas of the signals located at 1664 cm^{-1}, 1666 cm^{-1} and 1676 cm^{-1}. Finally, a signal at 1619 cm^{-1} is ascribed to β aggregates.[18,23,24,34,35]

The α-helix (18.6%), β-sheet (33.8%) and β-turns (29.9%) are the major elements of the secondary structure of the purified protein. A minor contribution of aggregates (7.3%) and random (10.5%) structures were also recognized in the Amide I signal. A similar distribution appears when the pure protein is immobilized over the oxide support. The results of the analysis of 0.05 $CALB_{P30}/TiO_2$ and 0.08 $CALB_{P30}/TiO_2$ indicate that the secondary structure is independent of the surface coverage.

The well known Novozym® 435 is a commercial immobilized lipase B of *Candida antarctica* adsorbed on a macroporous resin called Lewatit VP OC 1600. This particular biocatalyst also contains seventeen substances, including five major components: glycerol, benzoic acid, 2-hydroxyethyl benzoate, 2-hydroxyethyl sorbate, and sorbic acid.[33] The source of such substances is ascribed to additives of the enzyme formulation to prevent microbial growth and to stabilize the lipase (in the particular case of glycerol). The higher contribution of the α-helix structure (26.4% versus 18.6%) and the lower amount of aggregates (0.2% versus 7.3%) in the commercial biocatalyst compared with the pure CALB is an indication of the action of the additives. A similar secondary structure is observed for the commercial biocatalyst and the one synthesized with the crude extract 0.08 $CALB_{CE30}/TiO_2$ with the exception of the high contribution of β-turns observed in this last one. This conformational rearrangement is ascribed to the interaction with titanium dioxide and might contribute to diminish the catalytic activity of the immobilized protein as is discussed below.[18] The addition of the sorbitol–glycerol mixture in the preparation at 30°C and 37°C prevents the aggregation of the protein as can be concluded from the comparison of 0.08 $CALB_{P30}/TiO_2$ (7.9% of aggregates) with 0.06 $CALB_{GS30}/TiO_2$ (no aggregates) and 0.06 $CALB_{GS37}/TiO_2$ (2.8%).

The temperature of the preparation also influences the secondary structure of the protein. The increase in the contribution of the aggregates at 45°C in comparison with the preparation at 30°C (2.8% vs 14.2% of 0.06 $CALB_{GS30}/TiO_2$ and 0.08 $CALB_{GS45}/TiO_2$,

Table 3. Contribution of the aggregates, α-helix, β-sheet, random structure and β-turns to the secondary structure of the lipase B of *Candida antarctica* CALB, commercial Novozym® 435 and CALB immobilized over TiO_2 NPs at various coverage (expressed in micromols of protein per unit surface area of the oxide support)

	Percentage contribution				
Biocatalyst	Aggregates[a]	α-helix[b]	β-sheet[c]	Random[d]	β-turns[e]
Purified CALB	7.3	18.6	33.8	10.5	29.9
Novozym® 435	0.2	26.4	19.4	25.3	28.7
0.05 $CALB_{P30}/TiO_2$	9.1	13.1	29.0	22.6	26.4
0.08 $CALB_{P30}/TiO_2$	7.9	18.1	23.1	24.3	27.0
0.08 $CALB_{CE30}/TiO_2$	0.7	24.2	21.3	8.6	45.3
0.06 $CALB_{GS30}/TiO_2$	0.0	11.0	33.7	7.2	48.0
0.06 $CALB_{GS37}/TiO_2$	2.8	10.1	31.7	17.3	38.1
0.08 $CALB_{GS45}/TiO_2$	14.2	11.3	17.1	22.0	35.3

[a] Corresponds to the signal at 1619 cm^{-1}.
[b] Corresponds to the signal at 1654 cm^{-1}.
[c] Corresponds to the sum of the contribution of the signals at 1631 cm^{-1}, 1637 cm^{-1} and 1686 cm^{-1}.
[d] Corresponds to the signal at 1643 cm^{-1}.
[e] Corresponds to the sum of the signals at 1664 cm−1, 1666 cm^{-1} and 1676 cm^{-1}.
$CALB_{P30}/TiO_2$, material prepared with purified protein at 30°C.
$CALB_{CE30}/TiO_2$, material prepared with a crude extract of the protein at 30°C.
$CALB_{GS}/TiO_2$, material prepared with a crude extract of the protein with glycerol and sorbitol at various temperatures (30°C, 37°C and 45°C).

respectively) indicates a certain degree of denaturation of the protein.

Evidence of the promoter effect of polyols in the catalytic activity

Figure 3 shows the esterase activity in terms of specific activity (IU per mg of adsorbed protein) and the amount of protein adsorbed per unit surface area of the materials synthesized with the crude extract at 30°C as a function of the concentration of protein used in the preparation. The residual activity of the immobilized protein in comparison with a similar amount of free CALB is presented as a percentage on the same graph. The results clearly demonstrated that the higher the starting concentration of protein and polyols (that comes within the crude extract) in contact with the NPs, the higher the specific activity of the material even after the maximum dispersion limit of adsorbed protein was reached. In addition, comparison between the percentages of activity of the immobilized versus the free protein indicates that the oxide support induces a certain degree of inactivation. It has been demonstrated by Soderquist and Walton that changes in the secondary structure of albumin were stronger at lower protein loading onto silicon surfaces.[36] Alternatively, higher protein loadings (together with the presence of polyols) could exert a protective effect on the enzyme molecules, acting as 'added stabilizing proteins' themselves as well as biocatalyst.[37]

Consequently, it is clear that the higher the starting concentrations of protein and polyols (within the crude extract) in contact with the TiO_2 NPs the higher the retained activity of the immobilized lipase thus boosting the biocatalytic activity.

Kinetic resolution of ibuprofen: influence of a co-solvent, time of reaction, amount of ethanol and composition of the biocatalysts

Table 4 shows the conversion of R/S-ibuprofen to ethyl esters and the enantiomeric excess towards S(+)-ibuprofen in the esterification with ethanol in isooctane and just using the alcohol as

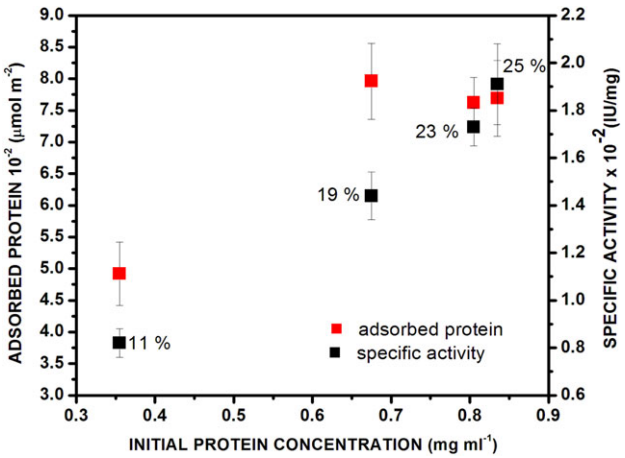


Figure 3. Amount of protein adsorbed per unit surface area of TiO_2 NPs and specific activity as a function of the concentration of protein in the starting solution in contact with the oxide support. The percentage values correspond to the difference in the esterase activity between the free and immobilized lipase.

reactant and solvent. The reaction was catalyzed with the materials synthesized in this investigation (0.08 $CALB_{P30}/TiO_2$ and 0.08 $CALB_{CE30}/TiO_2$) and the commercial biocatalyst Novozym® 435 for comparison purposes. Although the commercial biocatalyst is active under solventless condition the addition of a hydrophobic co-solvent boosts the catalytic performance five times. The catalytic activity of the immobilized lipase over the TiO_2 NPs is negligible when just ethanol is used as reactant and solvent in the reaction. This observation is attributed to the inhibitory effect of the alcohol reported previously in this esterification reaction in particular,[22–24] and extensively studied by different kinetic measurements, suggesting that the alcohol has the effect of a competitive inhibitor according to its concentration.[38,39]

The addition of isooctane greatly favors the catalytic performance of the biocatalyst synthesized with crude extract. Moreover,

Table 4. Conversion (X %), enzymatic specific activity (ibuprofen converted per time and amount of immobilized protein) and enantiomeric excess towards the S(+)-enantiomer eeS(+) in the kinetic resolution of racemic ibuprofen with ethanol (with and without co-solvent added) catalyzed with the commercial Novozym® 435 and immobilized CALB over titania NPs. The reaction was carried at 45°C for 48 h

Biocatalyst	Co-solvent	X %	Specific activity (μmol min^{-1} mg^{-1})	eeS(+) %
0.08 $CALB_{P30}/TiO_2$	no	----	----	----
	isooctane	3.6	8.17 10^{-3}	----
0.08 $CALB_{CE30}/TiO_2$	no	4.5	1.03 10^{-2}	5.5
	isooctane	52.3	0.12	36.0
Novozym® 435	no	14.5	2.03 10^{-2}	14.3
	isooctane	68.7	9.61 10^{-2}	50.0

Table 5. Conversion (X %) and specific activity as a function of the amount of enzyme (enzymatic specific activity) and the specific surface area of the TiO_2 NPs (bio-catalytic specific activity) in the esterification of racemic ibuprofen with ethanol in various molar ratios catalyzed with the 0.08 $CALB_{CE30}/TiO_2$. The reaction was performed at 45°C, isooctane as co-solvent for 24 h

		Specific activity	
ethanol:Ibuprofen molar ratio	X %	Enzymatic (μmol min^{-1} mg^{-1})	Biocatalytic (μmol min^{-1} m^{-2})
1:1	21.0	9.31 ×10^{-2}	0.24
3:1	16.4	7.92 × 10^{-2}	0.18
5:1	11.0	5.30 × 10^{-2}	0.12
7:1	7.8	3.77 ×10^{-2}	8.63 ×10^{-2}

it is worth noting that the use of a co-solvent such as isooctane allows dissolution of the profen and consequently it is possible to tune the amount of ethanol to the lowest required for an equimolar (1:1) ratio of ethanol:profen.

Additional experiments varying the ethanol:ibuprofen molar ratios (with co-solvent added) were assayed in order to establish the influence of the amount of acyl-acceptor in the biocatalytic activity. Table 5 shows that the conversion and the specific activity in the esterification of ibuprofen catalyzed with 0.08 $CALB_{CE30}/TiO_2$ decrease upon increasing amounts of ethanol in the reaction media. The inhibitory effect of the alcohol on the lipase activity (previously detected in the solventless reaction) is further proved in these experiments.

The material synthesized with the crude extract undoubtedly shows higher activity than the one synthesized with pure CALB. Again this observation is evidence that the components of the crude extract co-adsorbed with the lipase exert a promoting effect on the activity. This observation is in accordance with the investigations reported by Triantafyllou *et al.* that demonstrated the promoting effect of sorbitol (a component of the crude extract of CALB) on the catalytic activity and stability of immobilized lipases.[40]

It is worth noting that the activity in the esterification of ibuprofen diminishes with increase of the temperature of preparation of the biocatalysts. In this context, the catalytic activity in the esterification of ibuprofen with ethanol in an 1:1 molar ratio, at 45°C with isooctane as co-solvent was 0.31 ± 0.01 μmol min^{-1} mg^{-1} for 0.06 $CALB_{GS30}/TiO_2$; 0.186 ± 0.005 μmol min^{-1} mg^{-1} for 0.06 $CALB_{GS37}/TiO_2$ and 0.125 ± 0.002 μmol min^{-1} mg^{-1} for 0.08 $CALB_{GS45}/TiO_2$. These observations show a correlation between the catalytic activity, the temperature of the preparation, the amount of adsorbed polyols, and the secondary structure of the

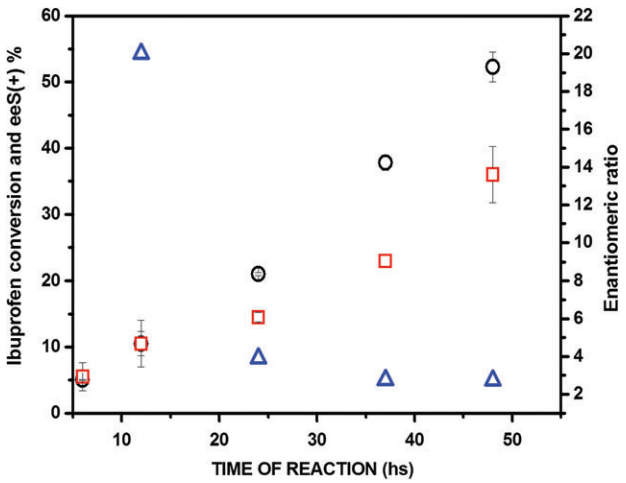


Figure 4. Conversion percentage (black circles), enantiomeric excess towards the S(+)-enantiomer (eeS(+)% red squares) and enantiomeric ratio (blue triangles) in the esterification of racemic ibuprofen with ethanol (1:1 molar ratio) catalyzed with 0.08 $CALB_{CE30}/TiO_2$ at 45°C, with isooctane as co-solvent up to 48 h reaction.

biocatalysts. In fact, the higher the temperature of the preparation, the lower the amount of adsorbed polyols, which in turn leads to a higher contribution of aggregates and lower catalytic activity.

A question arises about the possibility that sorbitol and glycerol might act as acyl-acceptors in the esterification of ibuprofen. Bearing this in mind, the esterification of racemic ibuprofen was assayed with 0.06 $CALB_{GS30}/TiO_2$ without the addition of ethanol. This experiment showed no conversion of ibuprofen, showing that the lipase does not accept either sorbitol or glycerol as substrates for esterification under the assayed conditions.

The kinetics of the enantiomeric esterification of the profen at 45°C with isooctane as co-solvent using 0.08 $CALB_{CE30}/TiO_2$, demonstrates that the conversion increases linearly with time of reaction, reaching 52% conversion and 36% enantiomeric excess at 48 h of reaction (see Fig. 4). The slight increase of the reaction rate along with the reaction time could be due to the concomitant increase in water content produced by the alcoholysis reaction. It has been observed that reaction rate of the alcoholysis (as well as hydrolysis) of chymotripsin in diisopropyl eter increased with the increase of the water activity a_w up to 0.9 and then suffered a little decrease.[41] A probable reason for this could be that the increasing water content favors the flexibility of the enzyme, thus its catalytic activity.

Figure 4 also shows that the enantiomeric ratio E equals 20 (at low conversion) that is considered acceptable for practical application of a biocatalyst.[42] A high E value indicates that the

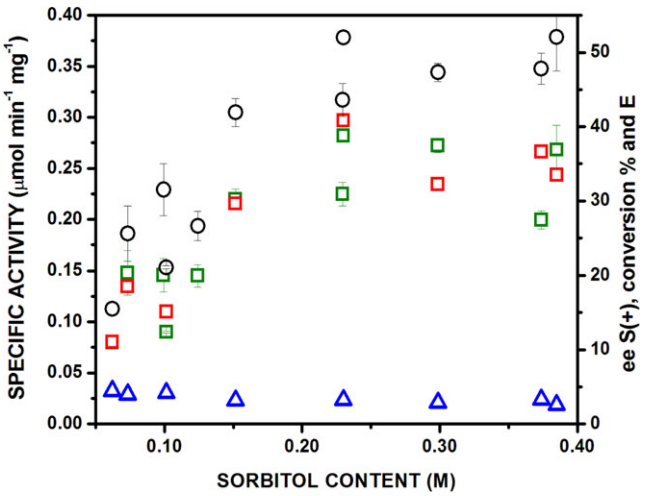


Figure 5. Specific activity (green squares), conversion percentage (black circles), enantiomeric excess towards the S(+)-enantiomer eeS(+) (red squares) and enantiomeric ratio (blue triangles) in the esterification of racemic ibuprofen with ethanol (1:1 molar ratio) catalyzed with 0.06 $CALB_{GS30}/TiO_2$ with various concentrations of sorbitol and glycerol added during the immobilization. The reaction was performed at 45°C, with isooctane as co-solvent up to 24 h.

rate of esterification of the R(−)-ibuprofen is higher than for the S-enantiomer. However, the observation that the E values fall (E ~3) above 20% of conversion is evidence that the presence of the R-ester is slowing down the esterification of the R-isomer. Therefore, a design of the reaction system towards scaling up of the biocatalyst must consider the continuous removal of the R-ester from the reaction medium or a continuous type of operation for an optimum result.

Further investigation of the catalytic performance up to 96 h of reaction indicates that the specific activity increases from 9.22 μmol h^{-1} mg^{-1} at 48 h to 10.64 μmol h^{-1} mg^{-1} (77.6% of conversion) at 96 h of reaction. In contrast, the enantiomeric excess falls to 4.1%, indicating that both enantiomers are esterified at the same time.

Finally, Fig. 5 shows the specific activity in the esterification of ibuprofen, the enantiomeric excess towards S(+)-ibuprofen and the enantiomeric ratio E of a series of 0.06 $CALB_{GS30}/TiO_2$ biocatalysts prepared with various concentrations of sorbitol and glycerol. It is worth noting that only the concentration of sorbitol is presented in the figure and that of glycerol is double that of sorbitol. These results provide further evidence of the enhancement of the catalytic performance of the immobilized protein over TiO_2 NPs due to the addition of the polyols. There is a linear increase in the specific activity and enantiomeric excess until those parameters level off at 0.23 mol L^{-1} of sorbitol (0.46 mol L^{-1} of glycerol).

The commercial counterpart Novozym® 435 is less active than 0.06 $CALB_{GS30}/TiO_2$ under similar reaction conditions. Novozym® 435 showed 34% conversion (0.091 ± 0.003 μmol min^{-1} mg^{-1}) and 16% enantiomeric excess compared with 52% conversion (0.31 μmol min^{-1} mg^{-1}) and 41% enantiomeric excess of the biocatalyst prepared in this contribution.

The use of polyols as enhancers of the enzymatic activity in organic solvents was reported earlier by many authors.[41,43–45] Different hypotheses were formulated in order to explain the positive influence of these additives in this kind of process. Polyols could act as 'water mimics', i.e. imitating the role of water as molecular lubricants of proteins in organic media and promoting the synthesis reaction over hydrolysis.[44] Yamane *et al.* and Aldercreutz suggested that sugar alcohols promote water retention, forming a protective atmosphere surrounding the immobilized enzyme and creating a tiny phase in which the catalytic reaction takes place.[41,45] The increase of water retained by this kind of compound would be responsible for a microenvironment formation that favors the flexibility of the enzyme, thus raising its activity. Moreover, glycols could act as plasticizers, since they could penetrate the structure of the lipase molecules and catalyze hydrogen-bond exchange, thus making them more flexible in the organic media.[46] Furthermore, in our case, the addition of sorbitol and glycerol during the process of adsorption of the protein onto the TiO_2 NPs would protect the enzyme from the negative effect that its interaction with the support and the subsequent lyophilization stage could produce in its native structure, minimizing the damage and preventing a severe activity loss. In this sense, polyols would be able to maintain the essential solvophobic interactions of the protein in organic solvents, as well as to disperse the enzyme molecules over the support surface, increasing the accessibility of active sites to the substrate.[40]

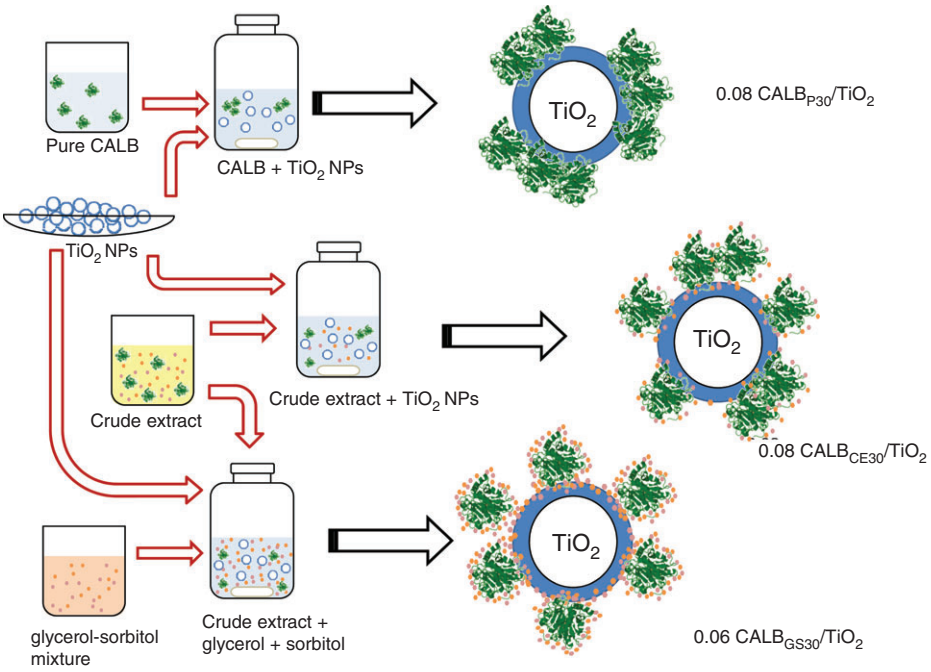
Stability of the immobilized lipase under reaction conditions

Desorption of the lipase immobilized over the oxide support in contact with the reaction media was investigated. The biocatalyst 0.06 $CALB_{GS30}/TiO_2$ was contacted with mixtures of ethanol–4.8% H_2O and ethanol–isooctane for 24 h as described earlier. Then the solid and the supernatant were allowed to react with ibuprofen for 24 h under the reaction conditions described earlier. Table 6 shows the results of the esterification in terms of conversion of the profen to the ethyl esters. The results demonstrate that the material maintains its catalytic activity even after being exposed to ethanol–water and ethanol–isooctane for an extended period of time.

This supernatant solution does not catalyze the esterification of ibuprofen points out that there is no desorption of protein.

Table 6. Conversion (X %) of ibuprofen in the esterification with ethanol (with and without co-solvent) catalyzed with 0.06 $CALB_{GS30}/TiO_2$ before and after being in contact with the reaction medium and the supernatant solution that was in contact with the biocatalyst

System	Treatment	Reaction medium	X % (24 h)
0.06 $CALB_{GS30}/TiO_2$	NO	0.12 mol L^{-1} ethanol in isooctane	43.2 ± 1.8
0.06 $CALB_{GS30}/TiO_2$	24 h with ethanol–isooctane at 45°C, 200 rpm	0.12 mol L^{-1} ethanol in isooctane	47.4 ± 3.9
supernatant	24 h with the biocatalyst at 45°C, 200 rpm	0.12 mol L^{-1} etanol in isooctane	1.1
0.06 $CALB_{GS30}/TiO_2$	NO	Ethanol with 4.8% H_2O	9.5 ± 1.1
0.06 $CALB_{GS30}/TiO_2$	24 h with ethanol–water at 45°C, 200 rpm	Ethanol with 4.8% H_2O	7.0 ± 1.8
supernatant	24 h with the biocatalyst at 45°C, 200 rpm	Ethanol with 4.8% H_2O	0.0



Scheme 1. Summary of the various sources of the lipase B of *Candida antarctica*, the procedure of adsorption onto TiO_2 NPs and models of the obtained biocatalyst.

However, further experiments using an aqueous buffer Tris–HCl at pH = 8.0 demonstrated that complete desorption of the protein from the NPs is achieved in 1 h at room temperature.

CONCLUSIONS

Scheme 1 summarizes the various sources of the lipase B of *Candida antarctica* and the procedure for adsorption onto TiO_2 NPs. Moreover, a model of each of the obtained biocatalysts is presented. The immobilization of the lipase B of *Candida antarctica* onto TiO_2 NPs was performed through the direct contact of aqueous solutions of either purified CALB or crude extract (with and without the addition of glycerol and sorbitol) with the oxide support at various temperatures. In this context, the maximum dispersion limit of protein over TiO_2 was 7.3×10^{-2} μmol m^{-2} regardless of the source of CALB.

The quantification with an electron microprobe combined with the typical Bradford method allowed the conclusion that carbon based substances (besides the protein) that compose the formulation of the crude extract such as polyols co-adsorb with the lipase onto the TiO_2 NPs. Actually, 30% of the total amount of carbon based substances that compose the biocatalyst prepared with solely crude extract is composed of those co-adsorbed species. The immobilization of the protein and the polyols over TiO_2 NPs at 30°C follows the Hill model therefore those species might compete for the adsorption sites and also exert a certain degree of cooperative binding during the adsorption. This observation allows the proposal that the polyols are adsorbed on the protein and the surface of the TiO_2 NPs as depicted in the model of 0.08 $CALB_{CE30}/TiO_2$ in Scheme 1. The addition of glycerol and sorbitol reaches 45% of the total carbon content of the biocatalysts (see the model of 0.06 $CALB_{GS30}/TiO_2$ in Scheme 1). Moreover, this molecular self-association exerts a protective effect since it prevents aggregation of the protein and protects its active conformation. It is clear that the material prepared with pure CALB such as 0.08 $CALB_{P30}/TiO_2$ contains the highest percentage of protein aggregates and the lowest activity in the esterification of the profen. In conclusion there is reliable evidence that the addition of the polyols promotes the catalytic activity of the lipase B of *Candida antarctica* immobilized over TiO_2 NPs and that it is possible to tune the catalytic behavior with controlled addition of glycerol and sorbitol.

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Supporting Information

Supporting information may be found in the online version of this article.

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