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Lipase immobilization on smectite nanoclays: Characterization and application to the epoxidation of α -pinene

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ABSTRACT

The immobilization of lipase B from *Candida antarctica* on smectite group nanoclays (Laponite, SWy-2 and Kunipia), as well as on their organically modified derivatives, was investigated. A combination of techniques, namely X-ray diffraction, thermal analysis, X-ray photoelectron and FT-IR spectroscopy, was used for characterization of the novel immobilized biocatalyst. Structural and biochemical characterization have revealed that the hydrophobic microenvironment created by the organo-modified clays induces minor changes on the secondary structure of the enzyme, resulting in enhanced catalytic behaviour in hydrophobic media. The immobilized lipase on such modified nanoclays can be effectively applied for the indirect epoxidation of α -pinene using hydrogen peroxide as substrate. The amount of α -pinene epoxide produced in a single-step biocatalytic process is up to 3-fold higher than that of free enzyme or enzyme immobilized in non-modified clays. Moreover, lipase immobilized in modified clays retains up to 90% of its initial activity, even after 48 h of incubation in the presence of oxidant, and up to 60% after four reaction cycles, while other forms of the enzyme retain less than 10%.

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1. Introduction

Lipases are currently attracting an enormous attention because of their biotechnological potential (Carboni-Oerlemans et al., 2006; Svedendahl et al., 2008; Lu et al., 2008). With the aim of improving activity, storage and operational stability, especially in low-water media, their immobilization on various organic/inorganic supports has been studied extensively (Petkar et al., 2006). On account of the relatively high surface hydrophobicity of lipases, their physical adsorption on suitably hydrophobic carrier materials is the most widely used method given its simplicity (Mena et al., 2008). It is well established that the structural characteristics of the host materials strongly affect the catalytic behaviour of the immobilized enzymes. In particular, the surface hydrophobicity of the support has been shown to strongly affect the activities of the different immobilized lipases through possible conformational changes caused by interfacial activation of a lipase upon binding on the solid interface (Petkar et al., 2006; Mena et al., 2008). Various hydrophilic or hydrophobic materials, including sol-gels (Yang

et al., 2009), resins (Yang et al., 2010), membranes functionalized with polymers (Ye et al., 2006) and nanoparticles (Deepak et al., 2009), have been reported in the literature for enzyme immobilization. Among them, nanostructured materials, such as smectite nanoclays, have emerged as alternative supports for enzyme immobilization, since they can enhance the biocatalytic efficiency by reducing diffusional limitations as well as by increasing enzyme loading because of superior surface area per mass unit (de Fuentes et al., 2001; Serefoglou et al., 2008). Due to their unique physico-chemical properties, such as swelling and cation exchange capability (CEC), as well as their mechanical and thermal stability, smectite nanoclays can be efficiently used as matrices for immobilization of biomolecules (de Fuentes et al., 2001; Gopinath and Sugunan, 2007; Serefoglou et al., 2008; Secundo et al., 2008).

Natural or synthetic smectite clays are layered materials consisting of nanometer-size aluminosilicate platelets. Their structure is composed of an octahedral alumina layer fused between two tetrahedral silica layers. The 1 nm thick platelets are negatively charged and neutrality is obtained, for example, by hydrated cations present in the interlayer galleries. The properties of the smectite nanoclays can be tailored using simple chemical methods such as intercalation with organic or inorganic guest molecules (Gournis et al., 2006). Their hydrophobicity can be increased through treatment with an organic surfactant (Litina et al., 2006). As a result, the

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presence of the surfactant expands the interlayer gallery rendering the nanoclay compatible with hydrophobic media. Due to their distinctive structure and properties, these organic–inorganic hybrid materials can be utilized in a wide variety of applications including, construction of modified electrodes (Kemmegne Mbougouen et al., 2006) and biosensors (Shan et al., 2006).

In the present work, we study the use of organically modified nanoclays of the smectite group as matrices for immobilization of lipase B from *Candida antarctica* (CaLB). The clays used are a synthetic trioctahedral hectorite (Laponite, LAP), and two natural dioctahedral montmorillonites (SWy-2 and Kunipia, KUN). The influence of the clays organomodification (through their interaction with octadecyl trimethyl ammonium bromide) on the adsorption and catalytic behaviour of the lipase was investigated. The resulting new hybrid biocatalysts were characterized by a combination of analytical techniques such as X-ray diffraction (XRD), thermogravimetric analysis (TGA), differential thermal analysis (DTA) and X-ray photoelectron spectroscopy (XPS). The conformational changes of lipase upon immobilization were investigated using Fourier transform infrared (FT-IR) spectroscopy. The structural and biocatalytic characteristics (synthetic activity, stability) of the novel lipase-clay conjugates were determined in low-water organic media. Moreover, the ability of the immobilized lipase to catalyze a synthetic reaction that is far from its natural role, such as the indirect epoxidation of α -pinene, via the formation of peroxycarboxylic acid using octanoic acid and hydrogen peroxide as substrates, was also investigated. α -Pinene is the main component of some essential oil (e.g. mastic oil) and gum turpentine, has low commercial value and is considered as a renewable raw material with a great potential to obtain pharmaceuticals, agrochemicals and other fine chemicals (Wender and Mucciario, 1992). Enzyme-mediated oxidative functionalization of terpenes such as α -pinene, represents an important way for valorization of these natural products, leading to the formation of biologically active compounds (Loutrari et al., 2004; Skouridou et al., 2003). The effect of various reaction parameters on the epoxidation activity, stability and reusability of the immobilized lipase on various nanoclays was examined and compared with that obtained for non-immobilized enzyme or commercially available immobilized preparation of the lipase.

2. Methods

2.1. Enzymes and chemicals

CaLB was from Fluka, Novozym 435® (immobilized CaLB) from Novozymes (Denmark). Urea hydrogen peroxide complex (UHP) was purchased from Aldrich (USA). H_2O_2 (30% w/w aqueous solution), α -pinene, as well as other chemicals and organic solvents were of the highest purity commercially available.

2.2. Supports

The support materials that were examined included three clays: a trioctahedral hectorite (Laponite, LAP) obtained from Laporte Industries Ltd., with a particle size of 200 nm and CEC of 50 meq per 100 g of clay, and two natural dioctahedral montmorillonites, Kunipia (KUN) with a particle size of 2000 nm and CEC of 120 meq per 100 g of clay, and Wyoming (SWy-2) with a particle size of 2000 nm and CEC of 80 meq per 100 g of clay, obtained from Kunimine Co. (Japan) and Source Clay Minerals Repository, University of Missouri, Columbia (USA), respectively. Natural clays were fractionated to less than 2 μm sizes by gravity sedimentation and purified by standard methods (Gournis et al., 2008). Organo-modified clays (ORLAP, ORKUN and ORSWy-2) were prepared through

intercalation with a cationic surfactant (octadecyl trimethyl ammonium bromide) (Litina et al., 2006).

2.3. Immobilization in layered materials

In a typical procedure, 50 mg of clay were dispersed in 10 ml of 50 mM citrate/phosphate buffer (pH 6.0), followed by addition of aqueous solution of the enzyme (CaLB). By varying the mass ratio of added enzyme to clay, derivatives with different protein loads were obtained: LAP-R, ORLAP-R, KUN-R, ORKUN-R, SWy-2-R and ORSWy-2-R (where R = 20, 50, 100, 200 mg enzyme per g of clay and prefix OR designates the use of organo-modified clay). The suspension was incubated at room temperature under mild stirring at 150 rpm for 24 h and then centrifuged at 2500 rpm. The precipitate was washed thoroughly with a buffer solution (50 mM citrate/phosphate, pH 6.0) and air-dried after spreading it on a glass-plate at 30 °C for 24 h. The amount of enzyme that was bound on clay particles was determined following the estimation of protein which remained on supernatant after centrifugation using the Bradford method (Bradford, 1976). The preparations stored at -20 °C before use.

2.4. Enzymatic reactions

2.4.1. Transesterification reactions

In a typical experiment, the enzymatic transesterification reaction of vinyl butyrate (50 mM) and 1-butanol (50 mM) in various organic solvents (5 ml) was carried out in stirred flasks in the presence of 50 mg of lipase-clay conjugate ($R = 100$ mg protein g^{-1} clay) or 10 mg of lyophilized preparation at 50 °C. To avoid possible drying or hydrating effects of the clays and the immobilized lipases, the organic solvent and the reactants were separately equilibrated to the same water activity (a_w) prior to being mixed with the enzyme. This was done by equilibration in sealed containers for at least 24 h, at 25 °C, with the vapour phase of a saturated salt solution of LiCl ($a_w = 0.11$) (Valivety et al., 1992).

2.4.2. Chemo-enzymatic epoxidations

Epoxidation of alkenes was carried out in a similar manner as described previously (Skouridou et al., 2003). In a typical reaction α -pinene (1 mmol) and octanoic acid (1 mmol) were dissolved in 5 ml *n*-hexane (previously dried with 3 Å molecular sieves) and the reaction was started by adding lyophilized (10 mg) or immobilized CaLB (50 mg, $R = 100$ mg protein g^{-1} clay). The reaction was initiated with the addition of 1.1 mmol of an oxidizing agent (H_2O_2 or UHP), and the mixture was stirred at 25 °C. Controls were performed for each reaction set in the absence of enzyme. Aliquots from the organic phase were withdrawn at different time intervals and analyzed by GC as described before.

Highly purified oxidized products were also obtained by semi-preparative HPLC using a Discovery C18 column (particle size 5 μm , length 250 mm, diameter 10 mm) in a similar manner as described elsewhere (Tziaila et al., 2008). Reaction products were analyzed by GC-MS (GC-CP 3900 interfaced with a Saturn 2000 MS) using a VF-5 ms column (30 m \times 0.25 mm i.d., 0.25 μm film thickness). Identification of products was based on analysis of commercially available standards (Tziaila et al., 2008).

2.5. Stability studies

2.5.1. Thermal and operational stability

Thermal stability of free or immobilized CaLB ($R = 100$ mg protein g^{-1} clay, 20 mg), were carried out by measuring the residual activity of the enzyme incubated in *n*-hexane at different temperatures (40–60 °C) for 72 h. The residual enzyme activity was determined based on transesterification lipase activity as described

before. The operational stability of CaLB was evaluated following repeated cycles of α -pinene chemo-enzymatic epoxidation using 1.1 mmol UHP as the oxidizing agent. Each reaction was carried out for 24 h at 25 °C as previously described. Between two consecutive catalytic cycles, the enzyme was separated by filtration and washed exhaustively with water, diethyl ether and/or *n*-hexane, until no reaction components (either reactants or products) could be detected in the filtrate. The residual enzyme activity was determined based on transesterification assay, as described before.

2.5.2. Effect of oxidant

Either free (10 mg) or immobilized biocatalyst ($R = 100$ mg protein g^{-1} clay, 50 mg), were incubated up to 48 h in *n*-hexane (1.5 ml) at 25 °C, in the presence of 1.1 mmol of the oxidant (UHP, or H_2O_2). After incubation, the enzyme preparations was filtered off and washed with water, diethyl ether and/or *n*-hexane to completely remove the added chemicals. The enzyme preparation was then dried under vacuum, weighed and residual lipase activity was measured using the epoxidation assay, as described before.

2.6. X-ray powder diffractometry (XRD)

The XRD patterns were collected with a D8 Advanced Bruker diffractometer operating at 40 kV and 40 mA, using $CuK\alpha$ radiation and a secondary beam graphite monochromator, as described elsewhere (Serefoglou et al., 2008).

2.7. FT-IR spectroscopy

Infrared spectra were determined on a FT-IR 8400 spectrophotometer (Shimadzu, Japan) equipped with a deuterated triglycine sulfate (DTGS) detector between 4000 and 400 cm^{-1} . Spectrum acquisition, data analysis of the Amide I region and band assignment were performed as described in previous work (Serefoglou et al., 2008). The data were manipulated using WinSpec software (LISE-Facultés Universitaires Notre-Dame de la Paix, Namur, Belgium).

2.8. Thermal analysis

Thermogravimetric (TGA) and differential thermal (DTA) analysis were performed with a Perkin Elmer Pyris Diamond TG/DTA, as described elsewhere (Serefoglou et al., 2008).

2.9. X-ray photoelectron spectroscopy

X-ray photoelectron spectra were measured using a SSX-100 (Surface Science Instruments) photoelectron spectrometer with a monochromatic Al $K\alpha$ X-ray source ($h\nu = 1486.6$ eV), as described elsewhere (Serefoglou et al., 2008).

3. Results and discussion

3.1. Lipase immobilization on clays

In the present work, three layered nanoclays of the smectite group (LAP, SWy-2 and KUN) and their organo-modified derivatives (ORLAP, ORSWy-2 and ORKUN) were studied as supports for the immobilization of CaLB. The effectiveness of the enzyme immobilization can be influenced by the nature and the composition of the support clay and depends on the strength of various non covalent interactions, such as van der Waals interactions, hydrogen bonding as well as hydrophobic and electrostatic interactions between the clay support and the amino acid on the surface of the protein (de Fuentes et al., 2001; Secundo et al., 2008). In this

work the lipase immobilization was carried out by adsorption at the isoelectric point of the enzyme (pH 6.0) (Anderson et al., 1998). At this pH value, the enzyme activity remains high while no net negative charge is created on protein molecule, and therefore the enzyme adsorption is not hampered by electrostatic interaction forces on the negatively charged surface of the clay nanoplatelets. Table 1 shows the effectiveness of the lipase immobilization on the different aluminosilicates and their organo-modified derivatives. In all cases, the immobilization yields from the adsorption of enzymes onto different supports were checked through the concentration of protein solutions before and after immobilization. As it can be seen, the protein content of the lipase-clay conjugates depends on the initial protein amount. The quantity of protein which could be retained by the support materials ranged from 65% to 96% of the initial load. As the enzyme to clay ratio increased, higher amounts of lipase remained attached to the phyllosilicates, which indicated that under the specific conditions applied in the present study, the saturation level of clays was not reached. The retention capacity of clays, which was observed, was higher than those reported when other support matrices or techniques were applied for lipase immobilization (Won et al., 2005). Comparison of the immobilization yields achieved for the same initial loads of CaLB showed that montmorillonites KUN and SWy-2 could adsorb equivalent amounts of enzyme, while hectorite (LAP), smectite clay with smaller particle size and lower charge density, exhibited the highest retention capacity among the clays examined. Interestingly, the organo-modified clays derivatives (ORKUN, ORSWy-2 and ORLAP), in which the intercalation of organic surfactants between layers imparts a more hydrophobic character to their surface, retained approximately 10% higher amounts of lipase than their unmodified precursors.

3.2. Characterization of nanoclays-lipase conjugates

The resulting nanoclays-lipase conjugates were characterized by a combination of techniques such as powder X-ray diffraction (XRD), thermogravimetric analysis (TGA), differential thermal analysis (DTA) and X-ray photoelectron spectroscopy (XPS).

X-ray diffraction (XRD) measurements provide a powerful tool to understand the changes in the interior of the clay microenvironment and thus to evaluate the different types of nanocomposites formed. Fig. 1 shows the XRD patterns of the (a) pristine sodium-laponite (LAP) and (b) hybrid biocatalysts with various enzyme loadings. The pristine Na-laponite shows a d_{001} -spacing of 12.2 Å which corresponds to an inter-sheet separation $\Delta = 12.2 - 9.6 = 2.6$ Å, where 9.6 Å is the thickness of the clay layer (Theng, 1974). On the contrary, the absence of the d_{001} diffraction peak, characteristic of the sodium-clay, in the patterns of the final hybrid biocatalysts (LAP-(20) and LAP-(50)) indicates that the ordered structure of the layered mineral is effectively destroyed after enzyme immobilization (Fig. 1a). This means that the immobilization of the enzyme lead to total exfoliation of the clay even at low enzyme loadings. Similar results were observed in the case of organo-modified laponite (Fig. 1b). The interaction between the clay and the surfactant led to a shift of the d_{001} diffraction peak of the organo-modified laponite toward lower 2θ values, implying the expansion of the interlayer space due to the alkylammonium intercalation. After the addition of the alkylammonium the d_{001} -spacing increases to 14.6 Å ($\Delta = 5$ Å), indicating that the alkylammonium molecules are intercalated in the interlayer galleries of the clay mineral. In the case of enzyme immobilization onto organo-modified laponite, the addition of 20 mg enzyme per g of the clay the d_{001} -spacing increases to 21.4 Å ($\Delta = 12.8$ Å), indicating that a portion of the enzyme is intercalated in the lamellar space of the clay mineral, while the rest is immobilized either on the external surfaces or the edges of the clay sheets which is in accordance to what

Table 1

Immobilization yields and transesterification activity observed for biocatalysts prepared on natural clays and their organo-modified derivatives with various loads of CaLB.

Clay	Initial protein load (mg g ⁻¹ support)	Immobilized protein (mg g ⁻¹ support)	Immobilization yield (%)	Specific activity (mM h ⁻¹ mg ⁻¹ protein)
LAP	20	17.0	85	0.85
	50	39.5	79	0.84
	100	76.0	76	0.84
	200	146.0	73	0.83
KUN	20	16.6	83	0.97
	50	37.5	75	0.95
	100	70.0	70	0.94
	200	132.0	66	0.94
SWy-2	20	16.6	83	0.85
	50	38.5	77	0.84
	100	69.0	69	0.82
	200	130.0	65	0.82
ORLAP	20	19.2	96	2.17
	50	47.5	95	2.24
	100	80.0	80	2.39
	200	183.8	92	2.40
ORKUN	20	18.7	94	2.22
	50	46.0	92	2.22
	100	81.0	81	2.22
	200	176.0	88	2.28
ORSWy-2	20	19.0	95	2.59
	50	45.5	91	2.68
	100	80.0	80	2.75
	200	180.0	90	2.78

was recently observed for β -glucosidase (Serefoglou et al., 2008). As it can be seen in Fig. 1b, further increase of enzyme loading lead to total exfoliation of the clay. Taking into consideration the dimensions of the enzyme molecule (30 Å × 40 Å × 50 Å) (Uppenberg et al., 1995), the molecule is not expected to intercalate between the clay nanosheets. At the same time, formation of a new d_{001} peak shifted to lower values is a conclusive evidence for intercalation. Probably, the side chains of different amino acid residues take part in intercalation while the polypeptide backbone is situated outside the pores. This is in accordance with previous works, where the adsorption of other enzymes as α -amylase, glucoamylase and invertase onto synthetic montmorillonite leads to intercalation of the side chains into the clay layers while the polypeptide backbone does not enter the interlayer space (Gopinath and Sugunan, 2007).

The DTA-TGA curves, obtained in air, for all composites revealed two exothermic peaks at 300 and 390 °C, which are related to the oxidation of the enzyme molecules (data not shown). The exothermic peak at 390 °C corresponds to molecules anchored on the surfaces or the edges of the clay nanosheets, and thus more protected, while the first peak (at 300 °C) is due to “loose” enzyme molecules not direct bound on the clay as discussed before. These molecules are attached to the previously adsorbed ones, rather than bound to the support surface.

XPS is a direct method to investigate how the enzyme binds to the siloxane interface in the hybrid systems. The presence of the amide groups of the immobilized enzyme was established by analysis of the binding energies of the N 1s core level spectra. In Fig. 2i–vi we present the N 1s core level photoemission spectra of the parent clays (SWy-2, KUN, LAP) loaded with 100 mg of CaLB (SWy-2-(100), KUN-(100) and LAP-(100)) and the same spectral region of the organoclays loaded with the same amount of enzyme (ORSWy-2-(100), ORKUN-(100) and ORLAP-(100)). The spectra for the

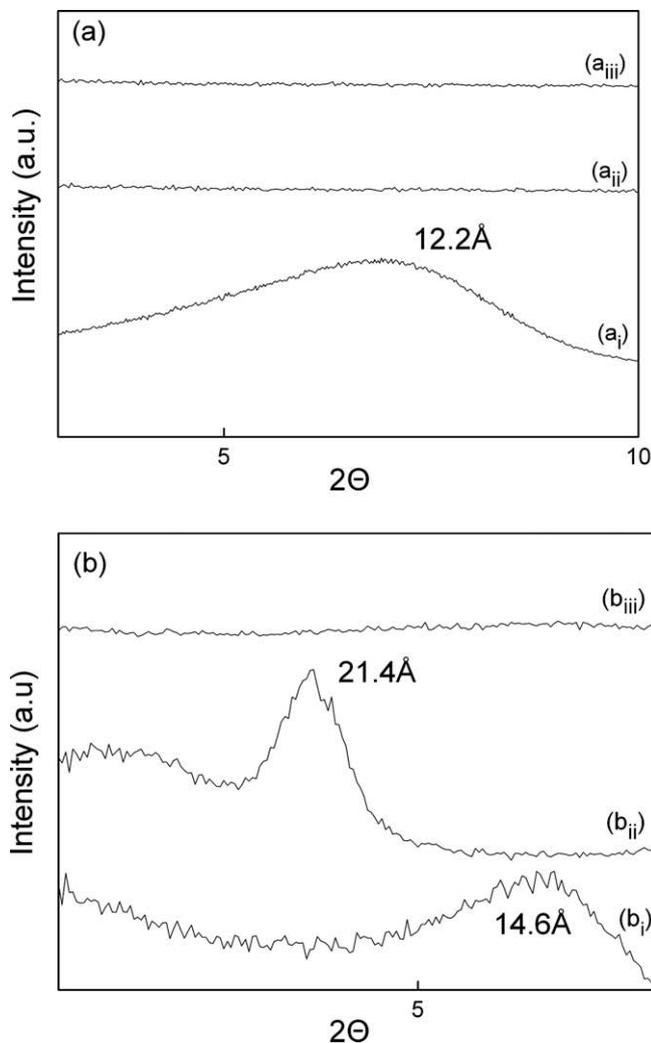


Fig. 1. XRD patterns of: (a) (a_i) LAP, (a_{ii}) LAP-(20), (a_{iii}) LAP-(50) and (b) (b_i) ORLAP, (b_{ii}) ORLAP-(20), (b_{iii}) ORLAP-(50).

clay-lipase conjugates (Fig 2i–iii) show only one component at 399.4 eV due to the amide group of the enzyme. In the case of organo-modified clays loaded with the enzyme (Fig 2iv–vi) this peak is shifted towards higher binding energy (400.0 eV) probably due to interaction of the amide groups of the enzyme with the surfactant of the organoclay (the peak at 403.0 eV binding energy is due to the quaternary ammonium group of the surfactant). In the case of ORKUN-(100) the intensity of the peak is higher compared to the other organoclay hybrids loaded with the same amount of enzyme indicating that larger amounts of enzyme might be immobilized on the organoclay surface. This seems reasonable if we consider that the amount of the organic surfactant in the case of Kunipia montmorillonite is higher due to its higher CEC (1.2 meq/g) compared with the other two clays.

3.3. Structural studies of immobilized lipase

The conformational changes of the enzyme upon immobilization onto parent or organo-modified clays compared to the structure in aqueous medium (pH 6.0, acetate buffer 0.1 M) were investigated by FT-IR spectroscopy. The analysis of the Amide I band at approximately 1600–1700 cm⁻¹ (mainly due to the C=O stretching vibration) makes it possible to obtain information on the effect of the immobilization on the secondary structure of

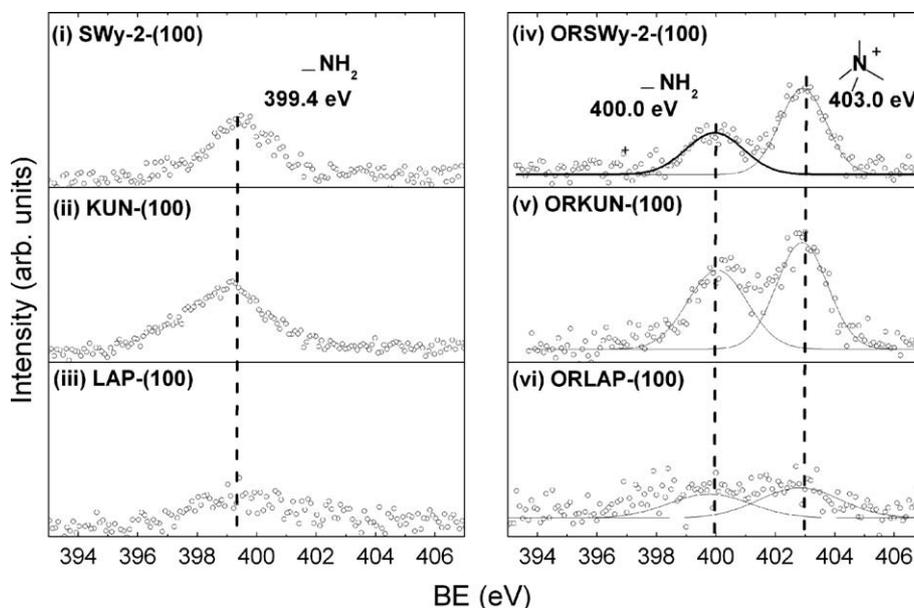


Fig. 2. X-ray photoemission spectra of the N 1s core level region for (i) SWy-2-(100), (ii) KUN-(100), (iii) LAP-(100), (iv) ORSWy-2-(100), (v) ORKUN-(100) and (vi) ORLAP-(100) nanocomposites.

the protein (Prestrelski et al., 1993; Secundo and Carrea, 2005; Secundo et al., 2007). The Amide I band consists of several overlapping components that are assigned to different secondary structure elements. These individual components were identified from the second-derivative spectra. The bands at $1610\text{--}1640\text{ cm}^{-1}$ and $1685\text{--}1695\text{ cm}^{-1}$ were assigned to β -sheet, at $1640\text{--}1650\text{ cm}^{-1}$ to random coil, at $1650\text{--}1660\text{ cm}^{-1}$ to α -helix and at $1660\text{--}1685\text{ cm}^{-1}$ to β -turns (Natalello et al., 2005).

In order to evaluate the differences among the spectra of the various lipase samples, we compared the correlation coefficients (r), according to previous works (Prestrelski et al., 1993; Secundo and Carrea, 2005) as indicated in Table 2. As it can be seen, there were significant differences between the Amide I spectra of free lipase in aqueous solution and immobilized onto parent non-modified clays ($r < 0.8$). When organo-modified clays were used as immobilization matrices, the correlation coefficient was higher than 0.9, which indicates that the structure of the immobilized enzyme is not significantly altered.

The component due to α -helix structures is a good indicator of the correct folding of the enzyme, (while an intact α -helix structure does not guarantee the presence of an active enzyme, the loss

of this secondary structure element usually leads to enzyme inactivation). As it can be seen in Table 2, a slight increase of α -helix content was observed in the case of immobilization into ORLAP and ORSWy-2 while a decrease was observed in ORKUN. On the contrary, a significant decrease on the α -helix content of the enzyme was observed after immobilization in non-modified clays (especially in LAP and SWy-2), indicating large changes on the secondary structure of the enzyme. The difference in secondary structure changes observed is probably related to transesterification activity expressed by the enzyme after its immobilization onto various clays (see Section 3.1 Table 1) and could be attributed to specific interaction of the enzyme with clay mineral and/or surfactant molecules (as indicated by XPS and XRD results, Section 3.2). The increased α -helix content of the enzyme in ORLAP and ORSWy-2 could be attributed to the increased hydrophobicity of these materials compared to the parent ones. Very recently, it was reported that an increase of hydrophobicity of the immobilization matrix results to an increase in the α -helix content and thus the fraction of properly-folded functional enzyme enhancing the biocatalytic activity (Mena et al., 2008). However, the mechanism by which matrix's hydrophobicity enhances the helical content of immobilized protein remains unsolved.

3.4. Transesterification activity of immobilized lipase in organic solvents

The ability of immobilized enzyme in various nanoclays to preserve its catalytic activity in low water-content organic media was confirmed using the synthesis of esters through transesterification of vinyl butyrate with 1-butanol in *n*-hexane as model reaction. As it can be seen in Table 1, the enzyme immobilization on different clays led to the preparation of active biocatalysts with all aluminosilicate materials used, for a broad range of initial protein loading (20–200 mg protein g^{-1} support). The initial amount of enzyme load influences the catalytic activity. In LAP, SWy-2 and KUN, as the initial enzyme load increases, specific enzyme activity slightly decrease, which is in accordance to that observed in other supports (de Fuentes et al., 2001; Secundo et al., 2008). This behaviour could be attributed to a possible adsorption of the new enzymes molecules onto the previously adsorbed ones, rather than onto the sup-

Table 2

Correlation coefficient (r) between the FT-IR spectra of CaLB immobilized onto clays and organo-modified clays and the spectra of the enzyme in water solution. Quantitative estimation (%) of the α -helix content of CaLB calculated by FT-IR analysis in Amide I region.

Enzyme preparation	r	α -Helix (%)
Aqueous solution	–	30.4
ORKUN	0.703	26.2
ORLAP	0.510	32.7
ORSWy-2	0.785	32.7
KUN	0.964	29.1
LAP	0.918	21.5
SWy-2	0.915	20.2

The correlation coefficient was calculated using the formula $r = \frac{\sum x_i y_i}{\sqrt{\sum x_i^2 \sum y_i^2}}$ (Prestrelski et al., 1993), x and y are the absorbance values of the lipase spectrum dissolved in water and immobilized, respectively, at the i th frequency position for the range $1600\text{--}1700\text{ cm}^{-1}$ (Amide I). For identical spectra, a value of 1.0 will be returned.

port surface, in a similar manner as discussed before (Section 3.2). In this case, the lower enzymatic specific activity observed with an increase of the enzyme loading is probably due to diffusional limitations of substrates to the active site of the enzyme. On the other hand, when organo-modified clays are used as immobilization supports, the transesterification activity of the immobilized enzyme was 2- to 3-fold higher for all clays used in almost all organic solvents tested. It is expected that the hydrophilic/hydrophobic character of the support matrix plays an important role in determining the high substrate accessibility of reactants and water in the enzyme microenvironment, affecting therefore the partition of substrates and products between the medium and the enzyme (Petkar et al., 2006). The hydrophobic microenvironment created in organically modified clays could affect the diffusion and distribution of substrates and products in the microenvironment of the immobilized enzyme increasing the accessibility to hydrophobic substrates and enhancing the transesterification activity as indicated in Table 1. Nevertheless, the increased catalytic activity of the immobilized enzyme in organically modified clays, could be also related to the relative stabilization of the enzyme conformation in these modified clays compared to extended structural changes observed in non-modified clays (Section 3.3, Table 2).

To investigate the effect of organic solvents on the synthetic activity of immobilized enzymes, various organic solvents with log *P* (the logarithm of the partition coefficient of a given compound in the standard octanol–water two-phase system) ranging from –0.33 to 4.8 were selected as reaction media. As it can be seen in Fig. 3, higher reaction rates were obtained when solvents with high log *P* value (log *P* > 2.5), such as toluene, *n*-hexane and isooctane were employed. These results are in accordance with those reported for the catalytic behaviour of Novozym 435® (Nordblad and Adlercreutz, 2008). For all organic solvents tested, the synthetic activity of the enzyme was significantly reduced when parent (non-organomodified) clays were used as immobilization matrices in a similar manner as described in Table 1.

It is interesting to note that the thermal stability of the immobilized CaLB in parent and organically modified clays is comparable and up to 3-fold higher to that observed for the non-immobilized enzyme. The higher residual activity (70% after incubation for 72 h in *n*-hexane at 60 °C) was observed for lipase immobilized in ORLAP and ORSWy-2. The high thermal stability of the immobilized lipase may arise from the conformational integrity of the en-

zyme structure after immobilization in these organically modified clays as indicated before (see Section 3.3).

3.5. Application of the immobilized lipase to the chemo-enzymatic epoxidation of α -pinene

In the present work, the ability of the immobilized lipase to catalyze a synthetic reaction that is far from its natural role, such as the indirect epoxidation of alkenes, via the formation of peroxy-carboxylic acid using H₂O₂ as substrate, was also investigated. The main drawback of the use of lipases to catalyze indirect epoxidation of alkenes using H₂O₂ as substrate, is that enzymes are not resistant to the drastic reaction conditions originating from the use of the oxidant (Svedendahl et al., 2008). In the present work, the capability of CaLB immobilized on various organically modified smectite clays to catalyze the indirect epoxidation of α -pinene, was investigated. In this biocatalytic process two common oxidants were used: 30% w/w aqueous solution of H₂O₂, as well as an anhydrous complex of H₂O₂ with urea (UHP) which releases the oxidant in a controlled manner (Ankudey et al., 2006). For all clays tested, the use of UHP as oxidant lead to higher conversion of α -pinene (up to 80% after 24 h of incubation), compared to H₂O₂, which is in accordance with the results recently reported for the chemo-enzymatic perhydrolysis of carboxylic acids in organic media using Novozym 435® (Ankudey et al., 2006). It must be noted that low amounts of verbenol and verbenone were also identified in the reaction mixture after 24 h of incubation, probably as products of the autooxidation of α -pinene.

Table 3 shows the reaction rate and the concentration of α -pinene oxide obtained after 24 h of the chemo-enzymic epoxidation of α -pinene catalyzed by the three biocatalyst immobilized onto (ORLAP, ORKUN and ORSWy-2) as well as by the non-immobilized CaLB and commercially available Novozym 435®, using UHP as oxidant. As shown in Table 3, the initial reaction rates for immobilized lipase in various organo-modified clays were comparable to that obtained for Novozym 435®. However, the use of organo-modified clays (especially ORLAP) as enzyme carriers, led to higher α -pinene conversion than that observed by Novozym 435® or non-immobilized lipase and up to three times higher concentration of α -pinene epoxide, after 24 h of incubation, than that observed with free enzyme or when parent (non-organomodified) clays were used as immobilization supports. This is probably due to the higher stability of the lipase immobilized on organo-modified clays, indicating that the nature of the carrier affects not only the enzyme activity as indicated in Section 3.4, but also the operation stability of the biocatalyst in the biocatalytic process. It must be noted that the reduced Novozym 435® stability observed in a similar process was attributed to a number of factors including oxi-

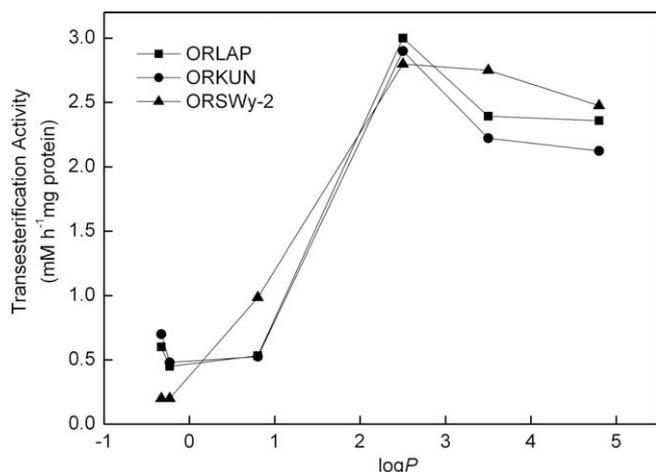


Fig. 3. Effect of solvent on transesterification activity of immobilized CaLB onto organo-modified clays. Reaction conditions as described in Section 2. Solvents (log *P*): acetonitrile (–0.33), acetone (–0.23), *tert*-butanol (0.80), toluene (2.5), hexane (3.5), isooctane (4.8).

Table 3

Initial reaction rates, conversion of α -pinene and α -pinene oxide concentration achieved after 24 h incubation at 25 °C during chemo-enzymatic epoxidation of α -pinene catalyzed by various immobilized forms of CaLB. Reaction conditions as described in Section 2.

Biocatalyst preparation	Initial reaction rate (mM h ⁻¹ mg protein ⁻¹)	α -Pinene oxide (mM)	Conversion of α -pinene (%)
ORLAP	2.7	453	80.3
ORKUN	3.6	360	65.5
ORSWy-2	4.5	359	65.0
LAP	0.9	125	29.0
KUN	1.3	198	40.0
SWy-2	1.5	135	30.3
Novozym 435®	4.1	211	41.7
Lyophilized CaLB	10.0	190	38.5

dativity damage by H₂O₂ and desorption of the active enzyme into the aqueous phase formed during the biocatalytic process (Orellana-Coca et al., 2005; Tornvall et al., 2007).

To further examine the effect of oxidants on the stability of the lipase, both immobilized and non-immobilized CaLB was exposed to either H₂O₂ or UHP in *n*-hexane. Fig. 4a shows the effect of UHP on the stability of various lipase preparations after 24 h and 48 h of incubation at 25 °C. As it can be seen, the residual activity of the immobilized lipase was significant higher than that measured for non-immobilized (lyophilized) lipase and comparable to that observed for Novozym 435®. The higher residual activity (90% after 48 h of incubation) was observed when ORLAP was used as support which can explain the higher conversion yield observed in Table 3. A similar protective effect of organo-modified supports on the enzyme stability was observed in the case of the enzyme incubation with H₂O₂ (data not shown). The hydrophobic organo-modified clays (ORLAP, ORKUN and ORSWy-2) used in the present work, are expected to adsorb a smaller amount of oxidant and water formed during the process, thus reducing their destabilization effect on the immobilized lipase. It is interesting to note that when more hydrophilic clays (LAP, KUN and SWy-2) used as lipase immobilization supports, the enzymic stability was dramatically reduced (less than 40% after 48 h of incubation).

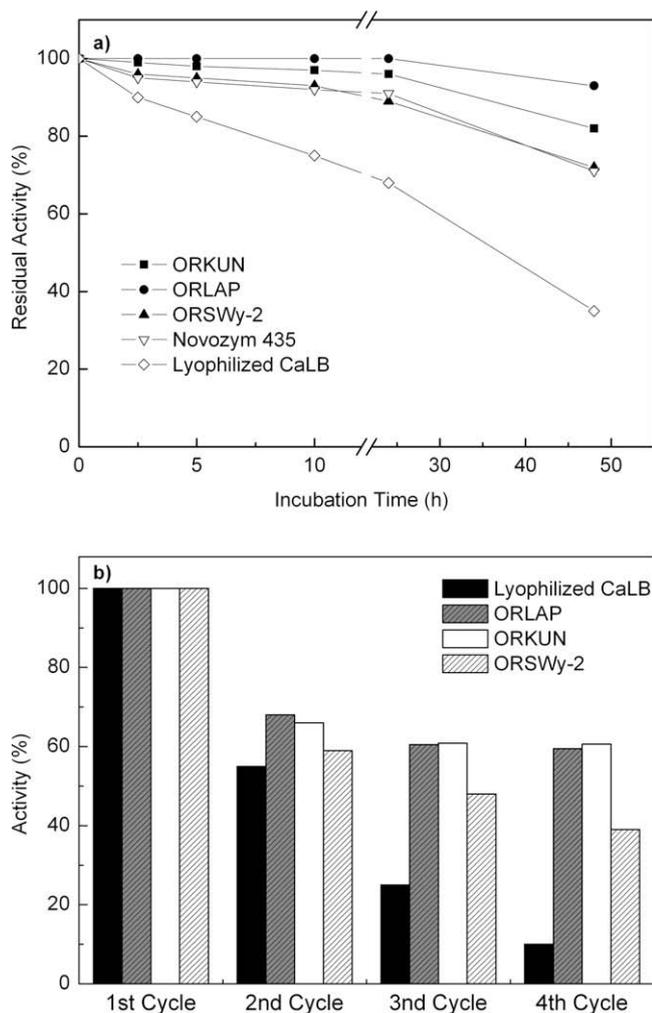


Fig. 4. (a) Effect of UHP on the stability of various lipase preparations as a function of incubation time at 25 °C. The reaction condition is described in Section 2. (b) Reusability of free or immobilized lipase in various organically modified clays. Each reaction cycle was performed for 24 h at 25 °C. Reaction conditions as described in Section 2.

3.6. Reusability of the immobilized lipase

Lipase, immobilized in various organo-modified clays (ORLAP, ORKUN and ORSWy-2) or lyophilized was recycled for the chemo-enzymatic epoxidation of α -pinene using UHP as oxidant. The reaction was performed for 24 h at 25 °C, after which the enzyme was separated, washed and added to the fresh substrate. As it can be seen in Fig. 4b, when four reaction cycles (96 h of total operation) were completed, the residual activity of the immobilized lipase is 60% for ORLAP and ORKUN and 40% for ORSWy-2. These results are probably due to the inactivation or of the enzyme caused by the oxidant and/or oxidizing products formed during the reaction. Moreover, when the non-immobilized enzyme was used, the residual enzyme activity was less than 10% at the end of the recycling process, indicating that the enzyme immobilization onto various organoclays significantly increases its operational stability. The operational stability observed here was significantly higher compared to that reported for Novozym 435® in chemo-enzymatic epoxidation of α -pinene using H₂O₂ as oxidant (Skouridou et al., 2003).

4. Conclusion

Organically modified smectite nanoclays (ORKUN, ORSWy-2 and ORLAP) can act as efficient supports for the immobilization of a lipase with numerous biocatalytic applications such the lipase B from *C. antarctica*. Structural and biochemical characterization of organo-modified clay-enzyme conjugates revealed that the hydrophobic microenvironment created by these modified clays stabilizes the enzyme structure, resulting in enhanced activity and stability in low-water media. The immobilized lipase effectively catalyzes a synthetic reaction that is far from its natural role, such as the epoxidation of α -pinene using hydrogen peroxide and a fatty acid as substrates. The activity and the operational stability of the enzyme in the presence of oxidant were of the highest reported in the literature, indicating the favourable effect of organo-modified clays on lipase catalytic behaviour.

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