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Analysis of the interaction of lipases with polypropylene of different structure and polypropylene-modified glass surface

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Abstract

The hydrophobic/hydrophilic characteristics of the surface exposed by a lipase support conditions the amount of adsorbed protein, and probably also the conformation of the immobilized lipase. In reference to polypropylene (PP) – hydrophobic – in this study the polymer obtained with metallocene catalysts (PP_{met}) showed the best characteristics for the immobilization of lipase from *Candida antarctica B* in terms of surface structure and particle size. On the other hand, commercial pellets of polypropylene obtained with Ziegler-Natta catalysts (PP_{ZN}) showed to have lower affinity for proteins, which we attribute to a combination of higher particle size and different exposed surface.

Despite its high affinity for proteins, low mechanical resistance of PP_{met} prohibited its use as lipase support in reactive systems with high mechanical efforts, such as strongly magnetically stirred batch laboratory reactors. Coating of glass balls with the polymer was attempted in order to confer better mechanical properties to PP_{met}. Mixed surfaces of PP_{met}/glass balls pre-treated with an acid/base protocol to generate surface OH successfully allowed biocatalyst recovery and reuse. However, the hydrophobic–hydrophilic surface generated could not resemble the strong active protein bonding achieved with powdered metallocenic polypropylene. Lipase adsorption over uncovered glass regions is proposed to be the reason for the differences found.
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Keywords: Lipase support; Adsorption; Exposed surface; Mechanical resistance; Polypropylene; Glass

1. Introduction

Synthetic reactions catalyzed by lipases may be performed in aqueous media, in organic solvents, in supercritical fluids [1], in ionic liquids [2] or, alternatively, in solvent-free systems [3–5]. Solvent-free systems (SFS) are highly concentrated media, economically and operationally interesting for industrial processes. In this kind of systems not only the cost of the solvent itself is avoided, but also its separation from un-reacted substrates and products, and the cost of recycle as well.

Candida antarctica B lipase (CALB) is an interesting lipase with potential application in a number of industrial process, such as the synthesis of optically active compounds in the pharmaceutical industry [6], pitch removal and de-inking processes in the pulp and paper industry [7], or the synthesis of esters used in the flavour industry [8]. CALB has been mostly used in an immobilized form commercially available from Novo Nordisk (Novozyme 435). However, this report concerns with the solvent-free synthesis of ethyl oleate catalyzed by CALB immobilized on a cheaper support like it is polypropylene powder obtained with metallocenic catalysts (PP_{met}).

Having previously verified the attractive activity developed by the immobilized biocatalyst (CA/PP_{met}) [9], this work is mainly focussed on the feasibility of immobilized lipase reuse. For the efficient scale up of enzyme-catalyzed processes biocatalyst reuse becomes essential. Then, the assay of the activity and stability of immobilized lipases in successive batch cycles is a study usually included in literature dealing with immobilized lipases. Not only for commercial immobilized lipases like Novozyme 435 or Lipozyme, but also for new immobilized lipase derivatives, reuse experiments represent an additional characterization tool.

However, one of the main problems for the reuse of any heterogeneous catalyst is its particle size. Depending on the mechanical resistance of the material, magnetically stirred lab-

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oratory reactors (something very common in lipase-catalyzed literature) usually exhibit important reduction of catalytic particles diameter during reaction. Attrition of catalytic particles may limit biocatalyst reuse by prohibiting immobilized lipase recovery.

In addition to severe biocatalyst particle reduction in magnetically stirred systems, lipase leaching also affects immobilized biocatalysts. Especially for weak lipase support interactions like the ones involved in adsorbed lipases, desorption is a common limitation for biocatalyst reuse. Although covalently immobilized lipases rarely present leaching problems, the formation of chemical bonds is known to reduce lipase activity. Covalent bonds involving aminoacids of the catalytic triad of lipases, or aminoacids of the access channel to the active site of the enzyme may reduce the activity of the immobilized lipase. Covalent binding also limits lipase flexibility, which is far superior in adsorbed lipases for which van der walls bonds are preponderant.

Getting back to the issue of initially low or reaction-reduced diameters of catalytic particles, some authors have tried to overcome the problem of biocatalyst recovery from reaction media by using magnetic solids added to the supports [10]. In the case of attritionable materials that have proved to be adequate for lipase immobilization, a previous work of our group proposed another way to overcome the problem of reduction of particle size. By coating the surface of non-attritionable glass balls of reduced size with the chosen material both, lipase interaction with the selected support and enhanced mechanical resistance of the biocatalyst would be achieved [11].

Recently, several reports dealing with the use of silica gels and xerogels as lipase supports have been published [12]. However, contributions in which the selected support is bare silica are rather scarce, probably due to lipase deactivation upon adsorption on this support. Oxidoreductases like catalase or peroxidase showed reduced activity when immobilized onto bare silica [13,14] Anyway, the use of functionalized silica as biocatalyst support and also as highly structured solid – mainly mesoporous as in the case of MCM36 – are becoming more common [15,16].

This manuscript presents the results of the synthesis ethyl oleate performed in a solvent-free system, and catalyzed by lipases immobilized onto polypropylenes of different structures and origins. Moreover, we present the results obtained with biocatalysts resulting from the adsorption of lipase from *Candida antarctica B* onto polypropylene-coated glass balls. In this last case, CALB adsorption may be not only restricted to the PP surface, but lipase could also bind to uncoated glass regions. There are reports of stable adsorption of CALB onto glass balls without functionalization of the glass surface [17].

In the last part of this study, we include a molecular modelling section aimed to analyze the thermodynamic fesibility of lipase adsorption onto the uncovered glass surface. Molecular modelling techniques were of use in the analysis of the interaction of different lateral groups of the aminoacids of lipases with polypropylenes and bare silica surfaces.

2. Experimental

2.1. Materials

Native lipase B from *Candida antarctica B* (5000 U/ml) was kindly supplied by Novozyme. Oleic acid (99%) was purchased from J.T. Baker. Absolute ethanol (99%) and sulphuric ether (99%) were both purchased from Dorwil. Buffer solution of pH 7 (di-sodium hydrogenophosphate) and potasium hydroxide were both from Merck.

Low-molecular-weight polypropylene powder, PP_{met} (30000 g/mol, BET area: 23 m^2/g), was obtained by polymerisation using metallocenes. Commercial polypropylene pellets produced with Ziegler-Natta catalysts, PP_{ZN} (400,000 g/mol, BET area: 1–2 m^2/g), were purchased from Petroquímica Cuyo, Argentina. Glass spheres of 1 mm diameter were purchased from Científica Nacional, Argentina.

Pre-treatment of glass balls surface involved fluorhidric acid (from Analytica), KOH (from Sigma), methylaluminoxane (MAO) obtained from Witco, 9-decen1ol (from Sigma), HCl (from Analytica), and $EtInd_2ZrCl_2$ which was purchased from Sigma–Aldrich.

2.2. Supports pre-treatments

Prior to contact with the lipase solution, PP_{met} and PP_{ZN} were pre-treated with ethanol following the method previously described for the immobilization of *Candida rugosa* lipase onto metallocenic polypropylene [18]. Polypropylene particles chosen for lipase immobilization were limited to the ones in the range of 590–1180 μm.

Coating of glass balls with PP_{met} required the pre-treatment of the glass surface. Two pre-treatments were assayed. First, we performed a soft pre-treatment in which 10 g of glass balls of 1 mm of diameter were contacted with 0.4 ml of HF 40% in 100 ml of distilled water (30 min, room temperature). Then, glass balls were washed with distilled water and contacted with 10.8 g of KOH in 40 ml of distilled water for 45 min at 45 °C. In the strong pre-treatment, the contact of 10 g of glass balls with HF solution was performed during 2 h at 80 °C. Contact with KOH solution was also performed at a higher temperature and for a longer time (2 h at 80 °C).

Next, the pre-treated glass balls were contacted with 5 ml of methylaluminoxane for 30 min at 70 °C. 0.1 ml of 9-decen1ol were then introduced and reaction was allowed to proceed for 30 min. Finally, 2 mg of $EtInd_2ZrCl_2$ were added with 2 additional millilitres of MAO (30 min – soft pre-treatment – or 60 min – strong pre-treatment – at 60 °C). The balls were washed with ethanol/HCl (98/2) and finally dried. With this procedure, a chemical bonding PP_{met}/glass was obtained.

2.3. Lipase immobilization on different PP supports

2.4 ml of the enzyme commercial solution (12000 U) were diluted up to 50 ml with standard buffer of pH 7 and contacted with 1 g of ethanol pre-treated polypropylenes (PP_{met}, PP_{ZN}

and PP_{met}/glass). The immobilization of lipase B from *Candida antarctica* was performed at room temperature for 7 h with 350 rpm stirring. Phosphate buffer kept neutral buffer and ionic strength equal to 0.014 M. After the desired contact time, the insoluble material was recovered by filtration and washed with distilled water. Finally, the biocatalysts (CA/PP_{met}, CA/PP_{ZN} and CA/PP_{met}/glass PP, respectively) were dried to constant weight at 45 °C.

2.4. Support and immobilized catalysts characterization

Characterization of the supports and the immobilized biocatalysts included SEM and XRD techniques. Scanning electron microscope (SEM) images were obtained using a JEOL 35CF microscope (operated at 15 kV), equipped with a secondary electron detector and energy dispersive X-ray microanalysis (EDX). Samples were coated with gold in a vacuum chamber. X-ray diffraction (XRD) was performed with a Diffractometer Philips PW1719 with Cu anode and monochromator of graphite, operated at 45 kV and 30 mAmp.

2.5. Determination of active lipase content of immobilized catalysts

2.5.1. Triolein hydrolysis assay

The hydrolytic activity of immobilized lipases was measured following the procedure described by Peled and Kenz, consisting of the hydrolysis of triolein in pre-established conditions [19]. Comparison of the hydrolyic activity of the immobilized catalysts (CA/PP_{met} and CA/PP_{ZN}) with the one developed by crude lipase from CALB, allowed the determination of the content of active lipase of the immobilized biocatalysts. The esterification of oleic acid with ethanol in pre-established conditions (see the following section) has also shown to be a useful assay for determination of active lipase content of immobilized biocatalysts [20].

2.6. Ethyl oleate synthesis

Immobilized biocatalysts were all assayed in the solvent-free synthesis of ethyl oleate by direct esterification of oleic acid and ethanol. In all experiments performed, reaction medium consisted of the stoichiometric mixture of substrates (3 g of oleic acid and 0.5 g of ethanol − 10.6 mmols – in all cases) and 0.6 g of water. Reaction was started by the addition of the biocatalyst (CA/PP_{met}, CA/PP_{ZN} or CA/PP_{met}/glass PP) to reaction mixture, which was kept at 45 °C in 10 ml vials stirred at 350 rpm. During reaction (up to 24 h) several samples were withdrawn and analyzed by titration for the residual acid content with a basic solution of potassium hydroxide. Phenolphthalein was used as the end-point indicator. The performance of each biocatalyst is reported in terms of the percentage of conversion of fatty acid, which was determined by the relative reduction in the acidity index of the samples withdrawn. The acidity index (AI) of a sample is defined as:

$$\mathrm{AI} = \frac{\text{KOH normality} \times \text{volume of KOH} \times \text{equivalent weigth of KOH}}{\text{Mass of sample}} \quad (1)$$

After each batch, the biocatalysts were recovered by filtration for further use in identical conditions. The solid recovered from reaction media was washed with ethanol, dried and reused. Due to the attrition experienced by PP_{met} recovering was much easier in the case of CA/PP_{ZN} and CA/PP_{met}/glass.

3. Theoretical study

3.1. Methodology

The semiempirical extended Hückel (EH) method has been extensively applied to study the electronic structure of molecules [21]. In the present work we have employed a modified version of this method, ICONC, developed by Calzaferri et al. [22] which does not involve great computational effort. Although this method does not calculate representative absolute energies, it can be employed to predict qualitative trends in model systems. ICONC has been applied to study the CH_3OH adsorption–oxidation process on V_2O_5 [23], to the analysis of Ziegler-Natta and metallocene catalysts [24], and also VO_x/Al_2O_3 catalyst [25]. Other details about extended Hückel molecular orbital (EHMO) calculation like parameters and other considerations can be found in the literature [25–27].

In reference to the study of the interactions of the aminoacids of lipase with polypropylene, considering the structure of PP the best method is not a semiempirical/ab initio, such as EHMO, but a molecular mechanics method, focused in the van der waals interactions more than in the consideration of electronic interactions at a semiempirical level, such as in the EHMO or PM3. In the last years, there has been an increasing demand for the large scale simulation of molecules that constitute hydrocarbon or carbon-based materials, such as fullerenes and nanotubes. However, most of the available parameterized tight binding total energy (TBTE) models are still limited for hydrocarbon systems, especially to model full structures based on van der waals interactions, instead of being predominant the covalent, ionic or metallic interactions [28]. On the other hand, methods based on molecular mechanics are getting more used for carbon-based materials, being their results important in terms of how these methods can represent actual structures, and also predict certain properties (under adequate constraints and considering carefully the inherent limitations) [29–31]. A previous work of our group thoroughly studied the interaction of the main aminoacids of lipases with polypropylene using MM2, a molecular mechanics method [32].

For both methods (EHMO and MM2) changes in steric energies (ΔEs) are calculated as the summatory of the energies of the products minus the summatory of the energies of the reactants for each adsorption reaction. In the case of the interactions between OH groups of the silica support and the lateral chains of lipase aminoacids, the energies are calculated as the energy of the adsorbed molecule on the surface minus the summatory

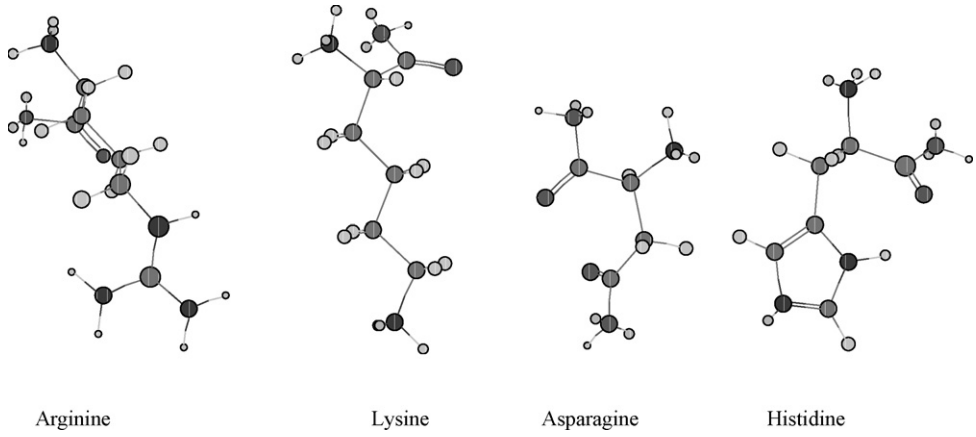

Fig. 1. Modelled aminoacids of lipase.

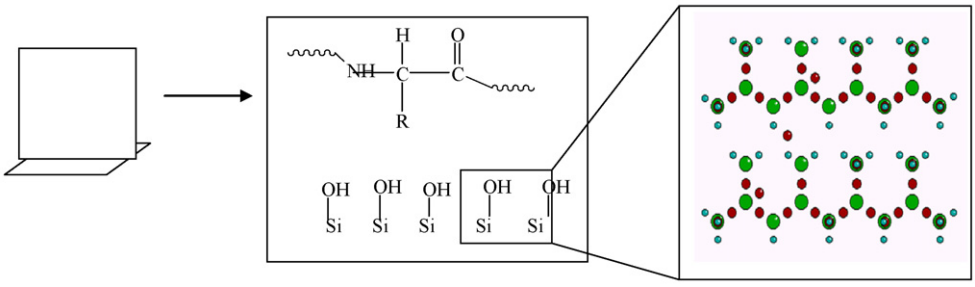

Fig. 2. Interaction of aminoacids with SiO_2. In the zoom square, the crystobalite (1 1 1) plane.

of the support and the aminoacid model energies (at infinite distance each other).

3.2. Structures used as support and lipase aminoacids models

3.2.1. SiO_2

The structure of amorphous silica is claimed to be similar to that of β-crystobalite. In the modelling, the (1 0 0) and the (1 1 1) faces were considered as the most abundant exposed planes, with 85% of the (1 1 1) plane. The (1 0 0) face presents geminal OH groups. Isolated silanol groups are present on the (1 1 1) face. SiO_2 (1 0 0) plane has 61 H, 30 O and 16 Si atoms. SiO_2 (1 1 1) plane includes 50 H, 39 O and 26 Si atoms.

3.2.2. Aminoacids

The aminoacids of lipase were modelled including the complete lateral chains, considering their interaction with the supports surface. Not all of them but some of the characteristic aminoacids of lipase were modelled (Fig. 1). The location of the amino and acid groups was considered far away from surface, being the lateral *R* group of the aminoacid located perpendicular to the support surface (Fig. 2).

4. Results and discussion

Free and immobilized lipase from *Candida antarctica B* showed high activities when used in adequate reaction media. Table 1 illustrates the performance of the different biocatalysts prepared in the synthesis of ethyl oleate performed at 45 °C, and in a system with initially 0.6 g of water. Due to the high substrate concentration of the solvent-free system even low/moderate fatty acid conversions lead to attractive catalytic activities and productivity. The reaction conditions chosen for biocatalyst assay were previously determined as the best for maximum ester production [9,33].

4.1. Activity and reusability of CA/PP_{met}

Due to the high activity exhibited by CA/PP_{met} (second data row of Table 1) reuse of this immobilized lipase was attempted in order to evaluate the potential of the biocatalyst in practical terms. Moreover, reuse experiments would give some insight on other chemical/physical problems of interest like lipase deactivation and protein leaching to reaction media. If immobilized CA/PP_{met} was nor desorbed neither deactivated, reusing of the biocatalyst in identical reaction conditions should produce the same conversion than the initial batch did.

Table 1
Performance of CALB and immobilized derivatives

Biocatalyst	Conversion – 7 h (%)	Yield (mmol of ester produced)
Native CALB	78	8.3
CA/PP_{met}	70	7.4
CA/PP_{ZN}	15	1.6
CA/PP_{met}/glass	17	1.8

Reaction conditions: $W = 20\%$, 45C, 300 U of CALB, 50 mg of immobilized catalysts. Measurements performed at 7 h of reaction.

Table 2
Effect of catalyst mass of CA/PP_{met} on the conversion of oleic acid measured at 6 h of reaction

Mass of CA/PP_{met} (mg)	Conversion – 6 h (%)
50	67
150	75
300	79

Reaction conditions: $W = 20\%$, 45C. Measurements performed at 6 h of reaction.

The reuse assay was performed as follows: 120 mg of CA/PP_{met} were used in the specified reaction conditions (Section 2.6). After 6 h of reaction, the solid was separated from the reaction mixture by filtration using special filters for powders with low size particle. Measured conversion resulted in 75% (data included in Table 1, was achieved with only 50 mg of CA/PP_{met}), which represents 7.95 mmols of ester produced in only 6 h of reaction. However, in spite of the attractive activity of the immobilized lipase, the nature of PP_{met} and its low mechanical resistance made difficult the total recovery of the solid, which showed a strongly reduced particle diameter caused by high magnetic stirring. In a second use of the recovered solid (just a fraction of the mass of catalyst initially added), the conversion measured after 6 h of reaction dropped to 35%.

The reuse experiment performed led to a first conclusion: if lipase deactivation/desorption from CA/PP_{met} actually occurs, the phenomenon does not involve all lipase. Otherwise, no catalytic activity would have been observed in the second use of CA/PP_{met}. However, the experiment performed did not give insight on the amount of desorbed/deactivated lipase in the first use. Because of recovering problems of low diameter particles the decrease of the activity could have been not only due lipase desorption/deactivation (reduction of the active lipase content of the immobilized catalyst); but also due to the use of a lower amount of total biocatalyst, although its lipase content might have remained constant. Finally, a combination of both effects could have taken place.

Data included in Table 2 depicts the influence of the mass of CA/PP_{met} on the conversion of oleic acid measured at 6 h of reaction in definite conditions. Considering that the mass of CA/PP_{met} recovered from the first batch was approximately 50–70 mg (presence of oily reactants in the biocatalyst matrix prohibited precise weighing of the catalyst recovered from the first reaction batch), Table 2 indicates that the decrease in the conversion measured in the first reuse of CA/PP_{met} cannot be purely assigned to the use of a lower mass of catalyst with constant lipase content (in the reuse experiment the conversion measured was only 35%). Then, together with the strong particle diameter reduction exhibited by the support, we verified a partial reduction of the active lipase content of CA/PP_{met}. Moreover, we may hyphothesize that a negative sinergic effect takes place: surfacial changes related to the change in particle size in the first use may have favored lipase desorption. Changes of the proportion of prefered exposed planes and surfaces is a known effect of stirring in solids prone to attrition.

4.2. Activity and reusability of CA/PP_{ZN}

The biocatalyst CA/PP_{ZN} did not show recovering problems from the reaction media. Commercial pellets of Ziegler-Natta polypropylene showed high mechanical resistance with no detectable particle diameter reduction during the reaction period. Consequently, after each batch the whole mass of catalyst added to reaction mixture could be separated by filtration allowing later reuse. However, as it is shown in the third data row of Table 1, the activity of CA/PP_{ZN} was much lower than the one exhibited by CA/PP_{met} in identical conditions. Using even 200 mg of CA/PP_{ZN}, measured conversion after 7 h was only 22%; a value far lower than that obtained with 50 mg de CA/PP ($X = 70\%$ in 7 h). From the total amount of lipase contacted with the support, which was the same in the case of PP_{met} and PP_{ZN}, it is evident from reaction results that the fraction of active lipase present in CA/PP_{ZN} is far lower than the one immobilized onto PP_{met}.

Triolein hydrolysis assay confirmed the hypothesis. The hydrolysis reaction revealed that whereas near 3500 U of CALB per gram of total biocatalyst were immobilized onto metallocenic PP, in one gram of CA/PP_{ZN} only 1950 U of *Candida antarctica B* lipase were found. The units reported here are the ones proposed by the provider. Comparison of the hydrolytic units found in the immobilized biocatalysts and those measured for free lipase, revealed that the amount of active lipase present in 1 g CA/PP_{met} is equivalent to 0.7 ml of CALB solution. On the other hand, 1 g CA/PP_{ZN} has a content of active lipase equivalent to just 0.39 ml of CALB solution. Lower amount of active lipase immobilized onto the PP obtained with Ziegler-Natta catalysts explains the lower activity exhibited by CA/PP_{ZN}.

Ethyl oleate esterification and triolein hydrolysis assays results suggest that the interaction of CALB with metallocenic PP or with Ziegler-Natta PP is different, favoring the generation of stronger or more lipase support bonds in the case of metallocenic PP. We believe that the kind of surface exposed by the polypropylenes conditioned the amount of lipase in active conformations effectively bonded to the support. XRD of both polymers reveal different structures of the polypropylenes. Metallocenic PP crystalizes in γ-structure, whereas Ziegler-Natta PP crystalizes in α-structure (Fig. 3, see the circles pointing

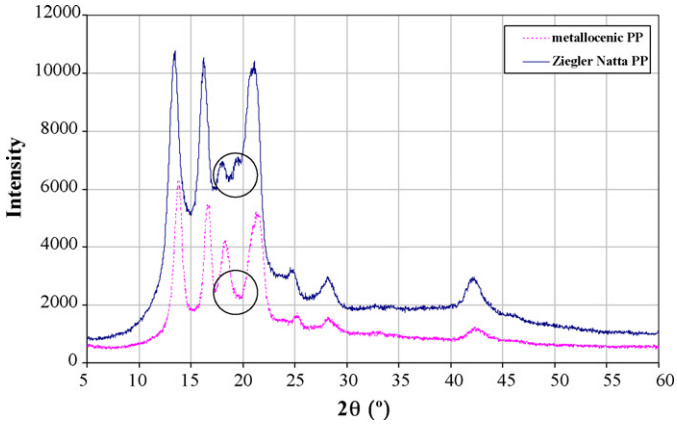

Fig. 3. X-ray diffractrometry of PP_{met} and PP_{ZN}.

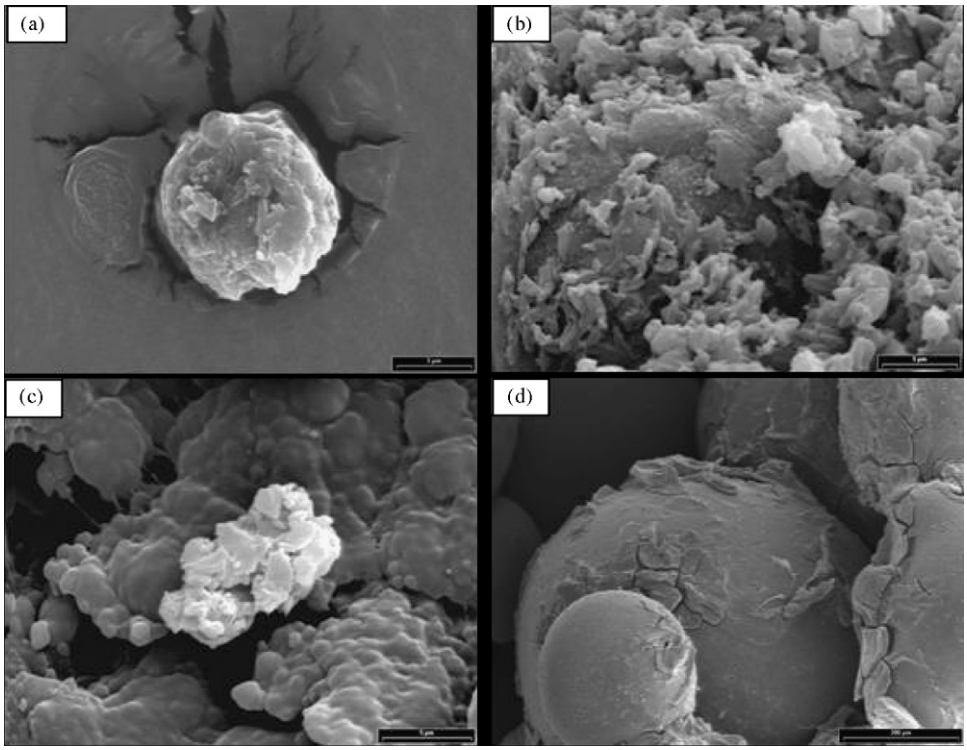

Fig. 4. SEM microphotographs of free and immobilized CA. (a) Free CALB (4000X), (b) CA/PP_{met} (4000X), (c) CA/PP_{ZN} (4800X), (d) PP_{met}/glass (150X).

out the differences in XRD spectra). Also, at the surface level, exposure of methyl groups might be different in space for both structures (methyl–methyl distances, surface concentration of methyl groups), justifying the different interaction with lipase molecules.

Fig. 4a–c show electronic scanning micrographies of CALB, CA/PP_{met} and CA/PP_{ZN}. Fig. 4b and c show the different topography of the polypropylenes used as lipase supports. While PP_{ZN} particles are round-shaped pellets with a smooth surface, PP_{met} particles look like flakes agglomerates. Although particles of similar diameter were considered, the rougher surface of the metallocenic PP leads to a higher interfacial area (BET area of 23 m^2/g versus 1–2 m^2/g for PP_{ZN}) favoring both, lipase-PP contact and also interfacial activation of the immobilized lipase upon adsorption onto a hydrophobic support. Then, the different topography of the studied polypropylenes may explain the differences found in terms of biocatalyst activity: higher interfacial area allows PP_{met} to contact more lipase, and also promotes its immobilization in active conformations. We have previously determined that PP_{met} favors the adsorption of lipases in very active conformations, especially for esterification reactions [32,34].

4.3. Activity and reusability of CA/PP_{met}/glass

In Section 4.1 the high activity of the biocatalyst obtained upon adsorption of CALB onto PP_{met}, was demonstrated. The structure of the polymer favored lipase immobilization in very active conformations. However, the strong attrition experienced by PP_{met} limited biocatalyst reuse, a major requisite for immobilized catalysts. Then, commercial pellets of Ziegler-Natta PP were assayed as lipase support. Increased mechanical resistance of PP_{ZN}, allowed biocatalyst recovery and reuse. However, the interaction established with lipase molecules showed to be different from the one achieved in CA/PP_{met}; with much lower catalytic activities in the case of CA/PP_{ZN}.

In an attempt to obtain a highly active and at the same time reusable PP-immobilized catalyst, we tried to confer better mechanical properties to PP_{met}. Coating of glass balls of low diameter with the polymer was attempted. The procedure followed was described in Section 2.2. If PP_{met}/glass surface mimicked PP_{met} surface, we would be able to obtain a biocatalyst with the desirable properties of activity and operational stability. Fig. 4d shows a SEM photograph of PP_{met}/glass balls.

The assay of CA/PP_{met}/glass in the synthesis of ethyl oleate revealed: (1) the high mechanical resistance of the prepared biocatalyst, and (2) the lower esterification activity of CA/PP_{met}/glass with respect to CA/PP_{met}. The maximum conversion achieved with 200 mg of fresh CA/PP_{met}/glass in standard operation conditions (Section 2.6) was 22% (fourth data row of Table 1). Evidently, although we accomplished our aim of conferring high mechanical resistance to CA/PP_{met}, the interaction of CALB with PP_{met} of the surface of the glass balls could not resemble the conditions found for CALB–PP_{met} bonding. Comparison of the conversion achieved with CA/PP_{met}/glass and with CA/PP_{met} demonstrates that the amount of effective lipase immobilized in an active conformation on the coated glass balls is far lower than in the case of plain PP_{met}; and very similar to the active lipase content of CA/PP_{ZN}. A possible explanation for the low content of active lipase of the coated balls is the existence of uncovered regions of the glass balls in which lipase might have been adsorbed to bare silica. There are reports of

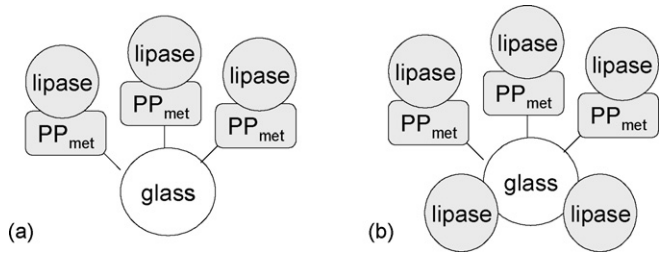

Fig. 5. Scheme of lipase distribution models: (a) on PP_{met} and (b) on PP_{met} and glass surface.

stable adsorption of CALB onto glass balls without functionalization of the glass surface [17]. Fig. 5 shows possible models for lipase adsorption onto PP_{met}/glass balls. Fig. 5a shows a model of lipase adsorption over PP_{met}-coated regions of glass balls surface. On the other hand, Fig. 5b depicts a model of lipase adsorption onto PP_{met} and also onto uncovered glass surface.

The immobilization of CALB onto exposed glass surface might have favored the immobilization of lipase in partially or completely non-active/closed conformations, leading to a far less active catalyst than the one achieved by lipase adsorption onto metallocenic powdered PP.

In reference to biocatalyst reusability, Fig. 6 shows the conversion measured at different times of ethyl oleate synthesis catalyzed by fresh and reused CA/PP_{met}/glass. For comparison purposes, the conversion profile measured in the non-catalyzed reaction has also been included. Being the ethyl oleate synthesis a spontaneous reaction, consideration of the extension of the uncatalyzed reaction is essential to distinguish low catalytic activity from non-catalyzed ester yield.

Although reuse of CA/PP_{met}/glass showed a progressive activity reduction, comparison of these data with the non-catalyzed reaction profile demonstrates that there is no total loss/deactivation of the lipase in the first use of the biocatalyst. However, the bond established seemed not to be strong enough for a third use of CA/PP_{met}/glass, in which conversion data measured can be purely attributed to the extension of the uncatalyzed reaction (Fig. 6, lowest continuous line). Then, even if no recovery problems were detected for CA/PP_{met}/glass, partial lipase deactivation/leaching from PP_{met}/glass was confirmed.

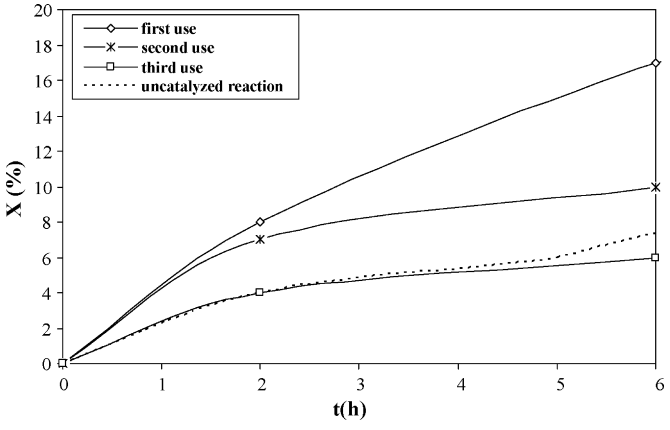

Fig. 6. Ethyl oleate synthesis catalyzed by CA/PP_{met}/glass balls in three sucessive uses. $W = 20\%$, 200 mg of CA/PP_{met}/glass, 45 °C, 350 rpm. Dotted line accounts for the non-catalyzed reaction.

4.4. Results of the theoretical study

In order to consider the thermodynamic feasibility of lipase attachment to glass and polypropylene surfaces, a semiempirical molecular approach was applied. Interaction of polypropylene and silica with the lateral chains of the aminoacids of the lipase was considered. Following the methodology described in Section 3, we have previously analyzed the effect of polypropylene in the stabilization of the opening of the lid of *Candida rugosa* lipase [34]. Moreover, the attractive forces involved in van der waals interactions established between polypropylene and the lateral chains of the aminoacids of lipase have also been analyzed with MM2 and PM3 molecular modelling methods (See reference [34] for the complete analysis).

In reference to HF/KOH pre-treated glass, the theoretical study showed that depending on the surface treatment strong interactions through H-bonding may exist. The KOH pre-treated surface is likely to have OH groups distributed on the "clean" glass, which may interact with the lateral chains of lipase aminoacids. Being these interactions very different from the ones established with PP_{met}, another kind of semiempirical method was used: EHMO (instead of MM2 used for PP_{met}). Lipase aminoacids were placed with the lateral *R* group directed to the silica surface.

Table 3 shows the minimum found for the adsorption energy of lateral chains of aminoacids with the model plane (1 1 1) of silica cristobalite. It is certain that arginine, lysine and asparagine have the highest adsorption energies on the silica surface. As it has been proposed, this implies a role for the H-bonding (distances are in the range that corresponds to an H-bonding). Energies involved in the adsorption of the aminoacids chosen over silica verified the thermodynamical feasibility of CALB immobilization on exposed glass surfaces. The feasibility of mixed adsorption on polypropylene and glass surfaces must have been detrimental of lipase activity immobilized onto PP_{met}/glass. Some possible reasons for the detrimental effect of exposed glass surfaces are: (a) adsorption of the lipase on the bare glass with extremely low OH concentration and desorption after first use; (b) adsorption of the lipase on the OH from glass and loss of activity because of higher molecular conformational restraint and hindrance; (c) deactivation of the remaining lipase on the PP_{met}; (d) low amount of lipase adsorbed in glass regions.

Table 3
EHMO results of the adsorption of lateral chains of aminoacids

Aminoacid	Energy (kcal/mol)
Arginine	−330
Lysine	−139
Asparagine	−62.1
Serine	−13.4
Histidine	−37
Cysteine	−10

5. Conclusions

The kind of surface exposed by the chosen supports conditioned the amount of adsorbed protein and probably also the conformation (open–closed) of the adsorbed protein. In the case of PP, the one obtained with metallocene catalysts presents the best characteristics as lipase support in terms of structure and particle size; whereas PP_{ZN} showed to have lower affinity for proteins. Higher particle size of the commercial pellets of the polymer and the different surface exposed, could have led to lower activity of the lipase immobilized on PP_{ZN}. Coating of glass balls of low diameter conferred high mechanical resistance to metallocenic PP. However, the prepared support material could not resemble the surface exposed by PP_{met} powder. The existence of uncovered silica regions might have generated a hydrophobic–hydrophilic (PP_{met}-glass) surface which was not able to induce a conformation of the adsorbed lipase similar to the one obtained in the highly active CA/PP_{met}. A mixed combined kind of interaction is proposed to take place in this situation, where rigidity of lipase upon adsorption probably plays a role.

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