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Investigation of the causes of deactivation–degradation of the commercial biocatalyst Novozym® 435 in ethanol and ethanol–aqueous media

Carla José[a], Rita D. Bonetto[a], Luis A. Gambaro[a], María del Pilar Guauque Torres[b], María Laura Foresti[c], María Luján Ferreira[b], Laura E. Briand[a,*]

[a] Centro de Investigación y Desarrollo en Ciencias Aplicadas-Dr. Jorge J. Ronco. Universidad Nacional de La Plata, CONICET, CCT La Plata. Calle 47 N° 257, B1900AJK La Plata, Buenos Aires, Argentina
[b] Planta Piloto de Ingeniería Química – PLAPIQUI, CONICET, Universidad Nacional del Sur, Camino La Carrindanga Km 7, 8000 Bahía Blanca, Argentina
[c] Laboratorio de Polímeros y Materiales Compuestos, Instituto de Tecnologías y Ciencias de la Ingeniería (INTECIN), Las Heras 2214, Facultad de Ingeniería, UBA - CONICET, Buenos Aires (C1127AAR), Argentina

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ABSTRACT

The effect of contacting neat ethanol and an ethanol: H_2O mixture on Novozym® 435 was investigated at room temperature and 45 °C, during various periods of time of interaction with the alcohol (from 40 min to 8 days) and at different biocatalyst: ethanol (ethanol/water) ratios. The alcohol dissolves the polymethylmethacrylate (PMMA) that constitutes the support of the *Candida antarctica B* lipase (CALB) regardless of the conditions investigated and diffuses into the biocatalyst's beads remaining strongly adsorbed (the desorption of the alcohol is evidenced only upon heating at 150 °C). The diffusion of the alcohol alters the inner texture of the beads generating channels and increasing the roughness of the polymeric material. Additionally, the ethanol (with or without water added) modifies the secondary structure of the enzyme by decreasing the α-helix contributions and increasing the β-sheet structure.

1. Introduction

Among the commercially available biocatalysts, there is no doubt that Novozym® 435 is the most commonly heterogeneous biocatalyst used in the enantioselective synthesis of optically active alcohols, amines and carboxylic acids. Novozym® 435 is the commercial immobilized *Candida antarctica* lipase B (CALB) produced by submerged fermentation of a genetically modified *Aspergillus* microorganism. The lipase is adsorbed on a macroporous resin called Lewatit VP OC 1600, according to the information given by the Novozymes Co. in their website (http://www.novozymes.com/en/MainStructure/ProductsAndSolutions/Biocatalysis/Commercial+products/Novozym+435/Novozym+435). This macroporous resin is a polymer of metacrylic acid cross-linked with divinylbenzene (DVB) and possesses certain hydrophobic nature [1].

The supplementary material accompanying this investigation summarizes the applications of Novozym® 435 that have been published in the last 10 years along with the temperature of reaction, conversion of substrate, enantiomeric excess of the substrate and/or the desired product and the composition of the reaction media (from references [2–22]). This literature survey demonstrates that, in most cases, the substrates are used in stoichiometric amount and a non-reactive co-solvent such as isooctane and n-heptane is added to the reaction media in order to dissolve the substrates. In contrast with that conventional operation modus, our research group demonstrates that the enantiomeric esterification of racemic R/S-ibuprofen is feasible with an excess of ethanol [9]. In this context, the alcohol operates as a substrate and also dissolves the ibuprofen, therefore no co-solvent is necessary. The investigation shows that the conversion of ibuprofen towards the ethyl esters and the enantiomeric excess towards S(+)-ibuprofen are greatly affected by the ethanol and the initial nominal water contents of the reaction media.

In this context, when an ethanol: R/S-ibuprofen molar ratio equals 141 (0.2 ml of ethanol per milligram of biocatalyst) was assayed, a low conversion (18%) and enantiomeric excess (12%) was achieved. However, a molar ratio equal to 7.0 (0.01 ml/mg) yields 62% conversion of ibuprofen with 50% of enantiomeric excess towards S(+)-ibuprofen at optimum operation conditions (see the application: Resolution of racemic ibuprofen within the supplementary material for details). The performance of the biocatalyst diminishes to half its initial activity upon several reuses [9,10]. In this context, there are several investigations that

* Corresponding author. Tel.: +54 221 4 211353/+54 221 4 210711.
E-mail address: briand@quimica.unlp.edu.ar (L.E. Briand).

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account for the deactivation of Novozym® 435 as observed in the supplementary material. Particularly, those investigations devoted to bio-diesel production observed that the excess of methanol or ethanol inhibits the transesterification reaction [2–5]. Similarly, the biocatalyst deactivates when 1-propanol, octanol, methanol, butanol, t-butanol and 1,2-propanediol are used as the acylating agent in esterification type of reactions [8,11,12,14,17,18]. Despite the reports of the deactivation/inhibition of Novozym® 435, none of the investigations have been focused on a reliable explanation of the causes of such behavior. In this context, the present investigation provides evidences on the deactivation of Novozym® 435 upon contact with ethanol (with or without water added) based on the effect of the alcohol on the secondary structure of *Candida antarctica B* lipase, the dissolution of the macroporous resin (the core of Novozym® 435 beads), the desorption of the enzyme and the surface interaction of ethanol with the macroporous support of the lipase.

2. Experimental

2.1. Materials

Candida antarctica lipase (CALB L) (batch LCN02102) and the commercial biocatalyst Novozym® 435 (batch LC200217) were obtained as a gift from Novozymes Brasil (Paraná, Brazil). The specific surface area (determined through the BET method) of the biocatalyst is 72 m^2/g.

Highly pure *Candida antarctica* lipase (35,500 g/mol) purchased from Sigma Aldrich Argentina (10.9 U/mg) was used as a reference for the infrared analysis purposes.

2.2. Procedures for the quantification of proteins

2.2.1. Procedure for the quantitative precipitation of Candida antarctica lipase

The concentration of *Candida antarctica* lipase (Lipozyme batch LCN02102) of the sample provided by Novozymes Brasil was determined through the precipitation and weighing of the total amount of protein. A known volume of the sample (1.00 ml) is cooled down with an ice bath and continuous stirring. Then 2.00 ml of a saturated solution of ammonium sulphate at 4 °C is added drop by drop and maintained for 5 h. The saturated solution of $(NH_4)_2SO_4$ possesses 533 mg per ml at 25 °C.

The precipitated enzyme is centrifuged for 15 min at 6000 rpm, dried for 16 h in a vacuum oven at room temperature and weighed. This procedure allowed us to determine that the sample contained 68 mg of protein capable of being precipitated per ml of the liquid simple.

2.2.2. Protein quantification through UV–vis spectroscopy

The amount of enzyme desorbed out of Novozym® 435 was determined through UV–vis spectroscopy. In this context, the absorbance of the supernatant solution remaining after each treatment with ethanol was studied in the 200–400 nm range with a Perkin Elmer Lambda35 equipment. Then, the absorbance at 256 nm was correlated with the concentration of CALB by means of a calibration curve performed with several solutions of known concentration of the CALB L enzyme in ethanol medium (the calibration was also performed in aqueous solution). In turn, the concentration of protein in the starting solution of CALB L was obtained through a precipitation method as described in Section 2.2.1.

2.2.3. Bicinchoninic acid assay

The quantification of protein through the bicinchoninic acid (BCA) was assayed both in a mixture of dimethylsulfoxide and Triton X-100 and in an ethanol: 4.76% H_2O mixture in order to establish the total amount of protein of Novozym® 435 (the removal of protein is described in Section 2.3) and the amount of protein desorbed upon interaction with ethanol, respectively.

The BCA method involves the preparation of three aqueous solutions that are called A, B and C for brevity. Solution A contains 0.4000 g of BCA (Fluka ≥ 90%), 0.8000 g Na_2CO_3 (Mallinckrodt), 0.1630 g $NaHCO_3$ (Anedra), 0.1581 g NaOH (Carlo Erba ≥ 97%) and 0.0641 g sodium tartrate dibasic dihydrate (Fluka ≥ 99% in 40 ml of distilled water. Solution B contains 4% (w/v) of $CuSO_4$ and solution C is a mixture of solution A and B in a 100/2 (v/v) ratio (in this particular case, solution C corresponds to 30.00 ml of solution A and 0.60 ml of solution B, and is prepared just before the quantification).

The standard solutions for the calibration curves were prepared with bovine seroalbumin (Sigma 99%, A3059 lot 12640255). The starting aqueous solution containing 1 mg/ml of bovine seroalbumin was diluted to 0.1 mg/ml with either an ethanol: 4.76% H_2O mixture or a mixture containing 35% (v/v) DMS:35% (v/v) Triton X-100:30% (v/v) H_2O (similar to the mixture used to remove CALB from Novozym® 435) depending on the particular application of the BCA method as discussed above. The standard solutions for calibration purposes were prepared by taking an appropriate volume of the 0.1 mg/ml of bovine seroalbumin solution then 1.00 ml of the solution C was added and incubated at 60 °C for 10 min (each solution was prepared by duplicate). The mixtures were allowed to cool down and the absorbance was measured at 562 nm (the spectrophotometer was described in Section 2.2.2). The calibration curves were performed in an interval containing from 0.694 μg/ml to 9.90 μg/ml.

The equation of the linear regression of the curve determined in DMS:Triton X-100 was: A (absorbance) = $-0.035 + 0.047 \times C$ (protein concentration in μg/ml) with a linear regression coefficient equals to 0.99. When the BCA method was applied to bovine seroalbumin in the ethanolic medium it was impossible to obtain reproducible results in four successive experiments.

2.3. Removal of protein out of Novozym® 435

The biocatalyst was subjected to the procedure described by Gross and coworkers in order to desorb the protein (lipase B from *Candida antarctica*) immobilized over the macroporous resin Lewatit VP OC 1600 [23]. Novozym® 435 (0.1000 g) was contacted at 37 °C for 30 min with 3.00 ml of dimethylsulfoxide (J.T. Baker, 100%) at 200 rpm in a shaker bath. The biocatalyst's beads were separated by filtration and washed with portions of 5.00 ml of dimethylsulfoxide. Then the recovered beads were contacted for 30 min with 3.00 ml of 5% Triton X-100 solution at 37 °C, filtered afterwards, washed three times with 5.00 ml portions of the same solution and finally washed with distilled water. This procedure allowed obtaining the support Lewatit VP OC1600 and to quantify the total amount of enzyme of Novozym® 435 through the bicinchoninic acid assay described in Section 2.2.3. Different attempts to precipitate the enzyme desorbed using ammonium sulphate were not fruitful using 1 g of Novozym® 435. This is probably related to the fact that the amount of CALB reported to be present in this commercial biocatalyst is from 30 to 55 mg CALB/gram.

2.4. Procedure for the treatment of Novozym® 435 with ethanol

The effect of ethanol on the biocatalyst was studied through four sets of experiments. In the first set, Novozym® 435 was contacted three consecutive times with ethanol (Carlo Erba, 99.8%) at 28 °C and 200 rpm in a shaker bath (Julabo SW22) for 40 min. These experiments were performed varying the ratio between the amount of Novozym® 435 and ethanol volume in 12:1 ratio

(1.1800 g of biocatalyst with 100.00 ml of ethanol) and 100:1 ratio (100 mg of biocatalyst with 1.00 ml of ethanol). This last experiment also contains an initial nominal water content of 4.76% (v/v).

A second set of experiments mimics the reaction conditions of the enantiomeric esterification of ibuprofen as discussed in the introduction of the present investigation. In this context, Novozym® 435 was contacted four consecutive times with a mixture of ethanol and 4.76% (v/v) H_2O (initial nominal water content), at 45 °C and 200 rpm in a shaker bath (Julabo SW22) for 48 h. The ratio between the amount of Novozym® 435 and ethanol volume was kept constant at 100 mg/ml. The above described experiments were performed in closed vials. A sample of the biocatalyst was withdrawn after each treatment for FTIR and ESEM analysis. The amount of desorbed lipase was quantified in the liquid media remaining after contacting with the biocatalyst. The procedure of precipitation of potential precipitable protein in supernatant using ammonium sulphate was unsuccessful. Additionally, the supernatant was allowed to dry for further FTIR analysis in order to determine the presence of dissolved biocatalyst.

Finally, 1.0000 g of the biocatalyst was contacted with 10.00 ml of a mixture of ethanol: 4.76% (v/v) H_2O at 45 °C and 200 rpm for 8 days (such period of time corresponds to 4 cycles of 48 h). Then, the beads were dried in a desiccator for 8 days (to dehydrate) and further heated at 150 °C for 10 min (to desorb the ethanol), cooled down and weighed. This last procedure was repeated eight times until constant weight. This procedure allowed establishing the total weight loss of the biocatalyst and the amount of adsorbed ethanol.

The solvent was allowed to dry and the remaining solid was dissolved with 2.00 ml of water, centrifuged to separate the non-soluble substances and recover the enzyme for further quantification through UV–vis spectroscopy as described in Section 2.2.2.

2.5. Ibuprofen adsorption kinetics on Novozym® 435

The adsorption of ibuprofen (Parafarm 99.23%) over Novozym® 435 was assayed at 298 K, 301 K and 305 K by contacting the biocatalyst (80–100 mg) with 20.6 ml of a solution containing 60 ppm of racemic ibuprofen in ethanol (Carlo Erba, 99.8%) for 40 min. The experiments were performed in sealed flasks placed in a shaker bath at 120 rpm and controlled temperature. The ibuprofen solution (200 μl) was withdrawn at 0, 5, 10, 15, 20, 25, 30 and 35 min during the experiment. The analysis of both enantiomers of ibuprofen and the ethyl esters was conducted by chiral HPLC analysis using a Nucleodex beta-PM (Macherey-Nagel) with an UV detector operated at 230 nm. The mobile phase (metanol/0.1% TEAA pH 4.0 (60/40, v/v)) was operated at a flow rate of 0.700 ml/min.

Additionally a control experiment was performed by following the adsorption methodology described above without the biocatalyst in order to evaluate the errors involved in the whole procedure. This experiment demonstrated that the quantification of ibuprofen in ethanol through chiral HPLC possesses a standard deviation of 0.74 ppm.

2.6. Determination of the secondary structure of lipase with infrared spectroscopy

The evolution of the secondary structure of Novozym® 435 upon successive treatments with ethanol was followed through infrared analysis. The samples were diluted with KBr and pressed in a conventional wafer for FTIR analysis. Spectra were collected in the 4000–400 cm^{-1} range (±2 cm^{-1} resolution) with a Bruker Vertex 70 equipment. The infrared analysis was recorded with 60 scans in the absorption mode. The contribution of the infrared spectrum of the support Lewatit VP OC1600 (obtained through the method described in Section 2.3) was digitally subtracted from the spectra of Novozym® 435. Similarly maximum absorption intensity of all the spectra was verified, which indicates that a constant optical path length was maintained during the analysis of the samples.

To estimate the secondary structure, peak fitting of the Amide I band (1700–1600 cm^{-1}) by Lorentzian-shaped components was performed on the non-deconvoluted spectra. The software used with this purpose was a special peak fitting module of Origin 5.0. The positions and number of the components were determined from the second derivative analysis of the spectra. The contribution of each component to the Amide I band was evaluated by integrating the area under the curve and then normalizing to the total area of the Amide I band.

2.7. Scanning electron microscopy analysis and fractal dimension calculation: surface roughness

Surface morphology of Novozym® 435 before and after contacting with ethanol was investigated through scanning electron microscopy analysis with an environmental ESEM FEI Quanta 400. The samples were prepared as ultra thin specimens by embedding the biocatalyst in a LR White resin and further sliced with a microtome. These specimens, covered with a conductive gold layer in order to avoid electrical charges on the surface, were observed in the microscope in the high and low vacuum modes (pressure of 0.98 Torr in the last case).

Images of the samples at 400× and 600× magnifications, with and without being in contact with ethanol, were taken and analyzed with the FERImage program to calculate the fractal dimension D and the d_{min} parameter by using the variogram [24–26]. The variogram, used to determine parameters that characterize the surface roughness, consists of a graph of the variance of variation of heights in a surface for different steps, as a function of such steps and at logarithmic scale. The slope of the graph is related to the fractal dimension D as $D = 3 - \text{slope}/2$.

The different signals produced by the SEM do not directly supply the elevation profile and a stereo pair of SEM images is necessary to obtain this profile. Another way to obtaining information about surface roughness by means of the variogram is from the texture study of SEM images. In the case of the fractal dimension, for instance, the gray levels in the digitized image of a fractal surface, shows the same fractal behavior as that of the fractal original surface [26].

For many images observed with the SEM, the variogram presents a fractal behavior at low scale and a behavior that seems to have an asymptotic tendency at high scale, but, if the vertical axis was expanded variance maximums and minimums appear. This periodic region was described by two parameters: d_{min} and d_{per} [27]. The $\mathbf{d_{min}}$ parameter will be also used in this work to characterize the roughness of the samples and it corresponds to the inferior end of the periodic scale region and is representative of the smallest cell size with enough statistic weight to produce periods.

2.8. Temperature programmed surface reaction of ethanol

The interaction of ethanol with the surface of Novozym® 435 was investigated through the adsorption of the alcohol at 32 °C followed by temperature programmed surface reaction (TPSR). Details of the equipment used in this investigation have been published before [28]. The sample (46.6 mg) was pretreated at 32 °C for 40 min under a flow of helium (35 cm^3(NTP) min^{-1}) prior to adsorption and TPSR analysis. Successive pulses of 0.5 μl of ethanol (Merck P.A., 100%) were dosed through a heated septum until the saturation of the Novozym® 435 was reached. The adsorption process was monitored *in situ* through a mass spectrometer and a conductivity cell that detects the non-adsorbed alcohol and/or the species desorbed from the sample. After the saturation, the sample was

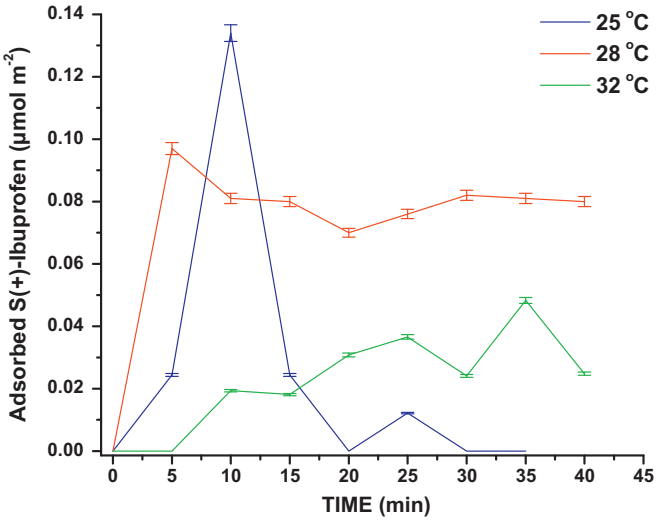


Fig. 1. Amount of S(+)-Ibuprofen in parts per million adsorbed on Novozym® 435 in contact for 40 min with S(+)/R(−)-Ibuprofen at different temperatures.

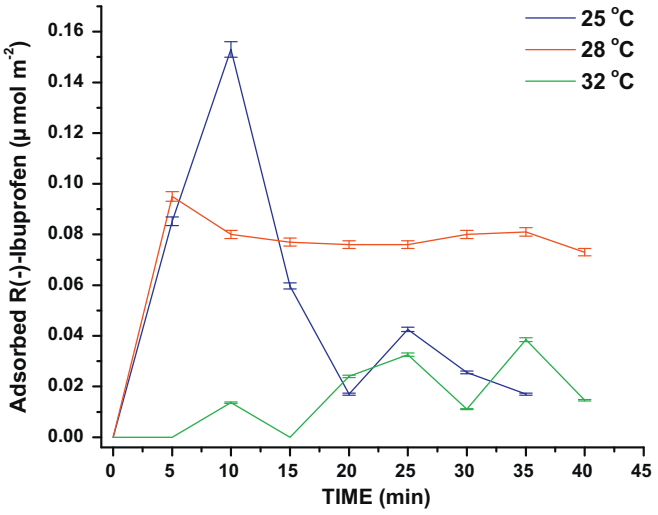


Fig. 2. Amount of R(−)-Ibuprofen in parts per million adsorbed on Novozym® 435 in contact for 40 min with S(+)/R(−)-Ibuprofen at different temperatures.

heated up to 200 °C at 10 K/min for the temperature programmed surface reaction experiment. The species resulting of desorption and/or reaction of the surface species are detected in the mass spectrometer and recorded in a computer. The following *m*/*e* ratios were employed to identify the desorbed species: ethanol C_2H_5OH, *m*/*e* = 31; acetaldehyde, *m*/*e* = 29; acetic acid, *m*/*e* = 31, *m*/*e* = 41 and *m*/*e* = 42; ethyl acetate, *m*/*e* = 43; ethyl ether, *m*/*e* = 31, *m*/*e* = 29 and *m*/*e* = 59; H_2O, *m*/*e* = 18; CO_2, *m*/*e* = 44; ethylene and CO, *m*/*e* = 28.

2.9. Temperature programmed desorption

Temperature programmed desorption analysis was performed over Novozym® 435 and Lewatit VP OC1600 after being treated with ethanol (as described in the *Ethanol Treatment Procedure* section). The samples (∼20 mg) are heated up to 400 °C at 10 °C/min under a flow of pure helium (35 cm^3(NTP) min^{-1}) for the temperature programmed desorption experiment. The species resulting of desorption and/or reaction of the surface adsorbed species are detected in the mass spectrometer and recorded in a computer. The equipment used in this experiment and the *m*/*e* ratios investigated were described in the preceding section.

3. Results

3.1. Adsorption of ibuprofen on Novozym® 435

Previous investigation on the stereo-selective esterification of racemic ibuprofen using ethanol as reactant and solvent (without additional co-solvents) demonstrated that Novozym® 435 losses 50% of its initial activity after reusing it for 4 successive cycles of 48 h. of reaction [9]. This behavior that might be attributed to the inhibition caused by the alcohol and/or the degradation of the biocatalyst was further investigated through the adsorption of an ibuprofen–ethanol mixture and ethanol, respectively.

Figs. 1 and 2 present the adsorption of S(+)-Ibuprofen and R(−)-Ibuprofen over Novozym® 435 as a function of time when the biocatalyst is in contact with a solution of the racemic ibuprofen in ethanol at different temperatures for extended periods of time. In general, the amounts adsorbed in those experiments are above the standard deviation of the procedure which ensures that the amounts adsorbed do not correspond to errors of the method (see Section 2.5).

The adsorption of ibuprofen on Novozym® 435 at 25 °C increases in the first 10 min of contact and slowly desorbs afterwards indicating a weak physisorption (the experiment was performed three times with the same results). An irreversible adsorption of S(+) and R(−)-ibuprofen is detected at 28 °C while at 32 °C the amount of adsorbed ibuprofen greatly diminishes to about 0.01 µmol m^{-2} on the biocatalyst. This behavior cannot be attributed to the surface reaction of ibuprofen with ethanol since the HPLC analysis did not evidenced esterification products at the investigated temperatures. Further experiments contacting Novozym® 435 with ethanol and an ethanol/water mixture were the key to understand those observations as will be discussed in the following sections.

3.2. Dissolution of Novozym® 435 support upon contact with ethanol

The effect of the amount of alcohol (with and without water added) on Novozym® 435 was investigated varying the ratio between the amount of the biocatalyst and ethanol volume, the temperature and the contact time (as described in Section 2.4). In this context, Fig. 3A and B compares the infrared spectra of highly pure CALB, Novozym® 435 (before contacting with ethanol), the resin Lewatit VP OC1600 and the solids phase recovered after drying the liquid medium in contact with the biocatalyst. Particularly Fig. 3B shows the solids recovered after three consecutive experiments of contacting 1.1800 g of Novozym® 435 with 100.00 ml of neat ethanol (12:1 mg/ml ratio); along with the solids recovered after contacting 100 milligrams of biocatalyst with 1.00 ml of ethanol (100:1 mg/ml ratio) containing 4.76% (v/v) H_2O at room temperature for 40 min and at 45 °C for 48 h. Additionally, Table 1 summarizes the band's position and the assignments of the infrared signals.

The infrared spectra of *Candida antarctica* lipase possesses an intense signal centered at 3331 cm^{-1} arising from the stretching vibration of the intramolecular hydrogen bonded N–H species that is superimposed with the stretching vibrations of O–H species [29]. Additionally, the lipase possesses the intense bands corresponding to the Amide I and Amide II signals centered at 1653 cm^{-1} and 1540 cm^{-1}, respectively [29,30]. The infrared signal known as Amide I arises from the stretching vibration of the carbonyl bond C=O of the backbone structure of the proteins. This vibration is not affected by the nature of the side chain but it is influenced by the secondary structure of the proteins. The infrared signal called

Table 1
Summary of the nature of the organic groups and the corresponding infrared signals that were detected on Novozym® 435, Lewatit VPOC, *Candida antarctica* CALB lipase and the solids recovered upon contacting Novozym® 435 with ethanol.

	ν(O–H)	ν(C–N)	ν(CH$_3$)	ν(C=O)	Amide I	Amide II	Aromatic ring vibration ν(C=C)	δ_{as}(C–H)	δ_s(C–H)	ω(CH$_2$) τ(CH$_2$)[a]	Out-of-plane δ(C–H)[b]
CALB lipase		3331 cm^{-1}	2928 cm^{-1}		1653 cm^{-1}	1540 cm^{-1}					
Lewatit VPOC	3436 cm^{-1}		2950 cm^{-1}; 2875 cm^{-1}	1731 cm^{-1}			1510 cm^{-1} 1454 cm^{-1}	1387 cm^{-1}	1361 cm^{-1}	1269–1145 cm^{-1}	991, 969 (s), 834, 800, 757, 709 cm^{-1}
Novozym® 435	3436 cm^{-1}		2950 cm^{-1}; 2875 cm^{-1}	1729 cm^{-1}	1656 cm^{-1}		1459 cm^{-1}	1387 cm^{-1}	1361 cm^{-1}	1269–1145 cm^{-1}	996, 966 (s), 840, 798, 763, 704 cm^{-1}
Recovered solids	3425 cm^{-1}		2964 cm^{-1}; 2864 cm^{-1}	1733 cm^{-1}	1644 cm^{-1}	1558 cm^{-1}	1456 cm^{-1}	1387 cm^{-1}		1271–1147 cm^{-1}	716, 672 cm^{-1}

[a] ω(CH$_2$) and τ(CH$_2$) wagging and twisting vibration of methylene groups.
[b] Out of plane bending of aromatic C–H groups.

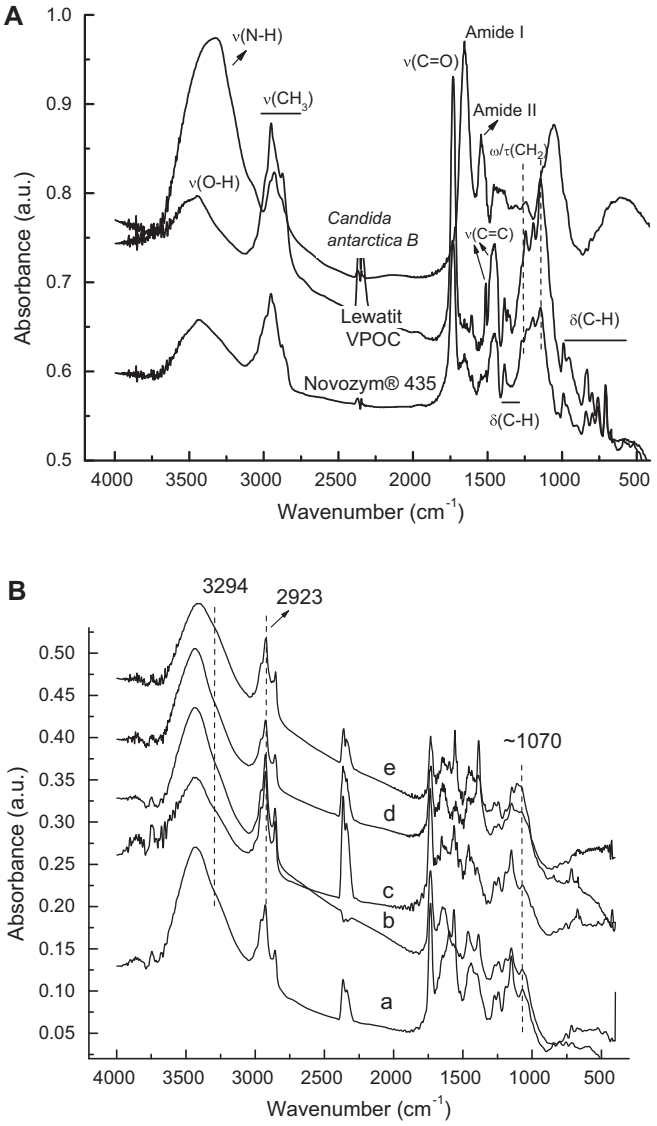


Fig. 3. (A) Infrared spectra of Novozym® 435, macroporous resin Lewatit VP OC 1600 and native *Candida antarctica B*. (B) Infrared spectra of the solids recovered from the supernatant solutions remaining after contacting ethanol with Novozym® 435 under the following conditions: (a), (b) and (c) correspond to the first, second and third cycles of treatment of 12 mg of Novozym® 435 with 1.00 ml of ethanol for 40 min at 28 °C. Spectra (d) and (e) correspond to the solids recovered after the treatment of 100 mg of Novozym® 435 with 1.00 ml of a mixture of ethanol with 4.76% (v/v) of water for 40 min at 28 °C and 48 h at 45 °C, respectively.

Amide II is attributed to the out-of-plane combination of the in plane bending mode of the N–H bond and the stretching vibration of the C–N bond of the protein.

The support Lewatit VP OC1600 possesses an intense signal at 1731 cm^{-1} due to the stretching vibration of the carbonyl groups of the poly-methylmethacrylate PMMA –(CH$_2$CCH$_3$)$_n$-CO-O-CH$_3$ [31,32]. Additionally, the intense signals belonging to asymmetric and symmetric stretching of the methyl groups (2950 cm^{-1} and 2875 cm^{-1}, respectively) along with asymmetric and symmetric bending vibrations (of weak intensity) of the methyl groups (1387 cm^{-1} and 1361 cm^{-1}, respectively) are observed [33]. These last two signals are characteristic of the C–H vibration of methyl and methylene groups attached to a tertiary carbon atom such as the one of the PMMA resin (see Fig. 10 for details of the molecular structure). The intense set of signals observed in the range from 1269 cm^{-1} to 1145 cm^{-1} are characteristic of twisting and wagging vibrations of methylene groups. The PMMA resin also has

divinylbenzene as a cross-linker as it was commented before. The presence of this substance is evidenced through the doublet of medium intensity at 1510 cm^{-1} and 1454 cm^{-1} due to the skeletal vibrations involving the carbon–carbon stretching within the aromatic ring. Additionally, a set of weak signals is observed from 834 cm^{-1} to 709 cm^{-1} that is characteristic of the C–H out-of-plane bending bands of the alkyl-substituted benzene [32,33].

The PMMA resin does not possesses O–H species therefore, the broad signal of medium intensity at 3436 cm^{-1} could be ascribed to adsorbed water molecules.

The commercial biocatalyst Novozym® 435 possesses both the signals belonging to CALB (a weak signal belonging to Amide I is observed at 1656 cm^{-1}) and the macroporous polymer as expected (see Table 1). Fig. 3B shows the infrared spectra of the solids recovered from the organic medium after being in contact with Novozym® 435 under various conditions (temperature, amount of ethanol, with and without water added and contact time were screened as described in 2.4).

The solids recovered from the organic medium show both the CALB enzyme and the species of the macroporous matrix as can be concluded from the similarity of the infrared signals of those three systems (see Table 1). Additionally, the infrared analysis provides evidences of the presence of molecularly adsorbed ethanol even though the alcohol was allowed to evaporate before the analysis of the sample (these new signals are indicated in Fig. 3B; not shown in Table 1). The signal observed as a shoulder at 3294 cm^{-1} belongs to the stretching vibration of the O–H of the alcohol. According to previous studies on the adsorption of ethanol onto a silica surface from ethanol–cyclohexane binary liquids, the signal at 3294 cm^{-1} corresponds to hydrogen-bonded clusters (polymers) that are formed between the adsorbed ethanol molecules [34]. The in-plane bending of the O–H species might also contribute to the intense signal observed at 1387 cm^{-1} formerly attributed to the bending of the C–H species. Additionally, the signals at 2923 cm^{-1} and 1070 cm^{-1} account for the stretching vibration of the methyl groups and the asymmetric stretching of the C–C–O species of the adsorbed ethanol [33].

The nature of the solid phase recovered from the ethanol that was in contact with Novozym® 435 evidences the dissolution (diggregation) of the biocatalyst exposed to the organic medium. The biocatalyst dissolves in the organic medium regardless of the amount of ethanol, the addition of water and the temperature and occurs even when the biocatalyst is in contact with the ethanol for a period of time as short as 40 min.

The dissolution of PMMA resin has been reported previously by González-Benito and Koenig, and Schubert and coworkers [35–37]. The authors demonstrated that PMMA dissolves in mixtures of acetonitrile/alcohol, CCl_4/alcohol (methanol, ethanol, and 1-propanol) and alcohol–water mixtures even at room temperature with a dissolution rate that increases in a parabolic way with time of exposure to the solvents. The dissolution process begins with the swelling of the polymer by the carbon tetrachloride that allows the alcohols to penetrate the PMMA. The alcohol interacts with PMMA through hydroxyl-carbonyl hydrogen bonds and separates the polymer chains. In this context, those observations also explain our findings on the modifications of the inner texture of Novozym® 435 beads that are presented in the following sections.

3.3. Desorption of protein from Novozym® 435 upon contact with ethanol

The results discussed above provide evidences of the dissolution–disaggregation of Novozym® 435 in contact with ethanol which in turn induces the loss of the surface protein (*Candida antarctica B* lipase). In this context, the biocatalyst was treated for extended periods of time (four cycles of 48 h each) at 45 °C mimicking the conditions of the esterification of ibuprofen with ethanol as reactant and solvent. It is worth notice that at first the quantification of protein was assayed through the bicinchoninic acid method which is reported as the less affected by interferences. However, the bicinchoninic acid method proved neither accurate nor reliable when assayed in ethanol medium. As a second attempt, the amount of desorbed CALB was determined through UV–vis spectroscopy (as described in Section 2.2.2). However, the analysis always provided a concentration of protein higher than the whole amount of enzyme of Novozym® 435 which allows to conclude that an interference was present or other unknown phenomena that makes the quantification method not useful. It is worth to notice that the total amount of protein determined through the method reported by Gross et al. (applied in the present investigation) is similar to the protein content based on HPLC analysis that was reported by Halling and coworkers for Novozym® 435 [23,38]. However, it does not match with other reports from the literature where contents as high as 10% of protein have been reported for Novozym® 435 [39].

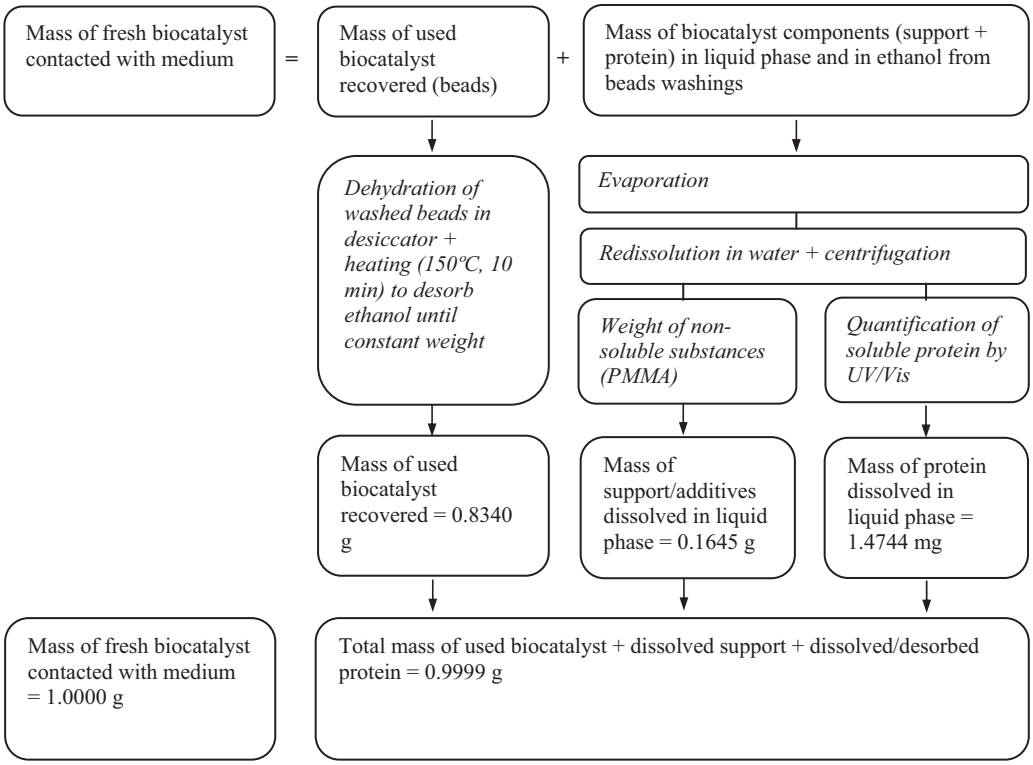
In order to avoid the interferences, the biocatalyst (1.0000 g) was allowed to interact with 10.00 ml of ethanol: 4.76% H_2O for 8 days as described in Section 2.4. After that period of time, the remaining liquid was separated by filtration with a GE nylon membrane filter (0.45 μm pore size) in order to retain the non-soluble substances as polymethylmethacrylate. The biocatalyst's beads were washed 4 times with 5.00 ml of ethanol and the solvent was separated by filtration (as described before) each time. The liquids remaining after each wash were added to the liquid phase initially separated after the 8 days treatment of Novozym® 435 and allowed to evaporate. The solids retained in the filters and the one precipitated from the liquid phase (after the ethanol was completely dried) were suspended in a minimum amount of water (1.50 ml) and further centrifuged in order to separate the non-soluble substances. Then the aqueous medium containing the enzyme was analyzed through UV–vis spectroscopy to quantify the amount of desorbed protein. This analysis evidenced that a 2.76% (mg of protein per mg of protein immobilized in 1 g of Novozym® 435) of the total amount of protein was desorbed.

The biocatalyst's beads were sequentially heated at 150 °C for 10 min, cooled down and weighed (see Section 2.4 for details). The cycle was repeated until constant weight which ensures that the ethanol was removed from the beads. The temperature choosed for the treatment (150 °C) corresponds to the temperature of the ethanol desorption found in the TPSR analysis as will be described in Section 3.5. This experiment evidenced that Novozym® 435 lost 16.6% of its initial mass and irreversibly adsorbed 38.8% of alcohol due to the contact with ethanol at 45 °C for 8 days. The Scheme 1 shows the mass balance of the above described experiments.

3.4. Evolution of the secondary structure of the Candida Antarctica B lipase

Novozym® 435 was also analyzed through infrared spectroscopy in order to investigate the effect of the ethanol on the secondary structure of CALB that remained adsorbed on the beads. The beads of biocatalyst withdrawn after each treatment with ethanol were allowed to equilibrate its humidity in a desiccator for four days at room temperature. A careful screening demonstrated that the intensity and profile of the infrared spectra of the Amide I are improved when that procedure was applied due to the elimination of physically adsorbed water molecules.

The analysis of the infrared spectra of enzymes in the 1700–1600 cm^{-1} region (the so-called Amide I region) provides qualitative and also quantitative information on the secondary structure elements that compose the protein [20,29]. The Amide I band originates from the stretching vibrations of the peptide car-



Scheme 1. Mass balance for Novozym® 435 before and after being in contact with a mixture containing ethanol and initial nominal water content of 4.76% (v/v). Desorbed protein, dissolved matrix and recovered beads amounts are included.

Table 2
Characteristic assignments of the infrared signals that composes the Amide I region of *Candida antarctica B* lipase of Novozym® 435.

Wavenumber range (cm^{-1})	Assignment
1618–1622	Intermolecular β aggregates
1629–1631	β-Sheet
1639–1642	β-Sheet, antiparallel β-sheet
1648	α-Helix
1650–1655	α-Helix
1662–1666	α-Helix
1671–1674	Turns
1676–1679	Turns
1684–1691	β-Sheet

bonyl groups in the backbone of the protein, whose frequency depends on the hydrogen-bonding and coupling along the protein chain, and is therefore sensitive to the protein conformation. The Amide I signals of the infrared spectra of the starting Novozym® 435 and after several cycles of ethanol treatment (for 40 min at 28 °C and for 48 h at 45 °C), along with pure *Candida antarctica B* lipase (as reference material), were de-convoluted in order to obtain evidences of the secondary structure changes of the enzyme. Table 2 shows the wavenumbers and assignments of the signals that were identified through the second derivative calculation performed on the Amide I band [29,30]. Additionally, Figs. 4 and 5 compare the percentage contribution of the α-helix and β-sheet obtained through the integration and further normalization of the corresponding signals. The percentage contribution of the α-helix structure was obtained by adding the area of the signals appearing in the 1650–1655 cm^{-1} range. Similarly, the contribution of the β-sheet structure corresponds to the addition of the areas of the signals located at 1629–1639 cm^{-1} and 1684–1691 cm^{-1}. Other structures such as β aggregates and turns do not show modifications therefore they are not presented in this discussion.

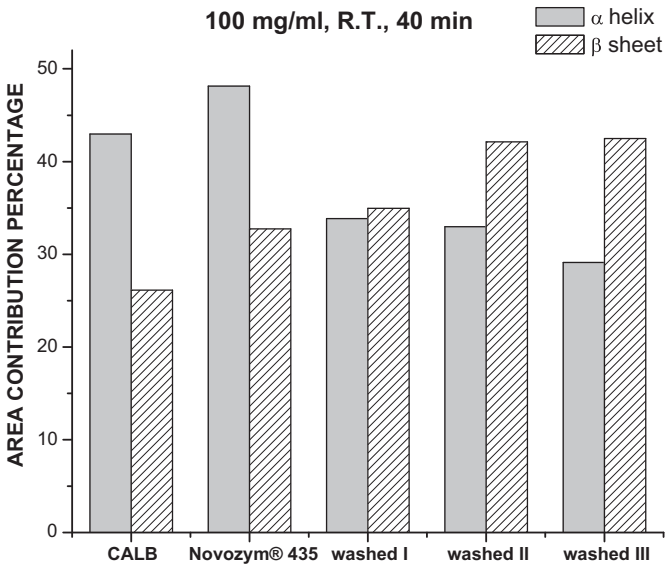


Fig. 4. Contribution of α-helix and β-sheet to the secondary structure of pure CALB (Sigma–Aldrich), Novozym® 435 before and after being treated three times (washed I, II and III) with ethanol: 4.76% (v/v) H_2O in a 100 mg/ml (milligrams of Novozym® 435 per milliliter) ratio, at room temperature for 40 min (each cycle of treatment).

The infrared analysis of highly pure CALB used as reference indicates a 43% contribution of the alpha helix and 26% of beta sheet structures which is in agreement with the literature. The secondary structure of the enzyme is not modified upon adsorption of the lipase on polymethylmethacrylate as observed in Novozym® 435 (see Fig. 4). However, the contact with ethanol at 28 °C in three cycles of 40 min. produces a gradual diminution of the alpha helix structure and increment of the beta sheet structure up to 43% (see Fig. 4). The same outcome

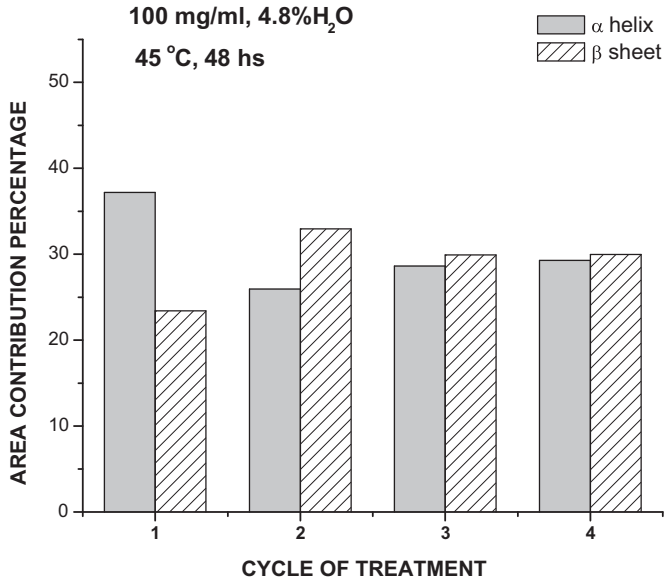


Fig. 5. Contribution of α-helix and β-sheet to the secondary structure of pure CALB (Sigma Aldrich), Novozym® 435 before and after being treated four times (1, 2, 3, 4) with ethanol: 4.76% (v/v) H_2O in a 100 mg/ml (milligrams of Novozym® 435 per milliliter) ratio, at 45 °C for 48 h (each cycle of treatment).

is observed when the biocatalyst is treated with ethanol at a higher temperature (45 °C) for extended periods of time (four cycles of 48 h. each) even in the presence of added water (see Fig. 5).

3.5. Surface interaction of ethanol with Novozym® 435

The interaction of ethanol with Novozym® 435 was investigated through the adsorption of gaseous and liquid ethanol followed by *in situ* temperature programmed surface reaction. Initially this investigation was performed over a sample of Novozym® 435 without any previous treatment with ethanol or used in the esterification of ibuprofen. However, to our surprise there was no adsorption of ethanol. Therefore, in a second stage a sample already used in the esterification of ethanol was analyzed. The adsorption was performed with successive doses of gaseous ethanol passing through the beads of Novozym® 435 and subsequently detected in a mass spectrometer as described in the experimental section. Fig. 6 shows seven pulses of the alcohol after leaving the "used" biocatalyst as a function of time. The area (indicated above each signal) of the pulses leaving the biocatalyst bed steadily increases. This observation indicates that the biocatalyst's surface adsorbs ethanol from the very beginning of the dosing and becomes saturated (is expected that the surface is completely covered with a layer of alcohol molecules) on the sixth dose. Moreover no desorbed species such as water, CO or CO_2 were detected which demonstrates that ethanol adsorbs without surface reaction at 32 °C which ensures that the surface adsorption sites are not modified during the analysis.

The temperature programmed surface reaction shows a broad signal of molecular ethanol and water desorption starting at 52 °C (see $m/e = 31$ in Fig. 7). The observation that neither ethylene, CO nor CO_2 are produced during the experiment evidences that ethanol do not react over the surface of the support of the biocatalyst. However, if there are PMMA chains not cross-linked with DVB, they may be solubilized by interaction with ethanol.

Further evidences of the strong adsorption of ethanol was obtained through the temperature programmed desorption performed on Novozym® 435 after being treated repeatedly with ethanol in the liquid medium at R.T. (as described in Section 2.4). Again molecular ethanol desorbs without surface reaction. Nevertheless, the process of desorption begins at 150 °C and shows a maximum at 184 °C (see Fig. 8).

Temperature desorption experiments (that is without previous adsorption of alcohol) were carried on fresh Novozym® 435 and the support (Lewatit VP OC 1600) in order to unambiguously demonstrate that molecular ethanol was detected in the previous analysis. These experiments demonstrated that water molecules are desorbed in different temperature ranges that are: 37–150 °C, 157–207 °C and a third desorption event that begins at 222 °C and continuous even above 400 °C as observed in Fig. 9. This desorption of water molecules at high temperature is accompanied by CO and CO_2 that is assigned to the decomposition of amino-acids and the PMMA support (the TPD of the support is not shown). This observation allows to confirm that the signal of $m/e = 31$ observed in the TPSR analysis at 184 °C corresponds to ethanol

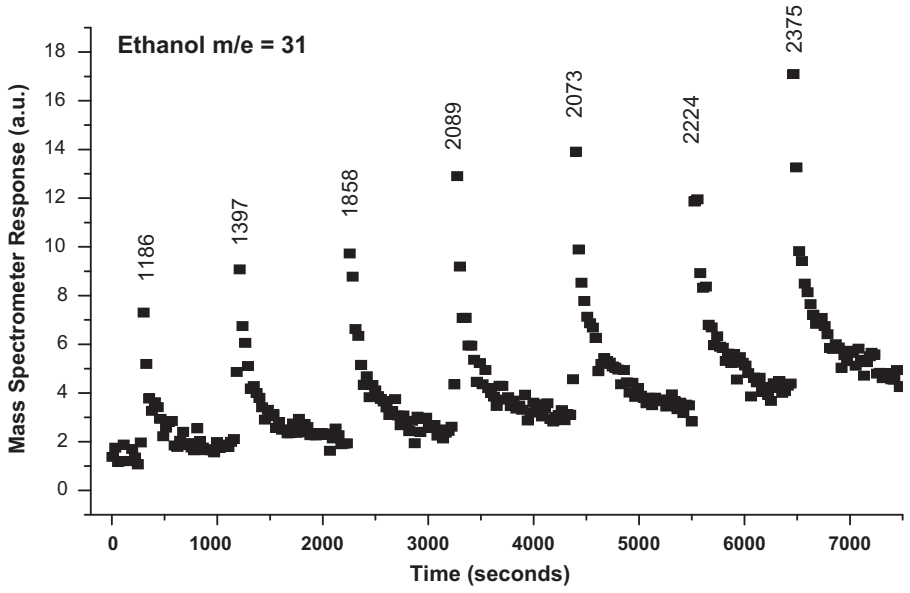


Fig. 6. Mass spectrometer signal of several doses of gaseous ethanol ($m/e = 31$) after interacting with Novozym® 435 beads that was previously used in the esterification of ibuprofen with ethanol.

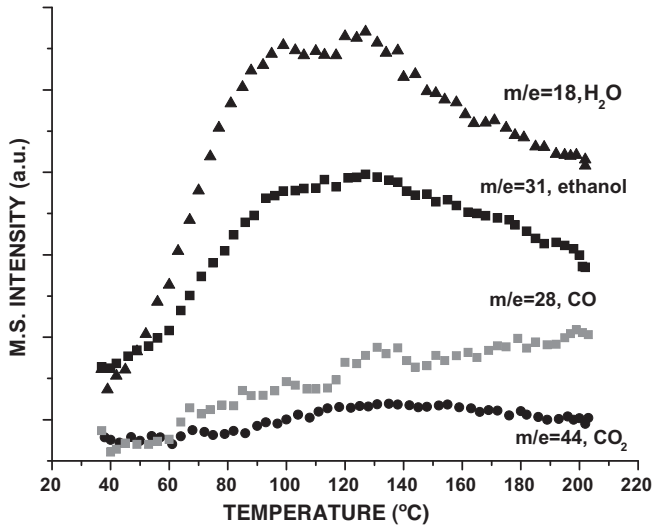


Fig. 7. *In situ* Temperature programmed surface reaction performed after ethanol adsorption on Novozym® 435.

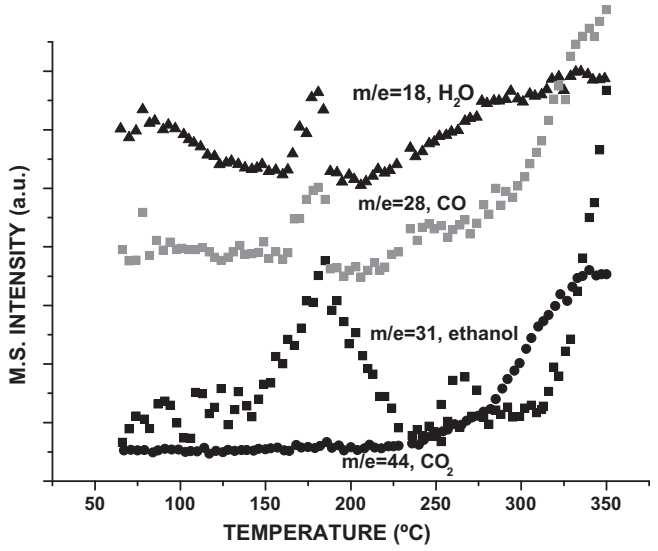


Fig. 8. Temperature programmed surface reaction spectra of Novozym® 435 after being in contact with liquid ethanol at R.T.

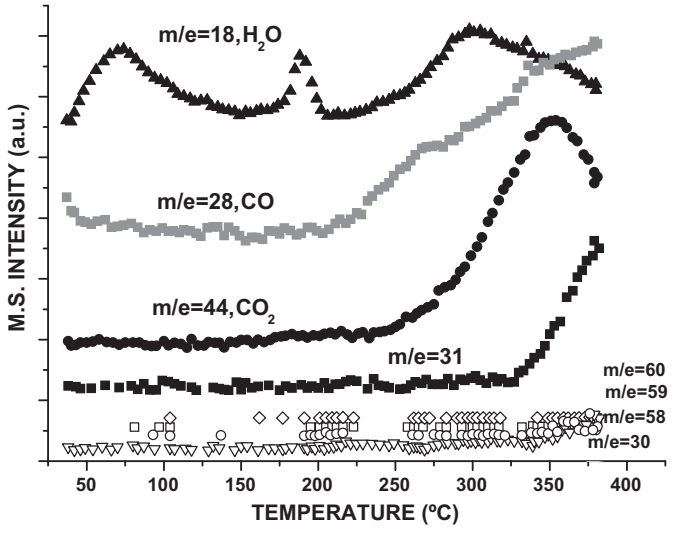


Fig. 9. Temperature programmed desorption spectra of Novozym® 435 as obtained from the commercial batch.

and do not belong neither to the CALB nor to PMMA thermal decomposition.

At the beginning of this section, it was pointed out that "fresh" Novozym® 435 does not adsorb ethanol. However, the observation that the biocatalyst exposed to ethanol and ibuprofen–ethanol mixture develops the capacity to adsorb the alcohol deserves a further discussion based on recent investigation reported by Zhao and Song [40]. The authors identified 17 substances (mainly glycerol, benzoic acid, 2-hydroxyethyl benzoate, 2-hydroxyethyl sorbate and sorbic acid) that migrate from Novozym® 435 towards aqueous solutions, organic solvents (such as methanol, acetone, acetonitrile, t-butanol and hexane) and ionic liquids. Although, the origin of those substances is not clear they suggested that might be introduced during enzyme formulation and immobilization onto the PMMA resin as preservatives or contaminants. Particularly, sorbic acid and benzoic acid are used as additives during the polymerization of acrylate. In general, the migration of those substances towards polar solvents happens in about 30 min at room temperature. Therefore, based on those observations, it is expected that the samples exposed to ethanol for at least 40 min (according to the procedure performed in the present investigation) do not possess the majority of such substances. Additionally, the present investigation demonstrates that also the PMMA resin is dissolved due to the contact with ethanol. Therefore, the exposure of Novozym® 435 to ethanol eliminated those interfering substances allowing further diffusion and adsorption of the alcohol in the inner portion of the beads as experienced in the TPSR analysis.

Fig. 10 shows the interaction of water with the PMMA surface and the interaction of the PMMA: water with ethanol. There are several compounds that may be exchanged by ethanol in liquid phase interaction. The lack of initial ethanol adsorption from the gas phase points out to the occupancy of the adsorption sites on the resin and even the unavailability of water to generate H-bonding adsorption of ethanol. If the additives/compounds are adsorbed on the water: PMMA surface, the PMMA surface is not available to the ethanol in gas phase or after short interactions with ethanol. However, looking at the interaction in the liquid phase, the relative solubility of these species in liquid ethanol makes available many adsorption sites for ethanol (by H-bonding to water and by H-bonding to the PMMA's oxygens). The hydrophobic interaction between the ethyl group of the ethanol and the CH_2-C(CH_3)– of the PMMA is important to understand the high temperature at which the ethanol is desorbed from the surface. Looking at Fig. 8, ethanol and water show peaks near 180 °C which is probably related to the similar strength of the bonding of both molecules when H-bonding is involved as the main interaction with the surface or other molecules and to the strength of the ethanol adsorption on the Lewatit from the liquid phase.

3.6. *Effect of ethanol on the outer and inner texture of Novozym® 435*

SEM micrographs of the entire biocatalyst's beads and the cross-sections before and after being in contact with liquid ethanol are shown in Figs. 11 and 12, respectively. The external texture of the beads exposed to ethanol show an evident scratched surface compared with the non-treatment biocatalyst as observed in Figs. 11 and 12A, respectively. In this context, the ethanol affected the inner texture of the beads also even though is not as visible as in the surface of the beads (see Figs. 11 and 12B). The inner structure morphology of the Novozym® 435, before and after interacting with ethanol, was studied with the estimator of the fractal dimension D and the d_{min} parameter, by using the variogram method, implemented on the FERImage program [24]. This program works with square images therefore, the central portion of the corresponding images that is typically a square of 132 × 132 μm

or $84 \times 84\,\mu m$ depending on the particle size was taken. If the image is anisotropic, both parameters, D and d_{min}, will not necessarily be constant in different directions, therefore, an average value of those obtained at six different directions, between 0º and 90º, was used. Figs. 13 and 14 show, as an example, graphs of variance versus step for images taken at 600× magnification of a slice of the cross-section of Novozym® 435 before and after interacting with ethanol, respectively. As can be seen in these figures, the inner structure is different for particles non-interacting or interacting with ethanol. Nevertheless, the inner structure for different particles in each one of the cases was similar and the same behavior was observed for the corresponding calculated parameters, D and d_{min} by working with high and low vacuum. In this context, five images corresponding to five different particles for each case were studied and their average values and its uncertainty are reported here. Fresh Novozym® 435 that is, without being in contact with ethanol possesses $d_{min} = 1.8 \pm 0.2\,\mu m$ and $D = 2.54 \pm 0.01$. In contrast, the contact with ethanol increases both parameters towards $d_{min} = 2.7 \pm 0.5\,\mu m$ and $D = 2.72 \pm 0.04$. The increase of the d_{min} parameter indicates that the alcohol increased the minimum internal structure of the enzyme support.

The D values ranging from $2 < D < 2.5$ indicate a persistent smooth surfaces while values $2.5 < D < 3$ are an indication of anti-persistence, being a completely rough surface when D is close to 3. The particular case of D equals to 2.5 corresponds to ordinary Brownian surfaces. The values in both types of analyzed samples (non-interacting and interacting with ethanol) indicate an anti-persistent fractal behavior ($D > 2.5$). The increase in the estimator of the fractal dimension D in the case of samples interacting with ethanol, indicate that their surface roughness increased.

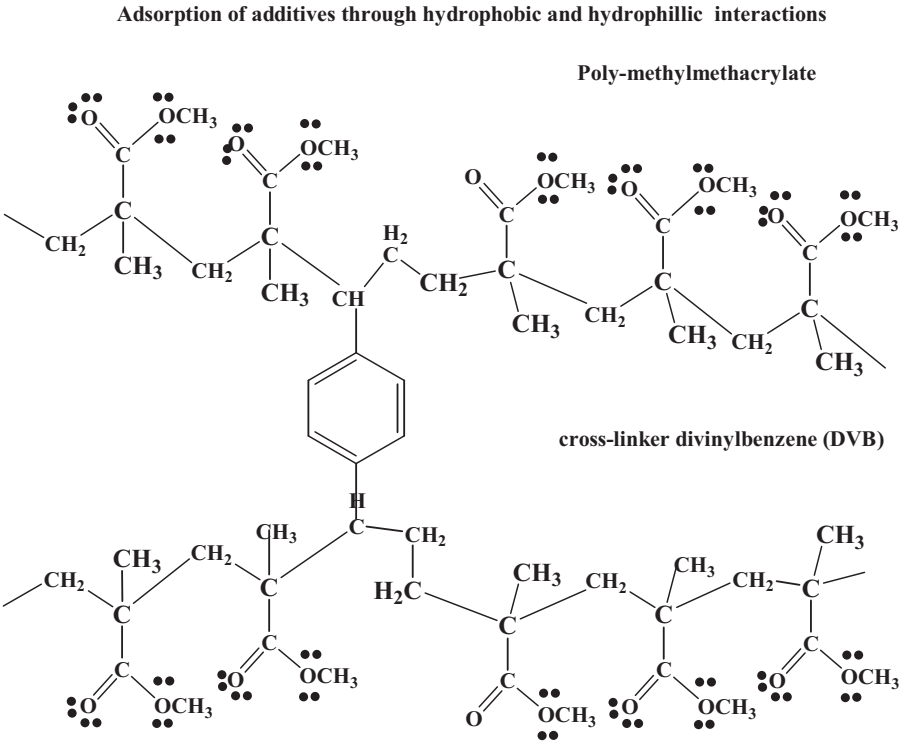


Fig. 10. Interaction of PMMA-Additives with ethanol.

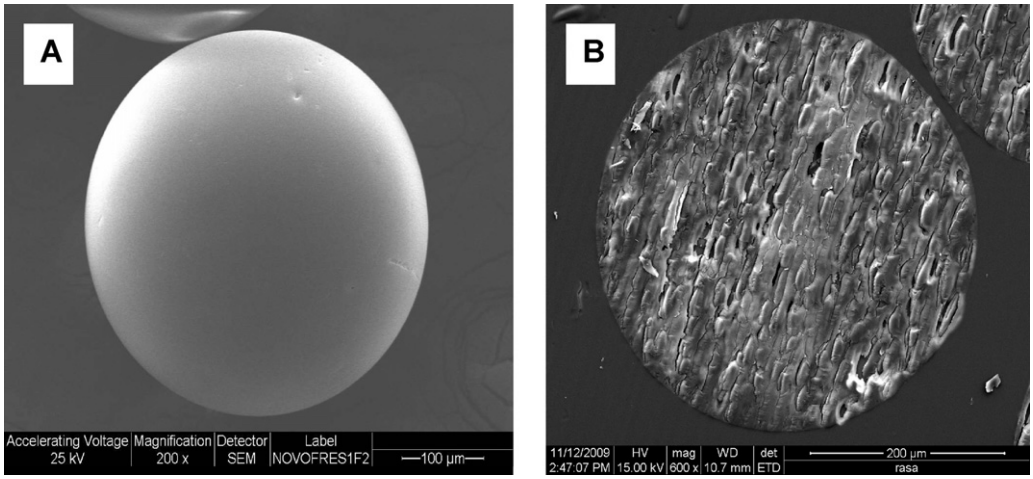


Fig. 11. Scanning electron micrographs of (A) a bead (magnification of 200X) and (B) the cross-section (magnification of 600×) of fresh Novozym® 435.

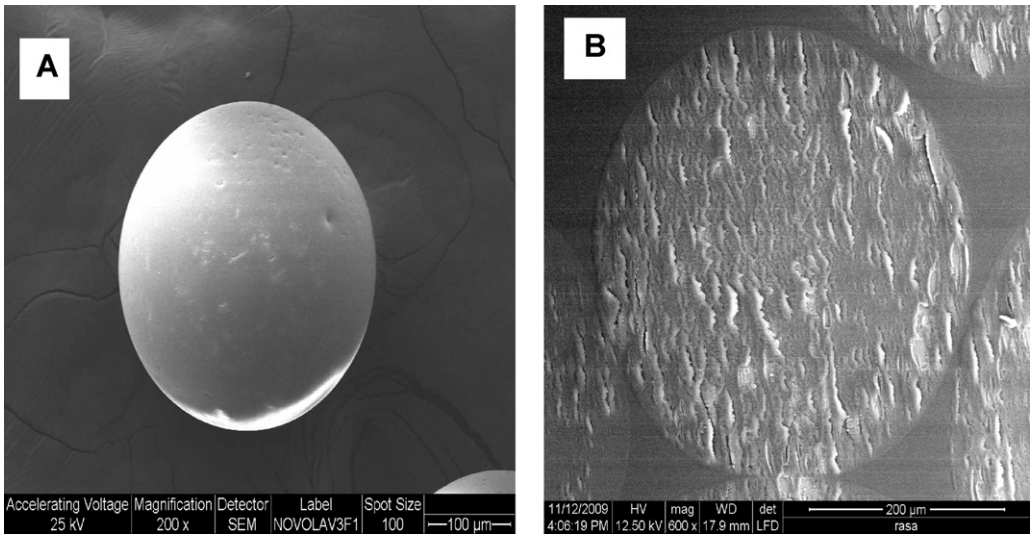


Fig. 12. Scanning electron micrographs of (A) a bead (magnification of 200×) and (B) the cross-section (magnification of 600×) of Novozym® 435 after being in contact with ethanol:4.76% (v/v) H_2O at room temperature.

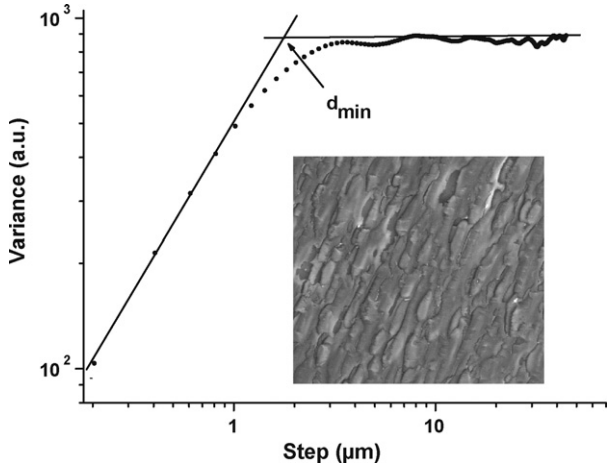


Fig. 13. Variogram corresponding to the 84 × 84 μm central portion of the image at 600× magnification of a slice of the cross-section of Novozym® 435 before contacting with ethanol. The image appears as an insert in the graph.

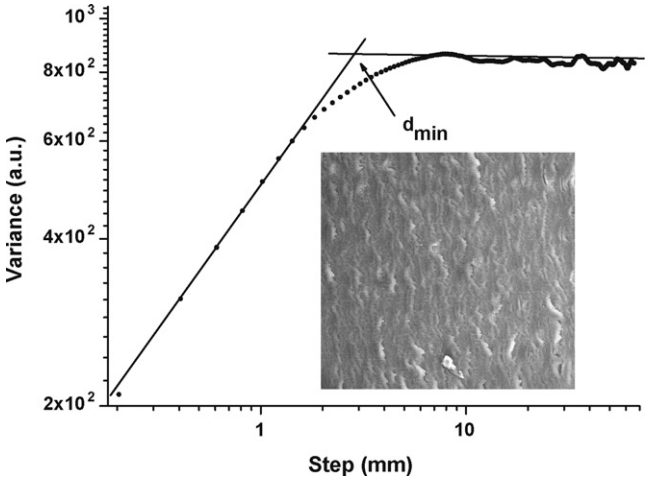


Fig. 14. Variogram corresponding to the image taken at 600× magnification of a slice of the cross-section of Novozym® 435 after contacting with ethanol. The image appears as an insert in the graph.

4. Discussion

The motivation of the present investigation was to elucidate the causes of the deactivation of Novozym® 435 after several reuses in the enantiomeric esterification of ibuprofen with ethanol as reactant and solvent (without the addition of a co-solvent). The exhaustive examination of the literature of the last 10 years devoted to the application of that biocatalyst shows several reports of its deactivation/inhibition under different reaction conditions however, there is not a clear and conscious explanation of that phenomenon. In this context, the present investigation demonstrates that there are not only one but several causes that account for the degradation of the biocatalyst in contact with ethanol (with or without water added). The most relevant effect of the solvent is the dissolution/de-aggregation of the PMMA that constitutes the core of the biocatalyst's beads. This effect is not restricted to ethanol since the literature also reports that PMMA dissolves in contact with mixtures of carbon tetrachloride and acetonitrile with alcohols of low molecular weight (methanol, ethanol and 1-propanol) regardless of the composition of the mixture [35–37]. The dissolution process exerted by the solvents leads to the migration of the polymer and other substances towards the liquid phase. In this context, Zhao and Song reported the appearance of 17 substances that migrated from Novozym® 435 towards organic solvents and ionic liquids. Additionally, some of those substances resulted active as acyl donors in the esterification and transesterification reactions in the presence of *Candida antarctica B* lipase [40].

Previous observations already reported in the literature and our investigation, are undoubtedly an evidence of the contamination and/or interference that happens when Novozym® 435 is used in organic media. This is a relevant observation that the researchers might not be probably aware of.

Moreover, the interaction of the ethanol with Novozym® 435 occurs not only at the surface but also diffuse into the biocatalyst's beads. Previously, Heinsman and coworkers found anomalous results of the enantiomeric ratio during the enantioselective esterification of 4-methyloctanoic acid with ethanol catalyzed with Novozym® 435 [41]. The authors demonstrated that ethanol produces the swelling of the enzyme support and that the 4-methyloctanoic acid strongly absorbs into the biocatalyst's beads. These two combined effects produce an increment of 175–200% of the bead's volume (from an initial volume of 58 ml to a final vol-

ume of 125 ml). The present investigation has further confirmed that ethanol diffuses into the beads through the parameterization of the SEM images of the cross-section of the beads. These calculations that, to our knowledge have never been performed before on a biocatalyst surface, evidence the modification of the internal texture of the beads. The increase of the length (the d_{min} parameter) of the statistic pattern that describes the texture of Novozym® 435 upon interacting with ethanol indicates that the alcohol increases the internal pore structure of the enzyme support. Moreover, the increase in fractal dimension D correlates with the increase of the surface roughness.

The adsorption of gaseous ethanol and further TPSR analysis provides an indication of the interaction of the alcohol with the outermost layer of the biocatalyst's bead where is located the enzyme (CALB is located in approximately 100 μm outermost layer of the bead according to the reference [34]). The desorption of molecular ethanol (only $m/e = 31$ was detected) allows to conclude that the alcohol adsorbs on the enzyme but does not further reacts upon heating. Additionally, the broad desorption profile of ethanol (from 60 °C to 200 °C) indicates a plurality of adsorption sites of the enzyme and probably the presence of exposed PMMA at the surface. In fact the TPSR analysis of the biocatalyst after interacting with liquid ethanol that, certainly diffuses into the beads as discussed before, demonstrates that the interaction of molecular alcohol with the PMMA is much stronger (again without reaction upon heating) since the desorption begins at 150 °C. The dissolution process of Lewatit may take place in the non-cross-linked portions of the PMMA, with low molecular weight. Low molecular weight resin probably dissolves by the interaction with ethanol, through swelling and disruption of Van der Waals forces between resin chains in the support. The competition that is proposed to take place (leading to partial dissolution) would be between the Van der Waals forces in the solid resin polymer and the H-bonding interaction of ethanol with the polar tails of the resin. This simple physical process does not involve breaking any covalent bond in the resin. Fig. 10 shows the differences between the DVB cross-linked PMMA and the PMMA chains interacting through Van der Waals forces – mainly hydrophobic but also hydrophobic plus H bonding – with ethanol. The exchange of the additives present on Novozym® 435 by ethanol is fast in a liquid media, probably affected by the relative solubility of the different additives present, exposing the adsorption hydrophobic surface to more ethanol. In the gas phase, this reaction is by far less favored due to the steric restrictions and strong interactions with the surface of the additives. The FTIR shows evidence of adsorbed water, but as expected the adsorbed amount is not high therefore it is not thought to be particularly important in the coordination of the ethanol.

These observations provide further evidence of the inhibiting effect that exerts the alcohol that was both experimentally and theoretically reported previously by some of us [9]. In this context, the molecular modeling calculations in a simple model allowed us to postulate that the formation of irreversible dead-end complexes between ethanol adsorbed on the serine (Ser 105) and histidine (His 224) residues of the catalytic triad of CALB, is thermodynamically feasible. Additionally, these complexes might be formed before the ibuprofen coordination which undoubtedly, leads to an inhibition of the enzyme's active sites in the esterification process.

The inhibition exerted by the alcohol along with the desorption of the protein out of Novozym® 435 caused a decrease of the active adsorption sites of the biocatalyst which in turn diminishes the amount of adsorbed ibuprofen as observed in the experiments discussed in Section 3.1. In this context, it is hypothesized that the different behaviors observed in Fig. 1 at 25 °C versus 28 °C and 32 °C could be attributed to the existence of two different pathways that take place according to the assay temperature. At 25 °C co-adsorbed additives that are known to be present in Novozym® 435 and that are plausible to be washed away in the ethanolic medium are not given enough time to abandon the support [40]. Then ibuprofen is adsorbed onto the additives instead than onto the enzyme and the support. As time goes by, the additives are washed away from the support and adsorbed ibuprofen is washed away with them. The previous could explain the dramatic decrease of ibuprofen concentration shown at 25 °C in Fig. 1. On the other hand, at higher temperatures (28 °C and 32 °C), additives might be washed away more rapidly and then ibuprofen would be allowed to become adsorbed directly onto Novozym® 435, leading to the irreversible adsorption illustrated in Fig. 1 for the mentioned temperatures.

The strong interaction of ethanol with the amino acids' residues might also perturb the hydrogen bonding between protein atoms which affects the tertiary structure (not investigated here) and in turn, might also be responsible for the modifications observed on the secondary structure of CALB. In this context, proteins in hydrophobic solvents are thought to retain their native structure due to stronger hydrogen bonding between the protein atoms and a more rigid structure in the absence of water. Conversely, proteins in polar solvents than can strip water from the surface of the protein and compete for hydrogen bonds between protein's atoms are thought to denature the structure to a largely unfolded state [42]. However, the investigations of Griebenow and Klibanov show a completely different behavior of the secondary structure of proteins (crystalline and lyophilized lysozyme and subtilisin) in aqueous–organic mixtures ranging from pure water to pure solvent such as, acetonitrile, tetrahydrofuran and 1-propanol [43]. The authors demonstrated that the α-helix content declined markedly in most water–solvent mixtures. This behavior was found to be kinetically controlled, i.e., to be due to inherent restrictions on protein conformational mobility in anhydrous, in contrast to aqueous–organic, media. Nevertheless, the influence of alcohols on the conformation and catalytic activity of immobilized lipases has been far less investigated. In this context, Gao and coworkers studied the effect of ethanol, n-propanol, isopropanol, n-butanol and t-butanol on the hydrophobicity of *Candida rugosa*, *Candida antarctica B* and *Porcine Pancreas* lipases in aqueous solutions containing alcohols and the specific activity of lipase immobilized on silica aerogels [44]. In the particular case of CALB, the addition of ethanol changes the folding of the enzyme in some extent which in turn, increases the exposure of more hydrophobic groups of the enzyme (the hydrophobic index increases). As presented before 16.6% of the Lewatit mass was lost and also the 2.76% of the total protein content of the biocatalyst. Considering 5.33 as the amount of protein present initially for 100 mg, this percentage implies 0.147 mg for a total mass loss of CALB in 16.6 mg of total mass difference and only the 0.88% of protein content in the dissolved Lewatit mass. The ethanol affects strongly the Lewatit through dissolution and the dissolved PMMA has not high affinity for the CALB compared with the average biocatalyst reported to have 5–10% of CALB.

5. Conclusion

The present investigation demonstrates for the first time in the literature that the commercial biocatalyst Novozym® 435 is dissolved by ethanol and an ethanol/water mixture in 16.6% of the initial mass of 1.0000 g, leading also to the loss of some protein (2.76% of the total protein content). In this context, the results on the protein quantification by different techniques and the problems found with their application emphasize the need of carefully designed and implemented quantification techniques.

The observation that ethanol is not adsorbed on the fresh biocatalyst (that has not been in contact with ethanol) suggests that the alcohol exposes the hydrophobic/hydrophillic surface of the PMMA that was previously hindered because of the presence of additives

and the cross-linker divinyl benzene. Moreover, the temperature programmed desorption studies undoubtedly demonstrated that the alcohol remains strongly adsorbed (most probably hydrogen bonded) to the biocatalyst's surface.

The action of ethanol over the integrity of the PMMA resin was also determined on the inner texture of the biocatalyst's beads. In fact, the microscopic analysis of the cross-section of the beads demonstrated the increase of their roughness and internal pore structure.

Finally, the ethanol impacts on the secondary structure of the *Candida antarctica B lipase* increasing the contribution of the β-sheet structure.

The conclusions discussed above demonstrate that the deactivation reported by ourselves and other researchers on the catalytic activity of Novozym® 435 in contact with alcohols cannot be explained straightforward since there is a multiplicity of effects that account for such phenomena. In this context and considering the variety of applications of Novozym® 435 and the future ones, the present investigation is a starting point for more studies on the stability of commercial biocatalysts in order to design reliable biotechnological processes. It then follows that, prior to its use, – and spite of the generally high activity shown by Novozym® 435 in a number of processes –, the stability of the biocatalyst *as a whole* should be investigated in the solvent/s before the development of a specific application. More importantly, the researchers should be aware that the dissolution of Novozym® 435 support in an ethanolic medium could certainly (i) damage/alter the performance of the analysis equipment used (i.e., chromatographic columns), and most important, (ii) targeted products could result contaminated with substances that migrated from the biocatalyst, which for medical or food applications is unacceptable since they could have a negative impact in human health.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2011.04.004.

References

[1] A. Ganesan, B.D. Moore, S.M. Kelly, N.C. Price, O.J. Rolinski, D.J.S. Birch, I.R. Dunkin, P.J. Halling, ChemPhysChem Special Issue: Biophys. 10 (2009) 1492–1499.
[2] T. Tan, J. Lu, K. Nie, L. Deng, F. Wang, Biotech. Adv. 28 (2010) 628–634.
[3] L. Fjerbaek, K.V. Christensen, B. Norddah, Biotech. Bioeng. 102 (2009) 1298–1315.
[4] C.-C. Lai, S. Zullaikah, S.R. Vali, Yi-Hsu Ju, J. Chem. Technol. Biotechnol. 80 (2005) 331–337.
[5] H.-M. Chang, H.-F. Liao, C.-C. Lee, C.-J. Shieh, J. Chem. Technol. Biotechnol. 80 (2005) 307–312.
[6] M. Trani, A. Ducret, P. Pepin, R. Lortie, Biotech. Lett. 17 (1995) 1095–1098.
[7] A. Ducret, M. Trani, R. Lortie, Enzyme Microb. Technol. 22 (1998) 212–216.
[8] M. Arroyo, J.V. Siniesterra, J. Org. Chem. 59 (1994) 4410–4417.
[9] M.L. Foresti, M. Galle, M.L. Ferreira, L.E. Briand, J. Chem. Technol. Biotechnol. 84 (2009) 1461–1473.
[10] C. José, L.E. Briand, Reac. Kinet. Mech. Cat. 99 (2010) 17–22.
[11] K. Won, J. Hong, K. Jim, S. Moon, Process Biochem. 41 (2006) 264–269.
[12] H.-Y. Zhang, X. Wang, C.-B. Ching, Chirality 19 (2007) 245–249.
[13] P. Torres, D. Reyes-Duarte, N. López-Cortés, M. Ferrer, A. Ballesteros, F.J. Plou, Process Biochem. 43 (2008) 145–153.
[14] T. Maugard, J. Tudella, M.D. Legoy, Biotech. Prog. 16 (2000) 358–362.
[15] B.A.C. van As, J. van Buijtenen, T. Mes, A.R.A. Palmans, E.W. Meijer, Chem.-A Eur. J. 13 (2007) 8325–8332.
[16] G.D. Yadav, A.D. Sajgure, S.B. Dhoot, J. Chem. Technol. Biotechnol. 88 (2008) 1145–1153.
[17] J.C. Santos, T. Bueno, P.C. Molgero, D. Rós, H.F. de Castro, J. Chem. Technol. Biotechnol. 82 (2007) 956–961.
[18] R.N. Patel, Coord. Chem. Rev. 252 (2008) 659–701.
[19] O. Pámies, J.E. Bäckvall, Chem. Rev. 103 (2003) 3247–3261.
[20] S. Gryglewicz, E. Jadownicka, A. Czerniak, Biotechnol. Lett. 22 (2000) 1379–1382.
[21] M. Kramer, J.C. Cruz, P.H. Pfromm, M.E. Rezac, P. Czermak, J. Biotech. 150 (2010) 80–86.
[22] B. Liu, X. Qian, Q. Wu, X. Lin, Enzym. Microb. Tech. 43 (2008) 375–380.
[23] B. Chen, J. Hu, E.M. Miller, W. Xie, M. Cai, R.A. Gross, Biomacromolecules 9 (2008) 463–471.
[24] F.D. Bianchi, R.D. Bonetto, Scanning 23 (2001) 193–197.
[25] J.L. Ladaga, R.B. Bonetto, in: W. Peter, Hawkes (Eds.), Advances in Imaging and Electron Physics, vol. 120, Academic Press, 2002, pp. 136–189, ISSN: 1076-5670-02.
[26] A.P. Pentland, IEEE Trans. Pattern. Anal. Mach. Intell. PAMI-6 (6) (1984) 661–674.
[27] R.D. Bonetto, J.L. Ladaga, Scanning 20 (1998) 457–463.
[28] L.A. Gambaro, L.E. Briand, Appl. Catal. A: Gen. 264/2 (2004) 151–159.
[29] A. Barth, Biochim. Biophys. Acta 1756 (2007) 1073–1101.
[30] M.L. Foresti, G.M. Valle, R. Bonetto, M.L. Ferreira, L.E. Briand, Appl. Surf. Sci. 256 (2010) 1624–1635.
[31] Y. Mei, L. Miller, W. Gao, R.A. Gross, Biomacromolecules 4 (2003) 70–74.
[32] R.M. Silverstein, G.C. Bassler, T.S. Morrill, Spectrometric Identification of Organic Compounds, J. Wiley & Sons, 1991, pp. 102–131.
[33] Infrared spectra database of the National Institute of Standards and Technology (http://webbook.nist.gov) of methyl acrylate $C_4H_6O_2$ (CAS: 96-33-3) and m-divinyl benzene $C_{10}H_{10}$ (CAS: 108-57-6).
[34] M. Mizukami, M. Moteki, K. Kurihara, J. Am. Chem. Soc. 124 (2002) 12889–12897.
[35] J. González-Benito, J.L. Koenig, Polymer 47 (2006) 3065–3072.
[36] J. González-Benito, J.L. Koenig, Macromolecules 35 (2002) 7361–7367.
[37] R. Hoogenboom, C.R. Becer, C. Guerrero-Sanchez, S. Hoeppener, U.S. Schubert, Aust. J. Chem. 63 (2010) 1173–1178.
[38] I. Petry, A. Ganesan, A. Pitt, B.D. Moore, P.J. Halling, Biotech. Bioeng. 95 (2006) 984–991.
[39] B. Sharma, A. Azim, H. Azim, R.A. Gross, E. Zini, M.L. Focarete, M. Scandola, Macromolecules 40 (2007) 7919–7927.
[40] H. Zhao, Z. Song, Biochem. Eng. J. 49 (2010) 113–118.
[41] N.W.J.T. Heinsman, C.G.P. Schroën, A. van der Padt, M.C.R. Franssen, R.M. Boom, K. vanît Riet, Tetrahedron Asymm. 14 (2003) 2699–2704.
[42] C. Mattos, D. Ringe, Curr. Opin. Struct. Biol. 11 (2001) 761–764.
[43] K. Griebenow, A.M. Klibanov, J. Am. Chem. Soc. 118 (1996) 11695–11700.
[44] S. Gao, W. Wang, Y. Wang, G. Luo, Y. Dai, Bioresource Tech. 101 (2010) 7231–7238.