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RESEARCH PAPER

Immobilization of CALB on lysine-modified magnetic nanoparticles: influence of the immobilization protocol

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Abstract Magnetic biocatalysts offer enormous advantages over traditional ones. Their ability to be isolated by means of a magnet, in combination with their extensive reuse possibilities, makes them highly attractive and competitive from the commercial point of view. In this work, magnetic biocatalysts were prepared by immobilization of *Candida antarctica* Lipase B (E.C. 3.1.1.3, CALB) on magnetite–lysine nanoparticles. Two methodologies were explored tending to find the optimal biocatalyst in terms of its practical implementation: I—physical adsorption of CALB followed by cross-linking, and II—covalent coupling of the lipase on the nanoparticles surface. Both procedures involved the use of glutaraldehyde (GLUT) as cross-linker or coupling agent, respectively. A range of GLUT concentrations was evaluated in method I and the optimum one, in terms of efficiency and operational stability, was chosen to induce the covalent linkage CALB-support in method II. The chosen test reaction was solvent-free ethyl oleate synthesis. Method I produced operationally unstable catalysts that deactivated totally in four to six cycles. On the other hand, covalently attached CALB (method II) preserved 60% of its initial activity after eight cycles and also retained 90% of its initial activity along 6 weeks in storage. CALB immobilization by covalent linkage using controlled GLUT concentration appears as the optimum methodology to asses efficient and stable biocatalysts. The materials prepared within this work may be competitive with commercially available biocatalysts.

Keywords Immobilized CALB · Magnetic nanoparticles · Glutaraldehyde · CALB

Introduction

Immobilization has consolidated as an indispensable technique to exploit enzymes' activity as commercial catalysts. Numerous protocols have been explored, trying to attach proteins in general through interactions of different nature, such as electrostatic attraction, generation of covalent bonds, or simple adsorption by weak intermolecular forces or formation of insoluble enzyme aggregates [1–4]. Most of the available research regarding this topic considers the linkage to a solid support as the most straightforward way of immobilization. The choice of the support material is usually based on the potential affinity with the target molecule [5]. Attention is far more focused on the possibility of chemical modification to promote the interaction support enzyme than the actual performance in the reaction medium. Questions about the catalyst's stability to stirring stress or solvents are rarely analyzed.

In this context, magnetite particles are considered as a suitable starting material for enzyme immobilization due to their chemical/mechanical resistance, surface reactivity and magnetism [6, 7]. This last property allows a rapid and simple magnetic separation of the biocatalyst from the reaction media. Besides, they are easily and economically synthesized by the chemical coprecipitation method [8–12].

Lipases are a special type of enzymes widely studied due to their versatility and ability to catalyze numerous reactions [13–17]. Among them, *Candida antarctica* lipase B (E.C.

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3.1.1.3, CALB) has been immobilized in several supports aiming to achieve efficient biocatalysts [6, 7, 18]. However, conformational changes and/or degradation of the protein and support materials are often limiting aspects for their practical application [19, 20]. The choice of this particular enzyme is based on its remarkable capability to catalyze the synthesis of a wide range of organic substances, such as nitrogenated compounds [21] or esters [22].

As a lipase, CALB exhibits a peculiar structure: the active site is covered by a mobile lid that opens before a hydrophobic environment. This phenomenon is known as interfacial activation [23]. Immobilization in its open form has been achieved using hydrophobic supports [24].

The potential of a biocatalyst should not be exclusively evaluated in terms of the conversion levels of the selected substrate. Their mechanical and chemical resistances in the operation conditions are key factors to avoid the contamination of the desired products. Besides, storage stability, cost of the starting materials and reusability capacity are also required features, besides selectivity when formation of secondary products is possible [25].

In our previous manuscripts, biocatalysts based on CALB immobilized on chitosan-coated magnetic nanoparticles were designed, with the covalent attachment of the lipase on the magnetic supports [18, 20]. Although efficient and active biocatalysts were achieved, phenomena such as leaching of the lipase as well as limited reusability were observed. These seriously affected the practical implementation of the biocatalysts. Besides, comparable results have been obtained by other authors studying enzyme immobilization in similar supports [26, 27].

From this scenario, it is clear that an improvement of the magnetic biocatalysts is required. The purpose of this investigation is to obtain a CALB-based magnetic biocatalyst capable of competing with the commercial ones.

In general terms, the features required to accomplish this goal are high activity, operational stability (including leaching minimization and resistance to stirring and solvents or substrates), reutilization capability, long-term storage stability and low fabrication cost. To attain these characteristics, a novel magnetic support was prepared based on magnetite nanoparticles coated with lysine.

Functionalization of magnetite requires the stable attachment of a reactive group. Amino acids emerge as a convenient option, since the carboxylate group is strongly coordinated with iron, while the free amino group provides wide possibilities of chemical modifications. Monomeric lysine contains an aminated side chain. This additional NH_2 group doubles the number of reactive sites on which the enzyme or a cross-linker molecule can attach. Being a low-cost and environmentally friendly reactant, this amino acid was chosen instead of other NH_2 providers such as 3-aminopropyltriethoxysilane [28, 29].

Although there is a vast literature devoted to magnetic catalysts, hardly any reports are found on amino acid-modified magnetite-based biocatalysts [30]. To the best of the authors' knowledge, the use of lysine-modified MNPs as support for CALB immobilization has not been reported earlier.

Two immobilization strategies were compared within this work to assess the above-mentioned features: I—the physical adsorption of CALB on the magnetic support followed by a cross-linking treatment using glutaraldehyde (GLUT); and II—covalent immobilization of CALB on GLUT-activated support. In the first approach, a range of GLUT concentrations was explored. The best GLUT concentration arising from procedure I was taken as reference to activate the aminated support in procedure II.

The obtained biocatalysts were characterized and evaluated in the solvent-free synthesis of ethyl oleate. Considering that fatty acid esters are common additives in cosmetic and food products (between others of high aggregate value), this is a representative test reaction for an industrial synthesis [22, 31].

The influence of immobilization protocols was analyzed on leaching, reuse capability and storage stability. The performance of the best biocatalyst in terms of its stability was compared with an immobilized CALB catalyst available in the global market. The obtained data allowed us to determine the optimum protocol to obtain not only active, but also reusable and stable biocatalysts.

Experimental

Materials

$FeSO_4.7H_2O$, $FeCl_3.6H_2O$, oleic acid and sodium dodecyl sulfate (SDS) were purchased from Anedra, Argentina. Ammonium hydroxide was from Merck. Novo Nordisk (Brazil) kindly donated the lipase broth Liposyme CALB-L batch number LCN02103. Heptane and ethanol (analytical grade) were from Dorwill, Argentina. Lysine chlorhydrate was from BDH Biochemicals, England. Glutaraldehyde (25% solution) was from Fluka.

Analysis of the commercial CALB broth

Protein purity was qualitatively checked using SDS–PAGE technique on the pure and diluted broth, following the protocol of Lerena-Suster et al. [32]. Denaturalization was performed by adding a buffer solution containing sodium dodecyl sulfate (SDS) and β-mercaptoethanol followed by heating at 100 °C for 10 min. The 12% polyacrylamide gel was prepared with stacking in a BioRad Mini Protean® III equipment and a Tris–glycine running buffer at a pH 8.8 was

used. A volume of 5 µl of each sample was analyzed along with molecular weight markers (from 14 to 90 kDa) LMW of GE-Healthcare. The electrophoresis was carried with a 30 mA current during stacking and 60 mA during resolution. After the electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 (USB) allowing visualization of the separated proteins.

To calculate CALB concentration, a novel methodology developed by the research group was implemented. This consists in determining the sulfur content associated with the CALB structure by atomic emission (AE-ICP) [33]. The calculation was performed considering that each CALB molecule contains 12 atoms of S. A 500 µL aliquot of the sample was diluted up to 5.00 mL with distilled water and measured in a Shimadzu Sequential 1000 model III equipment, with conventional nebulization and external calibration by certified patterns (Chem-Lab, Zedelgem B-8210, Belgium). The obtained result was 18 mg CALB/mL in the raw broth.

Preparation of magnetite nanoparticles (MNPs)

Magnetite was synthesized by the coprecipitation method. 7 mL of NH_4OH 5 M was slowly added to 33 mL of an aqueous solution of $FeSO_4$ (0.065 M), $FeCl_3$ (0.130 M) and sodium dodecyl sulfate (SDS, 8.4×10^{-3} M), at 80 °C under N_2 atmosphere with fast magnetic stirring (1000 rpm). The pH reached after the base addition was 12. The resulting precipitate was separated by magnetic decantation and re-suspended in distilled water. The procedure was repeated three times to remove unbound SDS [34]. Finally, an exhaustive washing with heptane under ultrasound was performed for 1 h to obtain bare, SDS-free, monodispersed magnetite particles. The final product was dried in an oven at 50 °C overnight.

Functionalization of MNPs with amino groups (amination)

Amination of the magnetite surface was achieved by reaction of the magnetic powder (typically 100 mg) with a 17 mg/mL lysine solution prepared in distilled water (5.8 mL, pH 5.7) during 7 h at room temperature. The molar ratio of magnetite/lysine was 0.64. The solid was decanted and washed three times with distilled water. These formulations were named MAG-NH_2.

Immobilization of CALB in MAG-NH_2

Method I: physical adsorption followed by cross-linking

Method I involved two steps: physical adsorption of CALB and cross-linking with GLUT. Physical adsorption of enzyme on the aminated support was carried out dispersing 100 mg of MAG-NH_2 in 17 mL distilled water (pH 5.7). The media was distilled water without buffer, aiming to minimize ionic strength. 582 µL of CALB broth (18 mg enzyme/mL) was added and the mixture was magnetically stirred for 7 h at room temperature. Excess of CALB was removed by washing three times with distilled water. The obtained material was called MAG-NH_2–CALB.

Post-adsorption cross-linking with GLUT was achieved by suspending 300 mg of MAG-NH_2–CALB in 5 mL of a GLUT solution of concentration ranging from 0.05 to 0.6% (weight of GLUT × 100 mL of solution). The suspension was stirred for 3 h at room temperature.

Method II: covalent attachment of CALB

To attain the covalent binding of CALB on MAG-NH_2, 100 mg of the support was left in contact with 1.6 mL of GLUT solution of 0.3% w/v for 3 h at 45 °C. This aldehyde-functionalized carrier was recovered by magnetic decantation, washed three times with distilled water and dried in an oven at 45 °C. For immobilization, it was suspended in 17 mL of distilled water and 582 µL of CALB broth was added. The mixture was maintained under magnetic stirring for 7 h at room temperature.

The Scheme 1 illustrates the two procedures to attain CALB biocatalysts.

Efficiency of biocatalyst

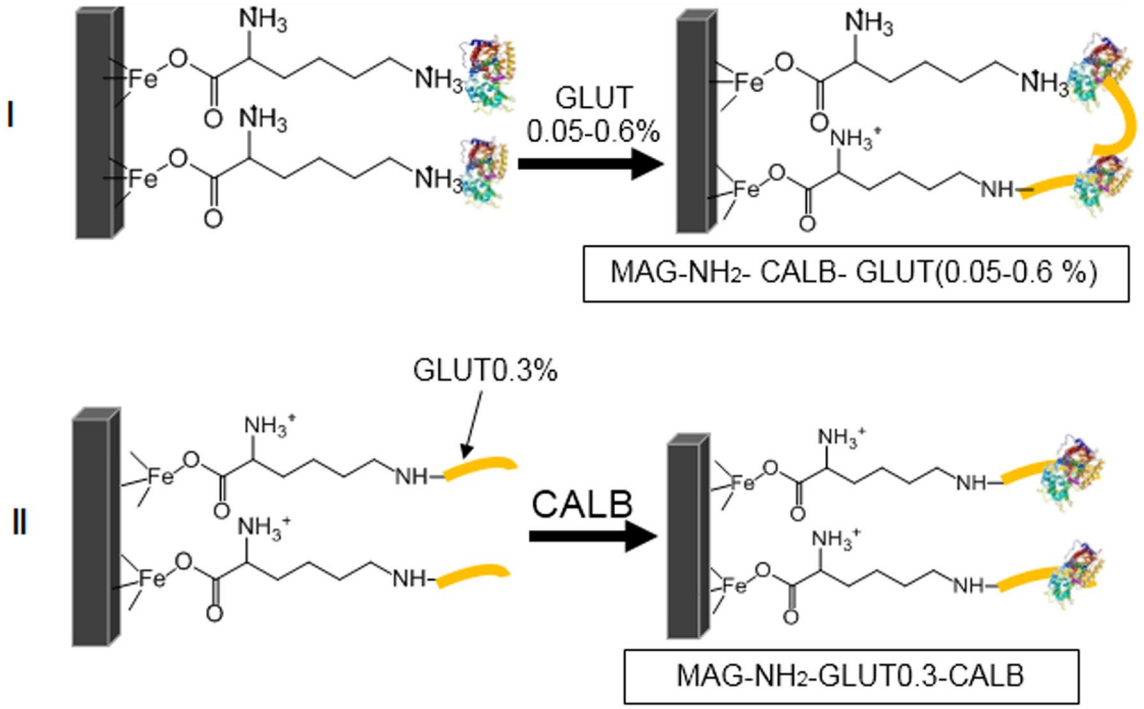
Test reaction, sampling and conversion determination as well as other experimental conditions related to these issues were taken from previous works using CALB-based biocatalysts [20, 35]. In brief, 1 g of oleic acid (OA), 200 µL of water, 150 µL of ethanol and 30 mg of biocatalyst were magnetically stirred at 45 °C for 3 h.

The conditions adopted for the test reaction (especially the addition of water to the reactants) were taken from previous exhaustive studies on this topic [36].

After the stipulated time, 1.5 mL of ethanol was added to convert the reaction medium into a single homogeneous phase. The catalyst was rapidly decanted with a magnet and two samples were withdrawn (about 250–300 mg each). They were weighted and then titrated against ethanolic KOH (precisely known concentration, typically around 0.0300 M) to determine unreactive acid. The conversion was calculated as:

$$X\% = \frac{\text{mass of converted acid} \times 100}{\text{initial mass of acid}}.$$

The difference between replicates was always lower than 1 unit.



Scheme 1 Representation of immobilization protocols. **I** Adsorption of lipase on aminated support, followed by GLUT cross-linking. **II** Covalent attachment of lipase on GLUT-activated support

Reusability assays

After each cycle, different procedures were implemented to recover the purified catalyst. In the first place, the biocatalyst was magnetically decanted. The liquid mixture was removed and preserved for leaching analysis. Afterward, in the same reaction vial, the solid was washed twice (unless otherwise specified) with a solution of heptane:ethanol 1:1 for 30 min. An additional washing was performed using pure ethanol under magnetic stirring.

In all the purification steps, the magnetic properties of the biocatalysts were exploited to asses an efficient separation of the residual washing liquids. Finally, the catalysts were dried at 37 °C overnight.

Leaching measurements

500 µL of the remaining reaction mixture after esterification was added to a fresh set of reactants, without catalyst, aiming to check enzyme desorption. Conversion was calculated following the methodology described in "Efficiency of biocatalyst", and was defined as leaching %.

Characterization

Diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) was performed using a Thermo Scientific Nicolet 6700 spectrometer, which was used to record spectra in the range 4000–400 cm^{-1}. About 3 mg of sample was manually mixed with 50 mg of dry KBr. The resulting powder was placed in the sample holder. The equipment was kept purged with nitrogen. Transmission electron microscopy (TEM, JEOL 100 CX II, Tokyo, Japan) was used to examine the morphology of the support nanoparticles. The TEM samples were dispersed in distilled water, placed on 200 mesh Cu grids and dried at room temperature.

The hydrodynamic diameter (Dh) of the support and catalysts was measured in distilled water (0.1 mg sample/mL, pH 5.7) by dynamic light scattering (DLS). The same suspension was used to determine the zeta potential (ζpot) by microelectrophoresis laser Doppler. A Zetaziser Nano ZS equipment (Malvern Instruments) was used for both techniques. ζpot results are averages of duplicates.

Results and discussion

Support and biocatalyst characterization

FTIR spectroscopy was employed to qualitatively assess the biocatalysts' composition. In Fig. 1, the FTIR spectrum of raw support (MAG-SDS) is compared with those corresponding to MAG-NH_2, MAG-NH_2–CALB and MAG-NH_2–CALB–GLUT. In the first spectrum, bands associated

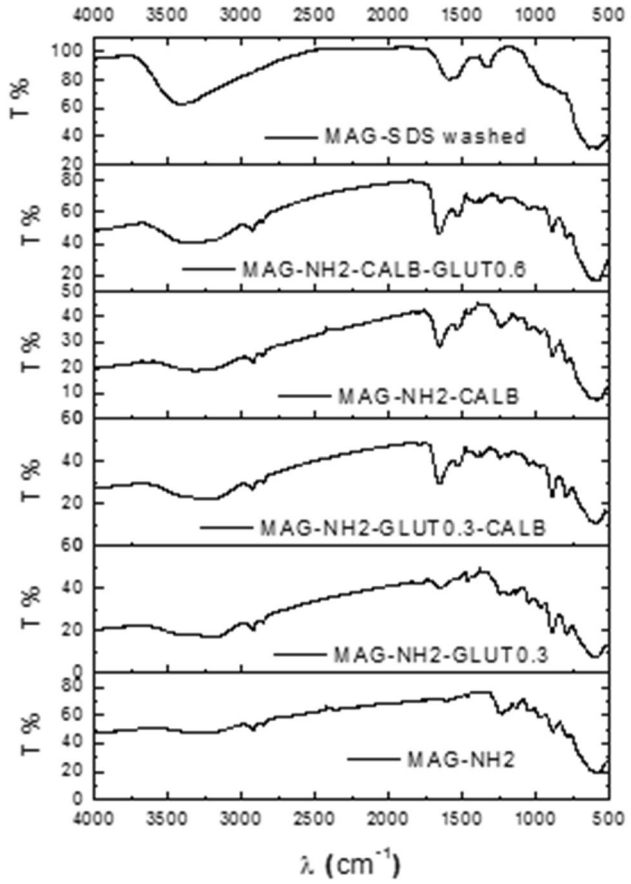


Fig. 1 DRIFTS spectra of selected biocatalysts and support materials

with water molecules adsorbed on the MAG surface are evidenced at roughly 3400 cm^{-1} (OH stretching band) and 1700 cm^{-1}. Those bands overlap with the corresponding functional groups of lysine (C=O and NH_2). A confirmation of the lipase incorporation was indicated by the presence of bands at 1720 and 1590 cm^{-1} assigned to N–H and C=O as well as signals at almost 3200–3400 cm^{-1}, associated with the stretching of N–H (from amide groups) and O–H (from ROH of COOH groups). Similar results were observed by Bezerra et al. when attaching *Thermomyces lanuginosus* onto aminated magnetite [6].

The incorporation of GLUT after physical adsorption of CALB may be appreciated by the decrease in the band associated with OH and NH in the region of 3200–3400 cm^{-1}. This represents an evidence of the cross-linking reaction between the coupling agent and the functional groups (NH and OH) of both the support and the lipase. Increase of C=N at 1635 cm^{-1} and the decrease of carboxylic group (–C=O^-) bands at 1730 cm^{-1} and –C–O^- at 1470 cm^{-1} may indicate the formation of Shiff base between the protein and GLUT.

Morphological characterization by TEM demonstrated that the support was composed of spherical and nearly monodisperse nanoparticles (MAG-NH_2), as shown Fig. 2a. The incorporation of the lipase by simple adsorption did not cause significant morphologic changes. However, particles' morphology seems to be sensible to GLUT concentration after immobilization. With regard to this, higher particle aggregation was evidenced, increasing GLUT concentrations after immobilization (see Fig. 2b–d).

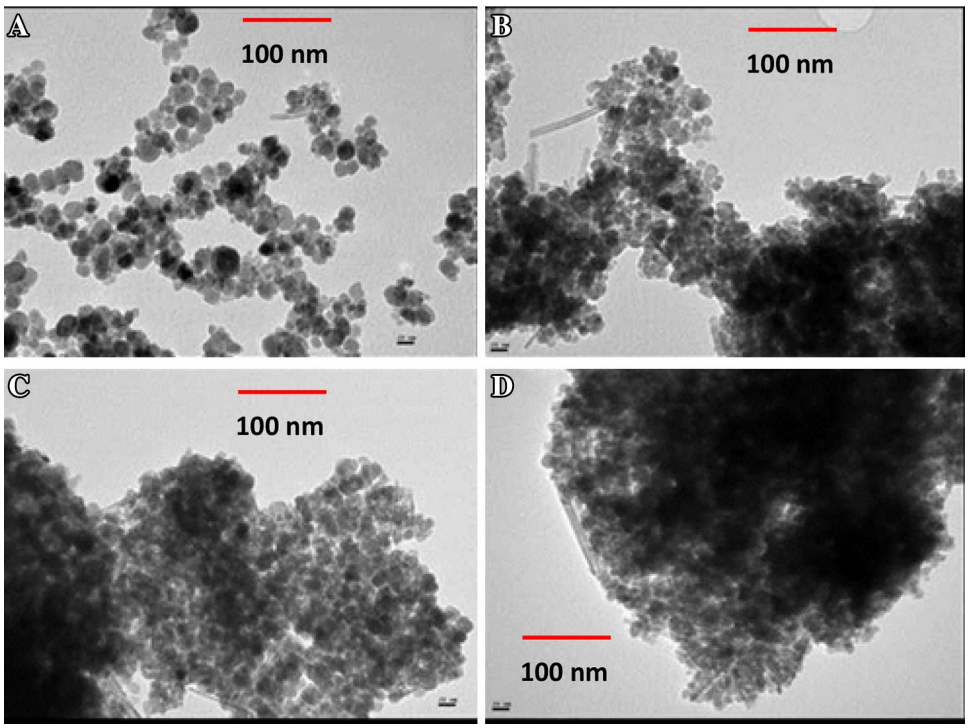


Fig. 2 TEM images of: **a** MAG-NH2, **b** MAG-NH2–CALB–GLUT0.05, **c** MAG-NH2–CALB–GLUT0.3, **d** MAG-NH2–CALB–GLUT0.6

The support particles and aggregates conserve the nanometric size, which is an important condition for increasing the surface area and promoting larger enzyme loadings.

Histograms, in Fig. 3, depict Dh distributions of the analyzed samples. Dh values are larger than magnetic core sizes estimated by TEM. This difference is the result of agglomeration of MAG nanoparticles in suspension. Lysine incorporation yielded significantly smaller MAG-NH_2 aggregates than the MAG-SDS precursor. CALB adsorption on MAG-NH_2 broadened Dh distribution and displaced sizes toward larger values. Cross-linking with GLUT (post-CALB adsorption) only raised the number of particles with greater sizes, whereas covalent immobilization on MAG-NH_2–GLUT also caused a major widening of the Dh range.

The ζ potential is a useful parameter for characterization in particle studies. This technique provides information on the surface charge on the particle. Hence, it is a valuable tool to infer the kind of interaction between, in this case, lipase and MAG-NH_2 or MAG-NH_2–GLUT and as complement of traditional techniques (such as FTIR, activity data) to verify lipase incorporation [1, 41–43].

Figure 4 compares ζpot of particles at different stages of modification. MAG-NH_2 has a positive potential since the amino groups of lysine have pKa > 8.9 [37], they remain protonated in distilled water ($–NH_3^+$). The sign of this potential is reversed in MAG-NH_2–CALB, indicating an effective incorporation of lipase. Catalysts conserve a negative potential after both immobilization protocols.

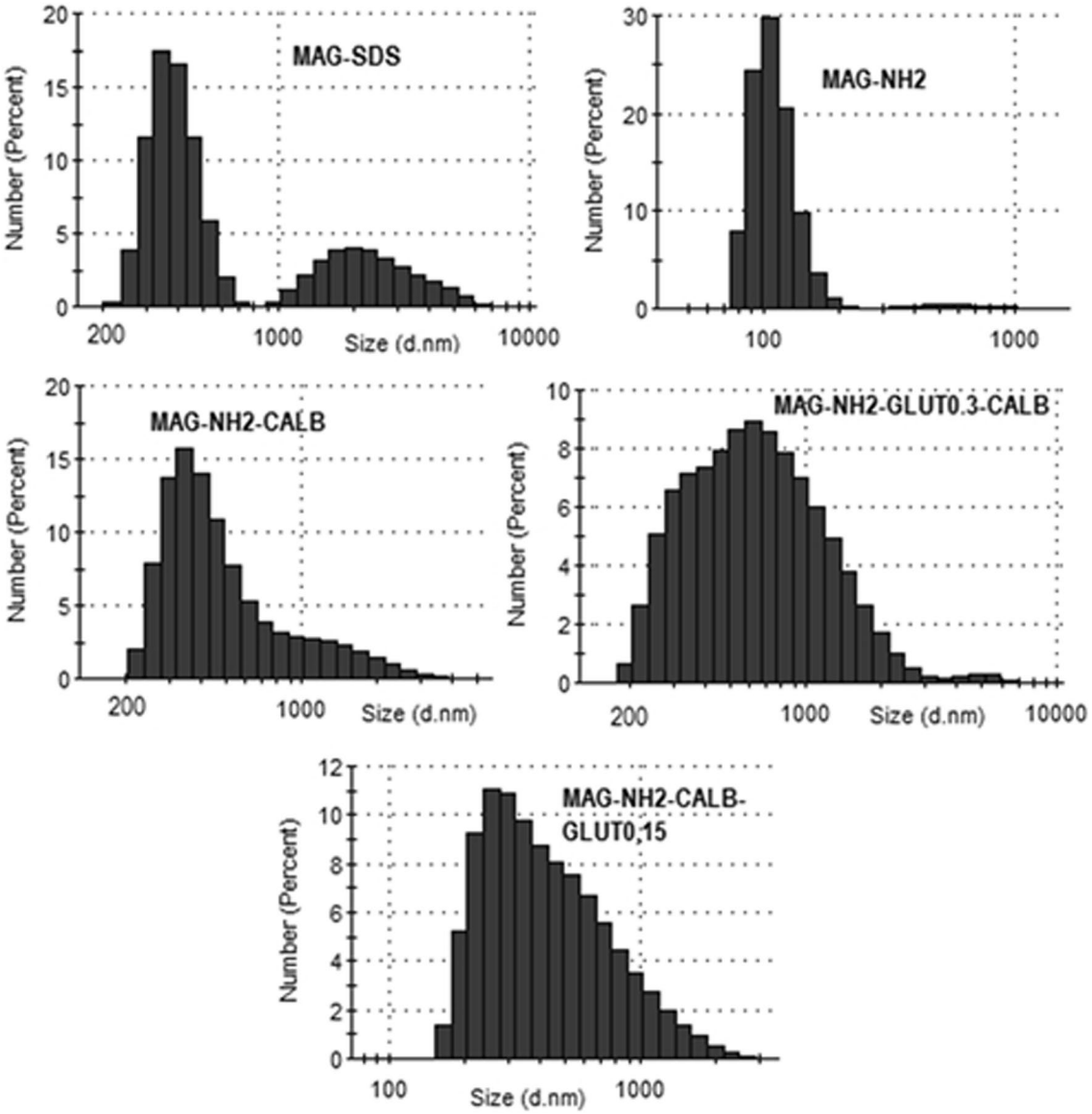


Fig. 3 Hydrodinamic diameter distribution of selected samples

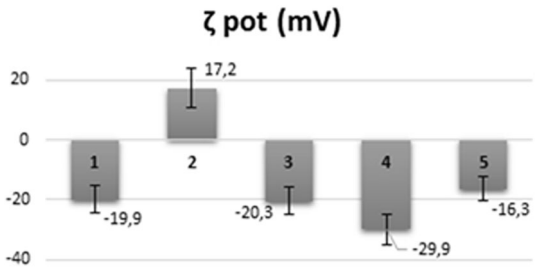


Fig. 4 Zeta potential of **1** MAG-SDS, **2** MAG-NH2, **3** MAG-NH2–CALB, **4** MAG-NH2–CALB–GLUT0.3, **5** MAG-NH2–GLUT03–CALB

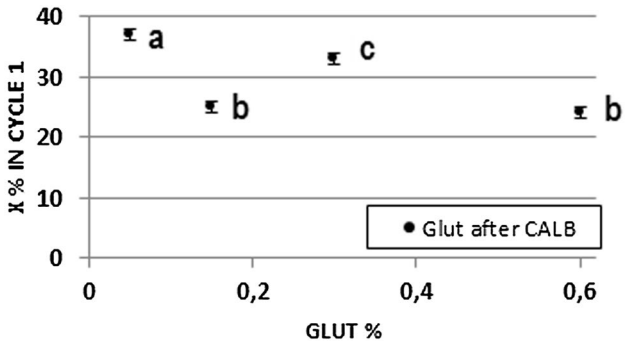


Fig. 5 Conversion percentage in the first cycle of post-adsorption cross-linked CALB. Letters next to points correspond to Tukey test results (95%)

Specific characterization related to the iron oxide core, such as determination of magnetic properties or crystalline pattern, were not performed because the synthesis and coating procedures were almost achieved under similar conditions than in previous works [18, 34, 38].

Efficiency of biocatalysts

Method I

The first step of this method involved the physical adsorption of CALB onto MAG-NH_2 particles. The pH of the adsorption medium needs to be controlled to promote this interaction. As it was mentioned in "Characterization", ζ potential of support at pH 5.7 became positive (+ 17.2 mV, see Fig. 4), suggesting protonated amino groups. CALB returned a barely negative surface charge (approx. − 2.18 mV) at that same pH. This difference was enough to promote electrostatic interactions favoring adsorption. This hypothesis is supported by FTIR, ζpot of catalysts and activity.

The efficiency of biocatalysts prepared with different GLUT concentrations is compared in Fig. 5 in terms of the conversion levels achieved in the first reaction cycle. Data on Fig. 5 suggested that the initial conversion was not greatly affected by the concentration of the cross-linker. However, Tukey test proves a statistical difference between the registered X% values; consequently, three groups may be distinguished (a, b and c in Fig. 5).

In method I, immobilization may be through a one-point or a multipoint interaction, and after this immobilization the support may continue increasing the number (or even the quality) of interactions involving new groups, as in the case for heterofunctional supports [5].

Values of conversion of oleic acid (X%) between 25 and 37% were reached as a function of GLUT concentration. As a general trend, a slight decrease in X% was observed when increasing GLUT concentration. This effect has been observed by other researchers [39–41].

To gain insight into the practical implementation of the biocatalysts, their reuse was studied. This analysis included the measurement of conversion and lipase leaching as a function of each cycle and is shown in Fig. 6.

From these data, it emerges that the cross-linking treatment post-adsorption enhances the enzyme stability, reducing the leaching levels when concentrations of GLUT above 0.3% are employed. Similar findings were reported by other authors using other systems [42]. It is important to mention that following the same protocol and employing an excess of GLUT (2.5%), the OA conversion registered in the test reaction was 0% (data not shown in Fig. 6). The selection of an optimal GLUT concentration is a controversial issue, judging by the literature articles devoted to enzyme immobilization. It appears to be highly reliant on the kind of support and the selected enzyme. It has been reported that by varying this parameter, a monolayer or bilayer of GLUT may be formed, depending on whether one or two molecules react per amino group. However, the chemical structure of the GLUT bilayer has not been fully determined [43].

The influence of GLUT concentration was also evidenced in leaching data. When low concentrations are employed (0.05–0.15%), leaching is observed and may be considered as one cause of deactivation between cycles. It is important to highlight that higher leaching levels were found in previous works using magnetic chitosan supports, even after covalent linkage with GLUT [18].

It is hypothesized that using low GLUT concentrations, a great number of CALB molecules remain weakly attached to the support. This occurs because the concentration of GLUT is not enough to cover the adsorbed lipase, avoiding the leaching. Conversely, higher GLUT concentrations play a fixative effect, since leaching was negligible when using GLUT concentration of 0.3 and 0.6%. However, this advantage was overshadowed by the total loss of the catalytic activity after seven and five cycles, respectively (see Fig. 6 GLUT0.3 and 0.6).

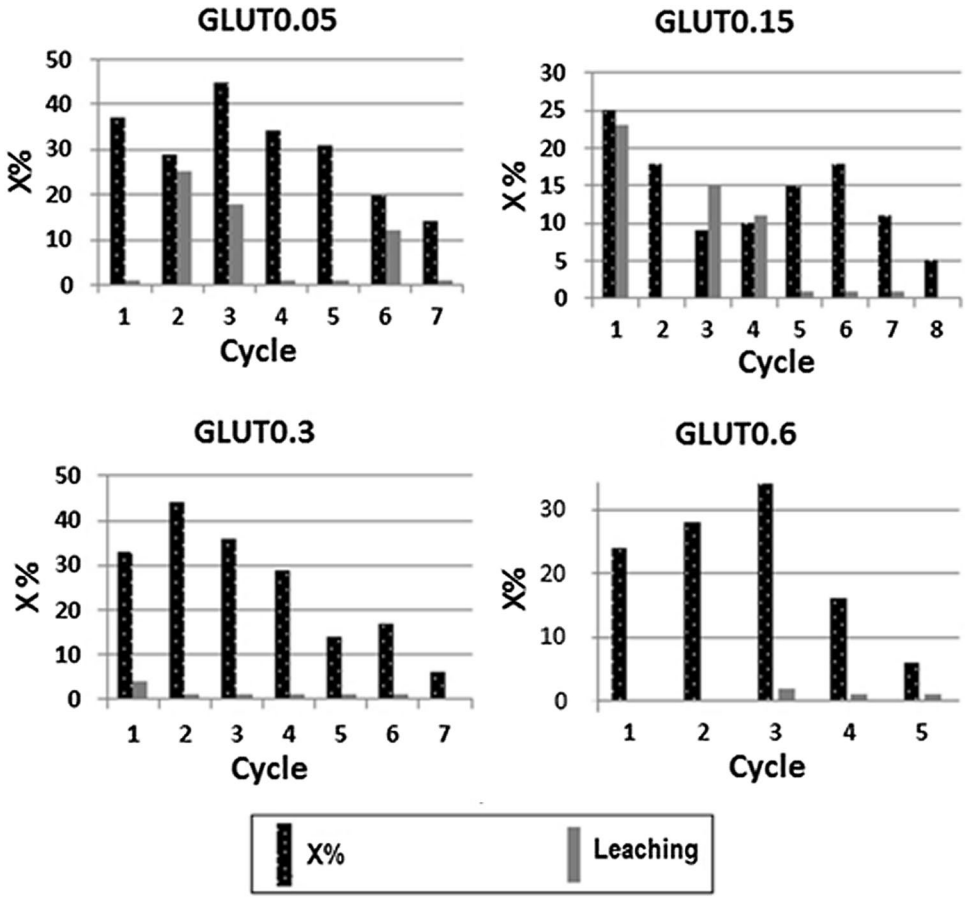


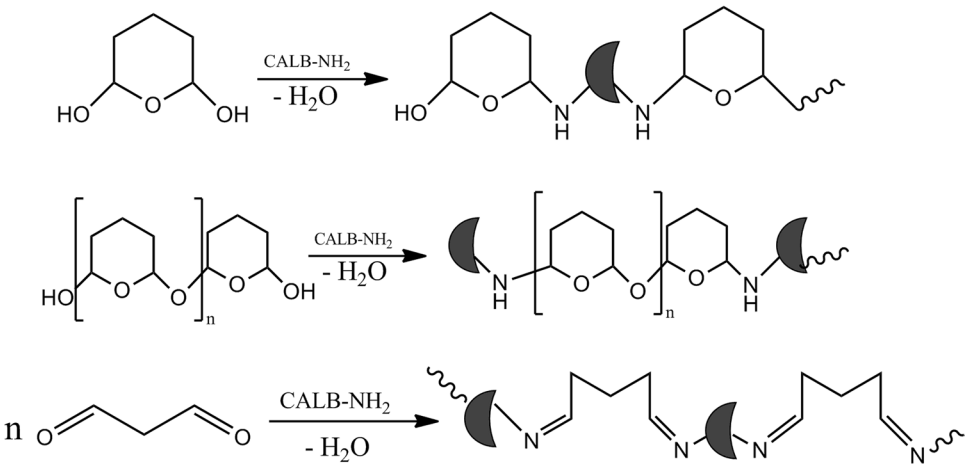
Fig. 6 Conversion and leaching levels in successive cycles of post-adsorption cross-linked CALB

In this context, it may be assumed that a relation is established between the operational stability provided by cross-linking and the impairment of enzyme denaturation. This dual effect of GLUT has been exhibited in different immobilization protocols. For example, D-amino acid oxidase was adsorbed on aminated Sepabeads® cross-linked with 0.2–2% GLUT. While the activity gradually decreased with the concentration of GLUT, the resistance to leaching was maximum at 0.5% [44]. A similar behavior was observed for *Fusarium moniliforme* CGMCC 0536 hydrolase [45] and CLEAs of *Thermomyces lanuginosa* lipase [46, 47] and CALB [48], respectively.

Considering the highly viscous and concentrated reaction medium, another feasible cause for inactivation is the inhibition from product/substrate deposited on the catalyst´s surface. This behavior may be observed, for instance, for GLUT0.05 catalyst in Fig. 6. The washing step after cycle 1 consisted in 5-min stirring in heptane plus 5 more minutes in ethanol. X% decreased noticeably in cycle 2. After this second reaction, washing was performed for 30 min in each solvent. X% increased in cycle 3. Therefore, exhaustive washing may help the correct elimination of the remaining compounds (ethyl oleate or OA) from the CALB surface and "reactivate" the catalyst [49].

On the basis of our findings and reports of literature, it is possible to postulate a mechanism for GLUT–protein interactions, involving the formation of several species. The proportion of such species depends on the pH of the medium. Under the conditions herein explored, stable secondary amines (from the monomeric and polymeric cyclic hemiacetal) could be formed. Schiff's bases stabilized by conjugation with C–C double bonds, from the poly-GLUT under basic conditions [50, 51] are not considered here since all GLUT treatments were performed in distilled water (pH 5.7). Hence, under such conditions, polymerization of GLUT is highly unlikely to occur. Scheme 2 illustrates the most probable products.

Since these products are stable under the conditions in which they were formed, no chemical treatments (such as reduction with sodium borohydride) were applied after immobilization. However, high leaching was measured along several cycles when using low GLUT concentration. These data may be justified in terms of the immobilization mechanism, assuming that the offered GLUT is involved in both the CALB–CALB and CALB–lysine cross-linking. In this concern, a combined immobilization mechanism is proposed involving covalent bond formation and physical adsorption of lipase molecules on lysine magnetic support. As it is



Scheme 2 Feasible immobilization mechanisms of GLUT–lipase interactions. Schiff base from monomeric GLUT is highly unstable

known, hydrophobic interaction among lipase molecules is recognized as most important. In fact, self-aggregation of many microbial lipases in solution has been reported [52]. This self-aggregation has been justified in terms of the surface hydrophobic characteristics of lipases. In this context, it is possible that CALB clusters may be the adsorbed species on the MAG-NH_2 support.

In test reaction system, a highly hydrophobic environment surrounds the biocatalyst. Together with the vigorous magnetic stirring, loss of enzyme toward the reaction medium is likely due to desorption of weakly adsorbed CALB as well as cross-linked aggregates. These aggregates may or may not be catalytically active depending on their denaturation level. This combined immobilization mechanism would explain the rather inconsistent fluctuation of X% and leaching values in Fig. 6, whose deviations are lower than ± 1%. For example, in the third cycle of GLUT0.15, leaching is greater than X%. This is easily attributed to the aggregation phenomenon of the immobilized CALB and its disaggregation upon desorption. Also, this being a magnetic nanomaterial, particle aggregation in the reaction medium may restrict substrate diffusion toward lipase´s active site, lowering the expected activity.

An illustration of the effect of the immobilization mechanism on the conversion and leaching levels is included in Scheme 3.

It is important to note that using 0.05% GLUT, after cycle 1, a short washing step was performed and X% decreased noticeably. The increase of X% in cycle 3 may be associated with the deeper washing implemented in the catalyst after cycle 2, to discard the possibility of inhibition by the substrate or product residues.

Even when the highest OA conversion was reached using the lowest GLUT concentration at the first cycle (Fig. 5), this trend was not mirrored when reuse results were analyzed (Fig. 6). The productivity found in reuse (as the total amount of ethyl ester of OA produced, shown in Fig. 6) appears to be the highest when intermediate GLUT concentrations were employed (0.3% GLUT), along with minimized leaching effect. According to this finding, the concentration of 0.3% GLUT (mass in volume of solution) was selected to assess the covalent CALB binding on the MAG-NH_2 support, as will be described later in method II.

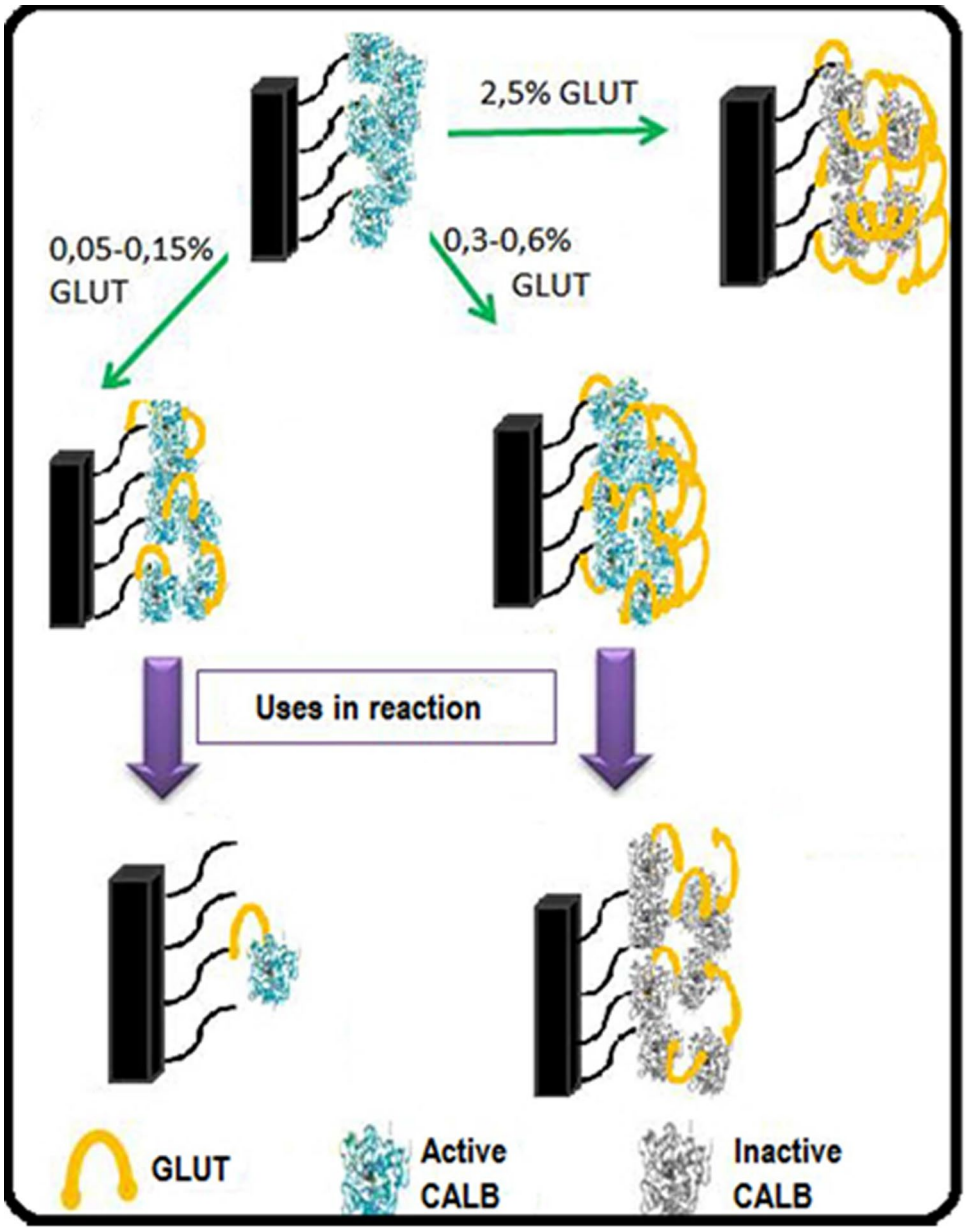
Method II: covalent immobilization on MAG-NH2–GLUT

Here, CALB is made to react with aldehyde groups and other functions like hemiacetal coating the magnetic core. Free amino groups of the enzyme attack carbonyls and other electrophilic zones. pH should be neutral or slightly acidic to prevent polymerization of GLUT, assuring availability of electrophilic sites.

The covalent linkage of CALB using 0.3% GLUT renders a highly efficient biocatalyst. Figure 7 shows the conversion and leaching data from this biocatalyst after a series of eight cycles of reutilization. The conversion reached almost 50% after the third cycle and remained almost constant (between 50 and 40%) up to the eighth cycle. The conversion in the first cycle is roughly twice that achieved when post-adsorption cross-linking (method I) is applied (see Fig. 5).

These data may be justified, in part, in terms of the nature of the support. It has been reported that aminated supports may react with GLUT moieties leading to amine–GLUT surface-exposed groups which exhibit different reactivities that free GLUT molecules. This would mainly restrict the GLUT polymerization capacity, avoiding a massive GLUT precipitation on the support [53].

As stated in the previous section, the combined immobilization mechanism (covalent linkage and CALB adsorption on the first lipase layers) allows explaining the loss of activity along the eight cycles in terms of the weakly adsorbed enzyme detachment. Leaching in the first two cycles appears to be noticeably larger than in GLUT0.3 (from method I).



Scheme 3 Representation of post-adsorption cross-linked CALB deactivation

This may be likely due to this double immobilization mechanism, involving the direct exposition of CALB to GLUT moieties in a double layer favoring its loss.

60% of the initial catalytic function is preserved up to at least the eighth cycle. Tukey test data reveal that X% in cycle 1 is significantly different from any of the subsequent reactions. However, the eighth cycle returns an X% comparable to the fourth. Considering that 60% of the initial catalytic function is preserved up to at least the eighth cycle, this catalyst possesses an acceptable operational stability.

Due to the high activity and operational stability of the catalyst obtained by method II, MAG-NH_2–GLUT0.3–CALB is considered to be the optimum one of all those prepared in this study.

It is important to highlight that the GLUT concentration was the other factor determining the integral efficiency of the biocatalyst (this is suitable conversion and leaching levels). The same GLUT concentration (0.3% GLUT) was employed in the two most effective biocatalysts (in terms of the total mg of OA converted and the level of leaching) independently of the immobilization protocol. This GLUT proportion fits with a proportional ratio between moles of cross-linker and the moles of NH_2 groups in the support; whereas the proportion of the lipase remained constant in all the formulations [54, 55]. As a main conclusion, the amount of GLUT that should be used in case of CALB to maximize activity and minimize leaching is lower than that traditionally reported in the literature for

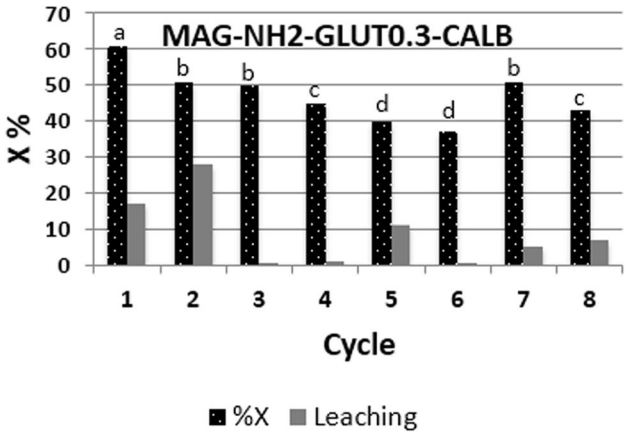


Fig. 7 Conversion and leaching levels in successive cycles of CALB immobilized on 0.3% GLUT-activated support. Letters next to points correspond to comparable means according to Tukey test (95%)

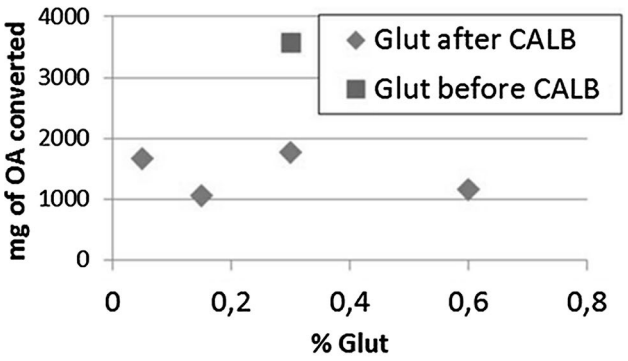


Fig. 8 Productivity of biocatalysts from methods I and II as a function of GLUT %. Method I (MAG-NH2–CALB–GLUT, rhombus), method II (MAG-NH2–GLUT0.3–CALB, square)

covalent bonding of CALB; i.e., 1.19% or more [30], 2.5% [56], 5% [57], 15% [42]. The choice of such concentrations is frequently related to a screening of GLUT concentrations taking into account the maximum catalyst activity. Considering that so dissimilar percentages are reported as useful, it may be inferred that activity depends on both the amination grade of the support and the GLUT proportion used to activate it.

Analysis related to the practical implementation of the prepared biocatalysts

The total conversions using the catalysts prepared from both immobilization procedures are compared in Fig. 8. The informed conversion involves the total milligrams of OA converted along all the performed cycles in each case. The biocatalyst prepared from method II was the most efficient, reaching a total conversion of already 3500 mg of OA along eight reaction cycles.

Although several CALB-based biocatalysts appear to be more efficient in terms of the conversion levels, many of them are not tested for their operational stability. Besides, after a brief survey of open literature, it is observed that others exhibit lower operational stability than the CALB biocatalyst proposed in this work. For instance, dos Santos et al. observed a 64% decrease of commercial CALB (Novozyme®435) activity from the first to the second cycle of oleic acid esterification in supercritical CO_2 [58]. To better illustrate this, a comparative analysis was performed by analyzing several CALB biocatalysts suitable for oleic acid esterification reactions. The collected data are included in Table 1.

Data in Table 1 reveal that magnetic CALB herein prepared is capable of reaching high conversion of a concentrated substrate (2.3 M). These levels are equaled by commercial Lipozyme435 and even surpassed when ultrasound is applied [58]. However, Lipozyme exhibits limitations associated with the reuse possibilities. In fact, resin support degrades and causes product contamination [62] and blockage in filtration operations and column reactors, in the presence of short-chain alcohols [14].

When considering silica-immobilized lipase [59], the biocatalyst showed an excellent reusability in cyclohexane. However, OA concentration and conversion were considerably lower than those of the magnetic biocatalyst. The amount of processed substrate was barely 0.7 g in a 12-h

Table 1 Experimental test reaction conditions and performance of CALB biocatalyst in oleic acid esterification

Catalyst	Solvent	[OA] M	% wt. CAT[a]	OA/alcohol	*T* °C	X% in 3 h	Reusability	References
MAG-NH_2–GLUT–CALB	None	2,3	3	1/1 molar	45	60	Good	This work
MAG-chitosan–GLUT–CALB	None	2,3	3	1/1 molar	24	64	Poor	[20]
SiO_2–CALB	Cyclo-hexane	0,5	3,5	1/ 1 molar	40	35 90 in 12 h	Excellent	[59]
Lipozyme 435	None	2,3	1,36	1/1 molar	40	65 85-US	Poor[b]	[60]
CALB-chitosan	SC-CO_2	1,4	10	1/6.5 molar	50	25	Poor	[61]

SC supercritical, *US* ultrasound

[a]Referred to OA initial mass

[b]Not evaluated in the cited article

cycle. It would take at least five more 12-h runs (a total of 60 h) to reach the productivity of eight 3-h cycles using MAG-NH_2–GLUT–CALB.

Covalently attached CALB on chitosan exhibited low activity and major deactivation in nearly supercritical CO_2 [61].

The effect of temperature on immobilization efficiency or on enzyme activity was not evaluated in this work. As one of the purposes of our research is to minimize energy consumption, all procedures were performed at room temperature or at the minimal one to promote reaction. CALB is an extensively studied enzyme and, comparing published works, 45 °C is low enough to save energy and high enough to increase conversion [26, 63, 64].

In the case of biocatalysts obtained from post-adsorption cross-linking (method I), the maximum productivity was observed when 0.3% of GLUT was employed after CALB physical adsorption. In such cases, roughly 1800 mg of OA was converted, which represents nearly 50% of the conversion achieved by the catalysts obtained from method II (see Fig. 8). Besides, MAG-NH_2–GLUT0.3–CALB was found to retain 60% of its initial activity up to, at least, the eighth use.

Operational stability (both activity retention and chemical/mechanical resistance) should be considered a much more important feature than conversion levels, since a low activity can be compensated with more cycles or larger catalyst amounts, and no contamination is produced by support degradation.

Additional studies devoted to gain insight into the storage stability revealed that MAG-NH_2–CALB–0.3GLUT retained nearly 100% of its activity after a month, whereas MAG-NH_2–0.3GLUT–CALB retained 91% after 6 weeks. It can be inferred then that both immobilization methods led to storage-stable catalysts, at least in the explored range of time.

When comparing the performance of the best biocatalyst obtained in this work with the commercially available one, it emerges that though the commercial Novozym® 435 is more active (in weight basis) [20], its chemical stability is recognized as a weakness. It has been widely reported that ethanolic media may induce the dissolution of Novozym® 435 support. Alaso, its reuse capability is lower and currently involves tedious separation pathways. In addition, the leaching is practically unavoidable since the enzyme is physically adsorbed [<link rid="bib65">65</link>]. An advantage of these biocatalysts is that magnetic decantation enormously facilitates the isolation of the MAG-NH_2–GLUT0.3–CALB catalyst, in contrast to the tedious filtration processes needed for conventional materials (especially in highly viscous media involving solvent-free esterification).

The costs associated with MAG-NH_2–GLUT0.3–CALB fabrication were estimated at a laboratory scale, indicating that they were 60% lower than those related to Novozym435, considering 1 g of both biocatalysts as reference. It is important to remark that this analysis considers prices available in the local market (Argentina in our case).

What is more, the value of the support MAG-NH_2–GLUT represents barely 3% of the total biocatalyst cost. Hence, this material by itself constitutes a potential carrier not only for lipase immobilization, but also for protein adsorption, isolation and purification.

Magnetic CALB is also a potential replacement of traditional catalyst for transesterification reactions, such as biodiesel synthesis [66–70], food additive preparation [71, 72], drug production [73] and other fine chemicals.

A simple batch reactor with mechanical stirring would be suitable for large-scale application of these magnetic catalysts. Decantation is easily achieved by an external strong magnetic field (Nd magnets, for example).

Concluding remarks

Biocatalysts with high chances of practical implementation were designed from immobilization of CALB on lysine-coated magnetic nanoparticles. Two different methodologies were explored to attain this goal, i.e., cross-linking post-adsorption and covalent linkage. In both cases, GLUT was used either as coupling or cross-linking agent, respectively.

The analysis of a range of GLUT concentrations for post-adsorption CALB cross-linking allowed finding out the optimal concentration of GLUT as coupling agent. This amount was used to modify the support and was selected considering the protein amount known to be immobilized by adsorption and not only the support surface.

Covalent immobilization of CALB on 0.3% GLUT-activated support was the optimum method in terms of catalyst activity and operational stability. The productivity of covalently coupled CALB doubled that of post-adsorption cross-linked lipase. Both methods ensure biocatalysts with elevated activity retention on storage, since over 90% of their initial activity was maintained after 4 or more weeks in storage.

Taking into account the low-cost starting material, simple preparation protocols, operational and storage stabilities, easy recovery and high efficiency, MAG-NH_2–GLUT0.3–CALB is a potentially attractive biocatalyst for solvent-free fatty acid esterifications.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

1. Wu L, Wu S, Xu Z et al (2016) Modified nanoporous titanium dioxide as a novel carrier for enzyme immobilization. Biosens Bioelectron 80:59–66
2. Yuce-Dursun B, Cigil AB, Dongez D et al (2016) Preparation and characterization of sol–gel hybrid coating films for covalent immobilization of lipase enzyme. J Mol Catal B Enzym 127:18–25
3. Lv JS, Liu XY, Xu JX et al (2013) Preparation and properties of adsorption material from corn stalks core when used for enzyme immobilization and the subsequent activities of the adsorbed enzymes. Ind Crops Prod 50:787–796
4. García-García MI, Sola-Carvajal A, Sánchez-Carrón G et al (2011) New stabilized FastPrep-CLEAs for sialic acid synthesis. Bioresour Technol 102:6186–6191
5. Santos JCS dos, Barbosa O, Ortiz C et al (2015) Importance of the support properties for immobilization or purification of enzymes. ChemCatChem 7:2413–2432
6. Bezerra RM, Neto DMA, Galvão WS et al (2017) Design of a lipase-nano particle biocatalysts and its use in the kinetic resolution of medicament precursors. Biochem Eng J 125:104–115
7. Cipolatti EP, Valério A, Henriques RO et al (2016) Nanomaterials for biocatalyst immobilization—state of the art and future trends. RSC Adv 6:104675–104692
8. Liu X, Chen X, Li Y et al (2012) Preparation of superparamagnetic Fe_3O_4@alginate/chitosan nanospheres for candida rugosa lipase immobilization and utilization of layer-by-layer assembly to enhance the stability of immobilized lipase. ACS Appl Mater Interfaces 4:5169–5178
9. Sui Y, Cui Y, Nie Y et al (2012) Surface modification of magnetite nanoparticles using gluconic acid and their application in immobilized lipase. Colloids Surf B Biointerfaces 93:24–28
10. Swarnalatha V, Aluri Esther R, Dhamodharan R (2013) Immobilization of α-amylase on gum acacia stabilized magnetite nanoparticles, an easily recoverable and reusable support. J Mol Catal B Enzym 96:6–13
11. Durdureanu-Angheluta A, Ignat ME, Maier SS et al (2014) Lipolytic biocatalyst based on recyclable magnetite-polysiloxane nanoparticles. Appl Surf Sci 292:898–905
12. Meng X, Xu G, Zhou Q-L et al (2014) Highly efficient solvent-free synthesis of 1,3-diacylglycerols by lipase immobilised on nanosized magnetite particles. Food Chem 143:319–324
13. Reis P, Holmberg K, Watzke H et al (2009) Lipases at interfaces: a review. Adv Colloid Interface Sci 147:237–250
14. Villalba M, Verdasco-Martín CM, dos Santos JCS et al (2016) Operational stabilities of different chemical derivatives of Novozym 435 in an alcoholysis reaction. Enzyme Microb Technol 90:35–44
15. Manoel EA, Ribeiro MFP, dos Santos JCS et al (2015) Accurel MP 1000 as a support for the immobilization of lipase from Burkholderia cepacia: application to the kinetic resolution of myo-inositol derivatives. Process Biochem 50:1557–1564
16. Lozano P, De Diego T, Carrie D et al (2003) Lipase catalysis in ionic liquids and supercritical carbon dioxide at 150 °C. Biotechnol Prog 19:380–382
17. Lozano P, De Diego T, Carrié D et al (2001) Over-stabilization of *Candida antarctica* lipase B by ionic liquids in ester synthesis. Biotechnol Lett 23:1529–1533
18. Nicolás P, Lassalle VL, Ferreira ML (2015) About the role of typical spacer/crosslinker on the design of efficient magnetic biocatalysts based on nanosized magnetite. J Mol Catal B Enzym 122:296–304
19. José C, Bonetto RD, Gambaro LA et al (2011) Investigation of the causes of deactivation–degradation of the commercial biocatalyst Novozym® 435 in ethanol and ethanol–aqueous media. J Mol Catal B Enzym 71:95–107
20. Nicolás P, Lassalle V, Ferreira ML (2014) Development of a magnetic biocatalyst useful for the synthesis of ethyloleate. Bioprocess Biosyst Eng 37:585–591
21. Gotor-Fernández V, Busto E, Gotor V (2006) Candida antarctica lipase B: an ideal biocatalyst for the preparation of nitrogenated organic compounds. Adv Synth Catal 348:797–812
22. Khan NR, Rathod VK (2015) Enzyme catalyzed synthesis of cosmetic esters and its intensification: a review. Process Biochem 50:1793–1806
23. Derewenda U, Brzozowski AM, Lawson DM, Derewenda ZS (1992) Catalysis at the interface: the anatomy of a conformational change in a triglyceride lipase. BioChemistry 31:1532–1541
24. Manoel EA, dos Santos JCS, Freire DMG et al (2015) Immobilization of lipases on hydrophobic supports involves the open form of the enzyme. Enzyme Microb Technol 71:53–57
25. Cao L (2006) Carrier-bound immobilized enzymes: principles, application and design, Wiley, Weinheim
26. Talbert JN, Wang LS, Duncan B et al (2014) Immobilization and stabilization of lipase (CaLB) through hierarchical interfacial assembly. Biomacromolecules 15:3915–3922
27. Ziegler-Borowska M, Siódmiak T, Chełminiak D et al (2014) Magnetic nanoparticles with surfaces modified with chitosan–poly[*N*-benzyl-2-(methacryloxy)-*N,N*-dimethylethanaminium bromide] for lipase immobilization. Appl Surf Sci 288:641–648
28. Kanimozhi S, Perinbam K (2013) Synthesis of amino-silane modified superparamagnetic Fe_3O_4 nanoparticles and its application in immobilization of lipase from *Pseudomonas fluorescens* Lp1. Mater Res Bull 48:1830–1836
29. Cui Y, Li Y, Yang Y et al (2010) Facile synthesis of amino-silane modified superparamagnetic Fe_3O_4 nanoparticles and application for lipase immobilization. J Biotechnol 150:171–174
30. Bezbradica DI, Mateo C, Guisan JM (2014) Novel support for enzyme immobilization prepared by chemical activation with cysteine and glutaraldehyde. J Mol Catal B Enzym 102:218–224
31. Zhong H, Fang Z, Zou B et al (2013) Studies on the lipase-catalyzed esterification of alkyl oleates in solvent-free systems. J Mol Catal B Enzym 90:114–117
32. Llerena-Suster CR, Briand LE, Morcelle SR (2014) Analytical characterization and purification of a commercial extract of enzymes: a case study. Colloids Surf B Biointerfaces 121:11–20
33. Nicolás P, Lassalle VL, Ferreira ML (2016) Quantification of immobilized *Candida antarctica* lipase B (CALB) using ICP-AES combined with Bradford Method. Enzyme Microb Technol 97:97–103
34. Azcona P, Zysler R, Lassalle V (2016) Simple and novel strategies to achieve shape and size control of magnetite nanoparticles intended for biomedical applications. Colloids Surf A Physicochem Eng Asp 504:320–330
35. Foresti ML, Ferreira ML (2005) Frequent analytical/experimental problems in lipase-mediated synthesis in solvent-free systems and how to avoid them. Anal Bioanal Chem 381:1408–1425
36. Foresti ML, Pedernera M, Bucalá V, Ferreira ML (2007) Multiple effects of water on solvent-free enzymatic esterifications. Enzyme Microb Technol 41:62–70
37. Metzler DE (2001) Biochemistry: the chemical reactions of living cells. Elsevier, Amsterdam
38. Nicolás P, Saleta M, Troiani H et al (2013) Preparation of iron oxide nanoparticles stabilized with biomolecules: experimental and mechanistic issues. Acta Biomater 9:4754–4762
39. Shu G, Chen H, Zhang Q, Dang Y (2013) The effect of glutaraldehyde cross-linking on the enzyme activity of immobilized β-galactosidase on chitosan bead. Adv J Food Sci Technol 5:932–935

40. Dinçer A, Becerik S, Aydemir T (2012) Immobilization of tyrosinase on chitosan–clay composite beads. Int J Biol Macromol 50:815–820
41. Liu K, Zhao G, He B et al (2012) Immobilization of pectinase and lipase on macroporous resin coated with chitosan for treatment of whitewater from papermaking. Bioresour Technol 123:616–619
42. Barbosa O, Torres R, Ortiz C, Fernandez-Lafuente R (2012) Versatility of glutaraldehyde to immobilize lipases: effect of the immobilization protocol on the properties of lipase B from *Candida antarctica*. Process Biochem 47:1220–1227
43. Monsan P (1978) Optimization of glutaraldehyde activation of a support for enzyme immobilization. J Mol Catal 3:371–384
44. López-Gallego F, Betancor L, Mateo C et al (2005) Enzyme stabilization by glutaraldehyde crosslinking of adsorbed proteins on aminated supports. J Biotechnol 119:70–75
45. Hua L, Sun Z-H, Zheng P, Xu Y (2004) Biocatalytic resolution of dl-pantolactone by glutaraldehyde cross-linked cells of Fusarium moniliforme CGMCC 0536. Enzyme Microb Technol 35:161–166
46. Zhang W-W, Yang X-L, Jia J-Q et al (2015) Surfactant-activated magnetic cross-linked enzyme aggregates (magnetic CLEAs) of Thermomyces lanuginosus lipase for biodiesel production. J Mol Catal B Enzym 115:83–89
47. Guauque Torres MP, Foresti ML, Ferreira ML (2013) Effect of different parameters on the hydrolytic activity of cross-linked enzyme aggregates (CLEAs) of lipase from Thermomyces lanuginosa. Biochem Eng J 72:18–23
48. Guauque Torres MP, Foresti ML, Ferreira ML (2014) CLEAs of *Candida antarctica* lipase B (CALB) with a bovine serum albumin (BSA) cofeeder core: Study of their catalytic activity. Biochem Eng J 90:36–43
49. Lage FAP, Bassi JJ, Corradini MCC et al (2016) Preparation of a biocatalyst via physical adsorption of lipase from Thermomyces lanuginosus on hydrophobic support to catalyze biolubricant synthesis by esterification reaction in a solvent-free system. Enzyme Microb Technol 84:56–67
50. Walt DR, Agayn VI (1994) The chemistry of enzyme and protein immobilization with glutaraldehyde. TrAC Trends Anal Chem 13:425–430
51. Migneault I, Dartiguenave C, Bertrand MJ, Waldron KC (2004) Glutaraldehyde: behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking. Biotechniques 37:790–802
52. Velu N, Divakar K, Nandhinidevi G, Gautam P (2012) Lipase from Aeromonas caviae AU04: isolation, purification and protein aggregation. Biocatal Agric Biotechnol 1:45–50
53. Betancor L, López-Gallego F, Hidalgo A et al (2006) Different mechanisms of protein immobilization on glutaraldehyde activated supports: effect of support activation and immobilization conditions. Enzyme Microb Technol 39:877–882
54. Fernández-Lorente G, Palomo JM, Mateo C et al (2006) Glutaraldehyde cross-linking of lipases adsorbed on aminated supports in the presence of detergents leads to improved performance. Biomacromolecules 7:2610–2615
55. Barbosa O, Ortiz C, Berenguer-Murcia Á et al (2015) Strategies for the one-step immobilization–purification of enzymes as industrial biocatalysts. Biotechnol Adv 33:435–456
56. Gunda NSK, Singh M, Norman L et al (2014) Optimization and characterization of biomolecule immobilization on silicon substrates using (3-aminopropyl)triethoxysilane (APTES) and glutaraldehyde linker. Appl Surf Sci 305:522–530
57. Silva JA, Macedo GP, Rodrigues DS et al (2012) Immobilization of Candida antarctica lipase B by covalent attachment on chitosan-based hydrogels using different support activation strategies. Biochem Eng J 60:16–24
58. dos Santos P, Rezende CA, Martinez J (2016) Activity of immobilized lipase from *Candida antarctica* (lipozyme 435) and its performance on the esterification of oleic acid in supercritical carbon dioxide. J Supercrit Fluids 107:170–178
59. Jiang Y, Zheng P, Zhou L et al (2016) Immobilization of lipase in hierarchically ordered macroporous/mesoporous silica with improved catalytic performance. J Mol Catal B Enzym 130:96–103
60. Ajmal M, Fieg G, Keil F (2016) Analysis of process intensification in enzyme catalyzed reactions using ultrasound. Chem Eng Process Process Intensif 110:106–113
61. dos Santos JCS, Bonazza HL, de Matos LJBL et al (2017) Immobilization of CALB on activated chitosan: application to enzymatic synthesis in supercritical and near-critical carbon dioxide. Biotechnol Reports 14:16–26
62. José C, Austic GB, Bonetto RD et al (2013) Investigation of the stability of Novozym® 435 in the production of biodiesel. Catal Today 213:73–80
63. Foresti ML, Ferreira ML (2007) Chitosan-immobilized lipases for the catalysis of fatty acid esterifications. Enzyme Microb Technol 40:769–777
64. Sun J, Jiang Y, Zhou L, Gao J (2010) Immobilization of *Candida antarctica* lipase B by adsorption in organic medium. N Biotechnol 27:53–58
65. Toledo MV, José C, Collins SE et al (2012) Esterification of R/S-ketoprofen with 2-propanol as reactant and solvent catalyzed by Novozym® 435 at selected conditions. J Mol Catal B Enzym 83:108–119
66. Séverac E, Galy O, Turon F et al (2011) Selection of CalB immobilization method to be used in continuous oil transesterification: analysis of the economical impact. Enzyme Microb Technol 48:61–70
67. Ngo TPN, Li A, Tiew KW, Li Z (2013) Efficient transformation of grease to biodiesel using highly active and easily recyclable magnetic nanobiocatalyst aggregates. Bioresour Technol 145:233–239
68. Mehrasbi MR, Mohammadi J, Peyda M, Mohammadi M (2017) Covalent immobilization of *Candida antarctica* lipase on core-shell magnetic nanoparticles for production of biodiesel from waste cooking oil. Renew Energy 101:593–602
69. Babaki M, Yousefi M, Habibi Z et al (2015) Preparation of highly reusable biocatalysts by immobilization of lipases on epoxy-functionalized silica for production of biodiesel from canola oil. Biochem Eng J 101:23–31
70. Fedosov SN, Brask J, Pedersen AK et al (2013) Kinetic model of biodiesel production using immobilized lipase *Candida antarctica* lipase B. J Mol Catal B Enzym 85–86:156–168
71. Ortega-Requena S, Bódalo-Santoyo A, Bastida-Rodríguez J et al (2014) Optimized enzymatic synthesis of the food additive polyglycerol polyricinoleate (PGPR) using Novozym® 435 in a solvent free system. Biochem Eng J 84:91–97
72. Ćorović M, Milivojević A, Carević M et al (2017) Batch and semicontinuous production of l-ascorbyl oleate catalyzed by CALB immobilized onto Purolite® MN102. Chem Eng Res Des 126:161–171
73. Ravelo M, Fuente E, Blanco Á et al (2015) Esterification of glycerol and ibuprofen in solventless media catalyzed by free CALB: kinetic modelling. Biochem Eng J 101:228–236