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SHORT COMMUNICATION

Development of a magnetic biocatalyst useful for the synthesis of ethyloleate

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Abstract *Candida antarctica* Lipase B was successfully immobilized on magnetite (Fe_3O_4) nanoparticles functionalized with chitosan and glutaraldehyde. The obtained magnetic catalyst was characterized and its performance was evaluated in solvent-free synthesis of ethyl oleate at room temperature. The performance of this biocatalyst was compared with the commercial Novozym 435, as a tool to estimate the efficiency of immobilization. It was found that using 33 mg of the biocatalyst it was possible to reach almost the same activity that was obtained using 12 mg of Novozym 435. Furthermore, this new biocatalyst presents the advantages of not being degraded by short alcohols, being easily recovered from the reaction media by magnetic decantation, and low fabrication cost. The possibility of reutilization was also studied, keeping a significant activity up to eight cycles. A special sampling protocol was also developed for the multiphasic reaction system, to assure accurate results. This novel biocatalyst is an interesting alternative for potential industrial applications, considering the above-mentioned advantages.

Keywords Magnetic nanoparticles · Chitosan · Glutaraldehyde · Oleic acid · *Candida antarctica* lipase B

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Introduction

Although lipases were designed by nature to cleave ester bonds with the concomitant consumption of water molecules (hydrolysis), they also catalyze the reverse reaction under micro-aqueous conditions [1] producing ester bonds between alcohols and carboxylic acids. Therefore, they are being extensively studied as potential biocatalysts for the fabrication of several products such as optically active drugs, food, cosmetic additives and biodiesel, besides their application in the treatment of industrial effluents among many other uses [2].

A major drawback using enzymatic biocatalysts is that it is virtually impossible to recover enzymes in their free form from the reaction medium. The need of easy recovering is related to the purity of products and also regarding to the "recycling" of the catalyst. Aiming to overcome this situation, immobilization has arisen as a relatively useful solution. Attaching the enzymes on a solid support, it is possible to separate them from reaction media by filtration or centrifugation/decantation procedures. In addition, this technique improves the resistance of many studied enzymes to aggressive environments [3]. As typical enzyme supports, large porous particles (100–200 μm) with high specific surface areas (which allow high enzyme loadings) are preferred. Polymeric matrix, ceramics, porous glass, etc. could be chosen to these purposes. The particles are packed in columns (or added batch wise to a reaction mixture) and recovered by centrifugation. Such large porous particles suffer from diffusion limitations [4] for the substrates and/or the products during the course of the enzymatic reactions. Therefore, their separation from the reaction media is difficult, and even worse if the reaction solution is dense or viscous. Moreover, fouling of the matrix pores presents a difficult task when reactions in

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suspensions are performed or, in fact, solvent-free systems are used. In this context, iron oxides, in particular magnetite Fe_3O_4 (MAG), are versatile materials for immobilization purposes because of its magnetic properties. These properties allow the separation of the biocatalyst from the reaction media by simple application of an external magnetic field [5, 6]. Even more, magnetic supports in the nano size present an extra advantage associated to its large surface-area-to-volume ratio, an aspect that improves its efficiency as carriers of biomolecules [7]. This feature has resulted in the development of many biomolecule-nanoparticle hybrids such as biocatalysts [8–11]. For instance, Kuo et al. [12] have used Fe_3O_4–chitosan nanoparticles for the covalent immobilization of lipase from *Candida rugosa* using N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS) as coupling agents. They have studied the optimal immobilization conditions and explored the factors affecting the immobilized lipase activity. They found that after 20 repeated uses, the biocatalyst retains over 83 % of its original activity. The immobilized lipase shows better operational stability, including wider thermal and pH application ranges, and remains stable after 13 days of storage at 25 °C.

Huang et al. [13] have covalently bounded lipase from *Candida Rugosa* onto Fe_3O_4 nanoparticles (12.7 nm) via carbodiimide activation. They found that the binding efficiency of lipase was about 100 % using weight ratios of lipase/Fe_3O_4 nanoparticles below 0.033. The lipase exhibited a 1.41fold enhanced activity, a 31fold improved stability, and better tolerance to the variation of pH than the free enzyme.

The main goal of this research is to design a novel biocatalyst using an unexplored support-lipase system from low-cost raw materials, stable, easy to remove from the reaction media, reusable, effective to the synthesis of ethyl oleate, and that could compete with commercial higher costs catalysts. To do this, nanoparticles of magnetite modified with chitosan have been employed as support, while *Candida Antarctica* lipase B has been chosen as enzyme. In a previous work, the synthesis and exhaustive characterization of MAG nanoparticles modified with chitosan (CS) and oleic acid (OA) have been developed [14].

Experimental

Materials

Oleic acid (OA) was from Anedra. *Candida Antarctica* lipase B (CALB L, batch LCN02103) and Novozym 435 were kindly donated by Novo Nordisk (Brasil). Glutaraldehyde (GLUT) was provided by Fluka as a 25 % w/w solution. CS (Chito Clear) was from Primex (Iceland). Analytical grade solvents purchased from Dorwill (Argentina, SA) were used in all the described procedures.

Methods

The first steps of the nanoparticles fabrication were carried out using co-precipitation and nano precipitation methods. These procedures have been reported in detail in our previous work [14]. The material used as support was MAG obtained in presence of OA and then coated with CS (MAG/CS w/w = 2/1).

Treatment of the support with GLUT

50.4 mg of the support was dispersed in 4 mL of water and 105 mg of GLUT was added (0.7 mL from a 150 mg/mL solution). The dispersion was stirred for 3 h at 45 °C. The supernatant was withdrawn and the solid was washed with distilled water and dried. The recovered solid was about 45.3 mg. The treatment with GLUT has been performed to avoid the segregation of CS moieties from the magnetic nanoparticles.

Immobilization procedure

A mass of support ranging from 50 to 100 mg was dispersed in an aqueous solution containing 10.3 mg CALB per mL. The mixture was magnetically stirred (800–900 rpm) at room temperature during 7 h, maintaining the mass ratio solid/lipase = 1/1.75. An excess of enzyme was employed to assure the catalyst activity. The solid was allowed to settle down. The supernatant was withdrawn after decantation of the biocatalyst. The obtained biocatalyst was washed three times with distilled water and air-dried at 37–38 °C.

Solvent-free synthesis of ethyl oleate

The reactants were added one by one in a 10 mL glass flask containing a stirring bar: 1.1 mL of a carboxylic acids mixture, 0.2 mL of distilled water, variable amounts of biocatalyst and 0.15 mL of absolute ethanol. Each weight was precisely registered. The vial was hermetically sealed with Teflon tape to avoid evaporation and the reaction was carried out for 3 h at 24 °C, with stirring at 800–900 rpm.

i-Sampling of the reaction catalyzed by Novozym 435® The Novozym 435® activity was tested in a wide range of catalyst/subtracts ratios, at room temperature (24 °C), following the procedure described in "*Solvent-free synthesis of ethyloleate*". To quantify the amount of carboxylic acid left in the reaction vial, a known mass of sample was withdrawn and titrated with KOH in the presence of

phenolphthalein; using a sampling method optimized in a previous work [15].

The sample can be assumed to be representative of the entire emulsion inside the flask, including the catalyst. The procedure is illustrated in Scheme 1.

ii-Sampling of the reaction catalyzed by the designed biocatalyst Different sampling strategies were employed and compared aiming to avoid mistakes in the reproducibility of the results

Method 1 Without adding any solvent. Sampling was repeated as described in above section.

Method 2 Adding heptane as solvent. At the end of the reaction, ~5 mL of heptane was added through the Teflon septum using a syringe, the flask was weighted and the sample was extracted with fast stirring, withdrawing biocatalyst as well.

Method 3 Adding ethanol as solvent. At the end of the reaction, ~4 mL of ethanol was added through the Teflon septum using a syringe, the flask was weighted, the whole mixture was stirred for a few seconds to homogenize, and the sample was extracted with fast stirring (with sampling of catalyst as well); or after total decantation of the catalyst on the stirring bar, without stirring.

Determination of conversion percentage (X)

The conversion percentage was defined as follows:

$$\text{Conversion percentage (X)} = 100 - \frac{\text{Titrated acid after reaction}}{\text{Initial amount of acid}} \times 100$$

To determine the initial amount of acids in the reactant, two different titrations were done:

A known mass of OA was dissolved in 10 mL of Ether/Ethanol 1/1, and titrated against KOH in ethanol. As a result, it was confirmed that 96.3 % (w/w) of the OA's weight was carboxylic acids.

The reaction was performed following the procedure described in the previous section but without any catalyst. After 3 h, two samples were taken and it was found that 2.7 % of the initial OA was consumed. Hence, only 93.4 % of the OA is assumed to be reactive acid with potential conversion assignable to the biocatalyst when it is used.

Characterization

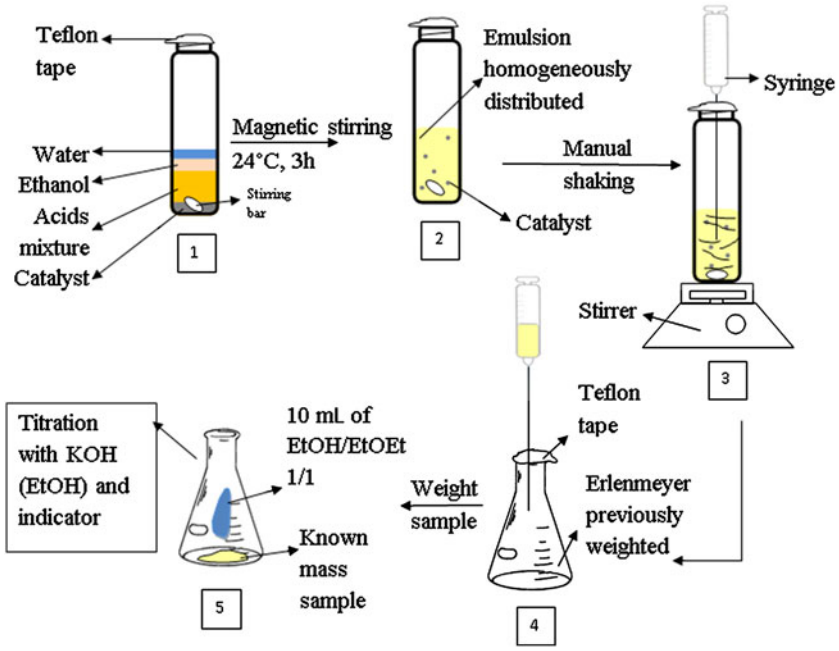
Particle hydrodynamic diameters were determined by Dynamic Light Scattering (DLS) at 25 °C using a Malvern Zeta sizer as well as Zeta potential (ζ). FTIR-DRIFTS spectroscopy analysis was conducted using a Thermo Scientific Nicolet 6700 spectrometer and UV–Visible with a Shimadzu 160 (Japan). Particle morphology was determined by transmission electron microscopy (TEM, JEOL100 CXII, JEOL, TOKIO, Japan, 1983 from CCT, Bahía Blanca, Argentina).

Results and discussion

Preparation and characterization of biocatalyst

Characterization of magnetic support and biocatalyst

Synthesis and characterization of magnetic nanoparticles used as precursors of this support have been reported in our



Scheme 1 Sampling procedure for the Solvent-free synthesis of ethyloleate

previous publication [14]. As CS-coated NPs tend to disperse easily in aqueous phase, their isolation from the immobilization media (and eventually from the reaction media) could be hindered. Although GLUT is a typical crosslinking reagent used for surface activation in enzyme immobilization [16], the purpose of this treatment was not to activate the support surface, but to avoid mass loss (mainly CS) and enhance decantation on the support [17].

The GLUT was incorporated on nanoparticles containing magnetite and also chitosan, as illustrated in Fig. 1. To determine the final content of GLUT in the support, the supernatant and washing solutions were analyzed by UV–Visible spectroscopy. Even though the band at 280 nm characteristic of GLUT appeared on the spectrum, it overlapped with a band of soluble chitosan of low molecular weight. This was a clear hint that some polymeric moieties remained dissolved from the particles. The absorbance value at the maximum was actually the sum of both dissolved CS and GLUT. Then it was not possible to detect the CS contribution (that even may be partially cross-linked and difficult to quantify) to make calculations. Regardless of this problem, the incorporation of the crosslinking agent was confirmed by FTIR and ζ_{pot} data (both shown later).

Comparative data of ζ_{pot} and hydrodynamic size of magnetic support, free CALB and biocatalyst are shown in Table 1. Analyzing ζ_{pot} values it is suggested that positive groups in the commercial lipase preparation (NH_3^+) interact with negative charges on the support surface. In addition, protein aggregates encapsulate the particles exposing different surface charge as a function of the level of aggregation and the lipase conformation, leading to the observed less negative net ζ_{pot}. The size of the biocatalyst, in terms of hydrodynamic diameter, does not change significantly after various uses.

In Fig. 2, FTIR-DRIFTS spectra of support (with and without Glut), and biocatalyst are compared. Bands attributed to MAG, GLUT and CALB are pointed out confirming the incorporation of each component to the biocatalyst. A contribution of the functional groups of the support should be considered. Nearly at 3,200–3,400 cm^{-1} signal associated to NH groups and OH groups of chitosan (from the magnetic support) and OH of the coupling agent (GLUT) overlap with the NH groups from the lipase. A similar situation could be appreciated in the C = O absorption region (between 1,720 and 1,590 cm^{-1}). In spite of this the incorporation of the enzyme could be verified by the increase in the intensity of such bands in NH and C = O regions in the spectrum c of the Fig. 2 assigned to the biocatalyst one.

The differences in morphology between the support and the catalyst are seen by electronic microscopy (TEM) as it is depicted in Fig. 3. The incorporation of CALB under the studied conditions led to a significant increase in the level of aggregation. Since the initial concentration of precipitable material (most of it proteins) in the immobilization media was very high (10.3 mg/mL, see sect "Immobilization procedure"), intermolecular interactions led to aggregates of variable sizes that resulted in attaching to the support's particles [18].

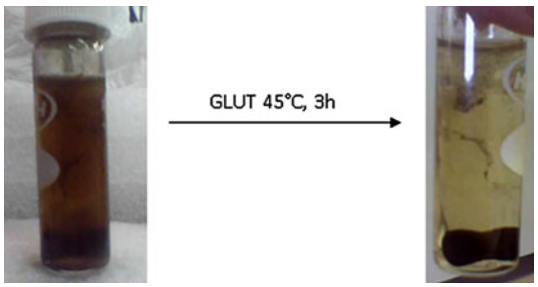


Fig. 1 Dispersion of magnetic nanoparticles used as support before and after the incorporation of GLUT

Determination of the immobilization efficiency

i-Using UV–visible based methods To determine the loading efficiency, UV–visible spectroscopy was used. It was intended to estimate the amount of CALB in the supernatant solution from the immobilization, and in the water from the washing solutions. For this purpose, a calibration curve was built using a free lipase solution. The spectrum of the lipase in contact with magnetite was different from the one corresponding to free enzyme. Based

Table 1 Comparative data of ζ (mV) and hydrodynamic diameter (Dh) of support, catalyst and free lipase in distilled water

	Dispersion concentration (mg/mL)	pH	ζ (mV)	Dh (nm)
Support	0.12	5	−23.4	614.5 (100 %)
Fresh biocatalyst	0.1	5.8	−7.55	637.7 (100 %)
Used biocatalyst after one cycle	0.14	5.8	−9.26	943.1 (90.5 %)
				5393 (9.5 %)
Used biocatalyst after eight cycles	0.1	5.8	0.293	782.8 (100 %)
Used biocatalyst free CALB	10.3	5.8	−2.18	Not measured

on registered absorbance results, it was found that roughly 90 % of the catalyst net weight was CALB. This huge error arises from the assumption that the molecular structure of lipase remained invariable after being in contact with the support. However, it is known that interactions with lipase-support induce conformational changes in lipases, modifying its molar absorptivity. Of course, any kind of methodology using a reactive substrate (Bradford, Lowry, Bicinchoninic acid) will have these and other mistakes [19]. Therefore, UV/visible based methods demonstrated to be unsuitable to these purposes and an estimation of active enzyme content was made by comparison between the prepared biocatalyst and a commercial one such as Novozym 435.

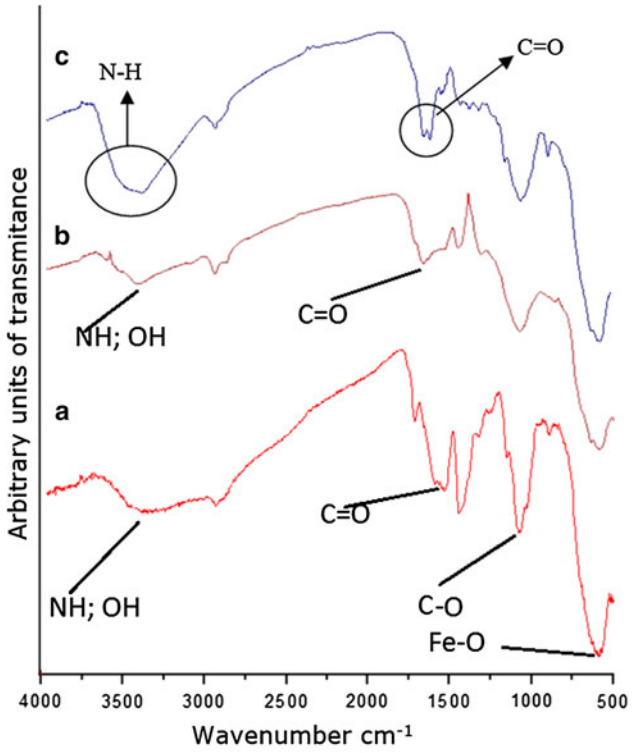


Fig. 2 FTIR spectra of (**a**) Magnetic nanoparticles, (**b**) Magnetic nanoparticles treated with glutaraldheyde, (**c**) Biocatalyst

ii-Estimation of enzyme loading using Novozym®435 The aim was to roughly calculate the enzyme loading by comparison with the conversions achieved by Novozym®435 and the magnetic biocatalyst (with the proper detection of the linear ranges conversion versus biocatalyst mass). The results corresponding to a range of OA/Novozym mass ratios from 1,000 to 22 are presented in Fig. 4.

The commercial catalyst contains 5.5 wt % CALB supported on a granulated resin (poly-methylmethacrylate) with high activity against a broad range of fatty acids. Taking into account that 33 mg of magnetic biocatalyst reaches the same conversion than 12 mg of Novozym®, it can be estimated that 1 mg of the prepared biocatalyst contains 0.02 mg of active CALB as present in Novozym®. These results are a very interesting first step in view of the further optimization of the biocatalyst preparation. Even more, Novozym® has been reported to be adversely affected by short alcohols, due to unexpected partial dissolution of the polymeric support [20]. This is an additional advantage of the magnetic biocatalyst designed within this work.

Performance of MAGCAT catalyst in test reactions: initial catalytic activity

In Table 2 conversion percentages achieved by different amounts of fresh magnetic biocatalyst in the solvent-free

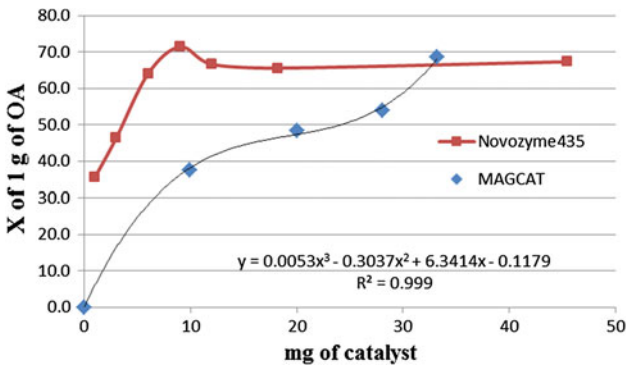


Fig. 4 Conversion (%) of Oleic acid versus mass of catalyst

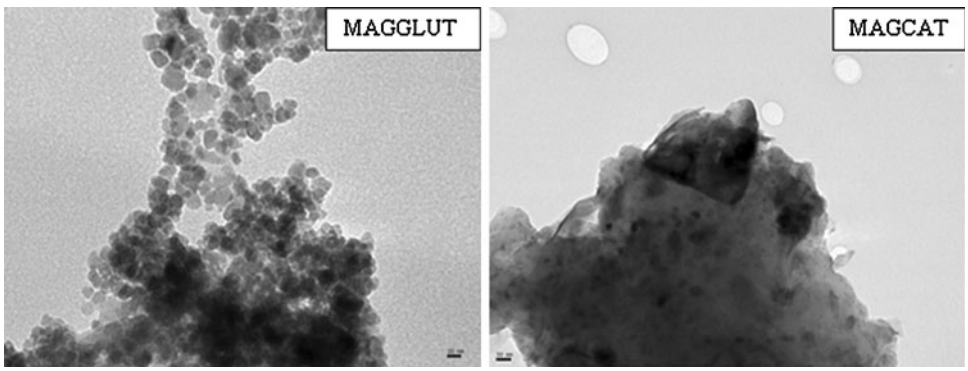


Fig. 3 TEM images of magnetic NPs and biocatalyst aggregates. The *scale bars* correspond to 10 and 50 nm respectively

synthesis of ethyl oleate are informed. Although the increasing tendency of X versus mass of catalyst is evident, another parameter that characterizes a catalyst and allows an easier comparison was considered. This parameter was the mass of converted substrate per mass unit of biocatalyst; then mg of OA converted per mg of catalyst was reported. Not surprisingly, the variation of this parameter with the mass of catalyst is quite opposite to X. Observe that 1 mg of the biocatalyst in a total mass of 9.9 mg converted 36.6 mg of OA, whereas 1 mg of biocatalyst in a total mass of 33.2 mg only converted 20.5 mg of substrate. The fact that this catalyst works better at low catalyst/substrate ratios arises from the agglomeration promoted by the particles' own magnetism, leading to bigger clusters as concentration increases. Larger aggregates related to high concentration- expose less active enzyme towards the reaction media and consequently, fewer enzymes moieties are able to be active.

Table 2 Conversion (X in %) and specific activity of the prepared biocatalyst during the synthesis of ethyl oleate as a function of the mass of biocatalyst

Mass of biocatalyst (mg)	X (%)	Specific activity[a]
33.2	68.5	20.5
28.1	53.9	17.7
20.0	48.3	22.9
9.9	37.5	36.6

[a] The value corresponds to mg of converted OA per total mg of used biocatalyst

Reuse of MAGCAT

In Fig. 5 the residual relative activity (RRA) for successive cycles is plotted. It is important to highlight that two different washing conditions were employed, i.e.: (a)-Using heptane and washing for 3 min; and then using ethanol and washing for 3 extra minutes (short washing step), (b)-Using heptane and washing for 60 min and then using ethanol and washing for 5 extra minutes (long washing step). Both methods were carried out by magnetic stirring in the same reaction flask after removing the remaining reactants from ethyl oleate synthesis. This operation is very simple because the biocatalyst decants on the stirring bar, and no filtration steps are needed.

The increase in the RRA of the short-washed biocatalyst is due to the minor amount of biocatalyst left in the reaction vial after withdrawing the sample for titration. As explained in previous paragraphs, a low catalyst/substrate ratio favors enzymatic activity. The registered fall in activity was caused by the long washing steps. This observation supports the conclusion that inhibition by product or substrate is not a cause for biocatalyst deactivation. Desorption of enzyme is then a much more likely explanation for deactivation, especially after the seventh use with mild washing step and by third use with exhaustive washing step (see Fig. 5).

Although magnetic biocatalysts capable of being reusable for up to 12 or even 30 cycles are reported in the literature, it is important to highlight that in those cases the activation of the support was achieved using different agents such as glutaraldehyde [21, 22].

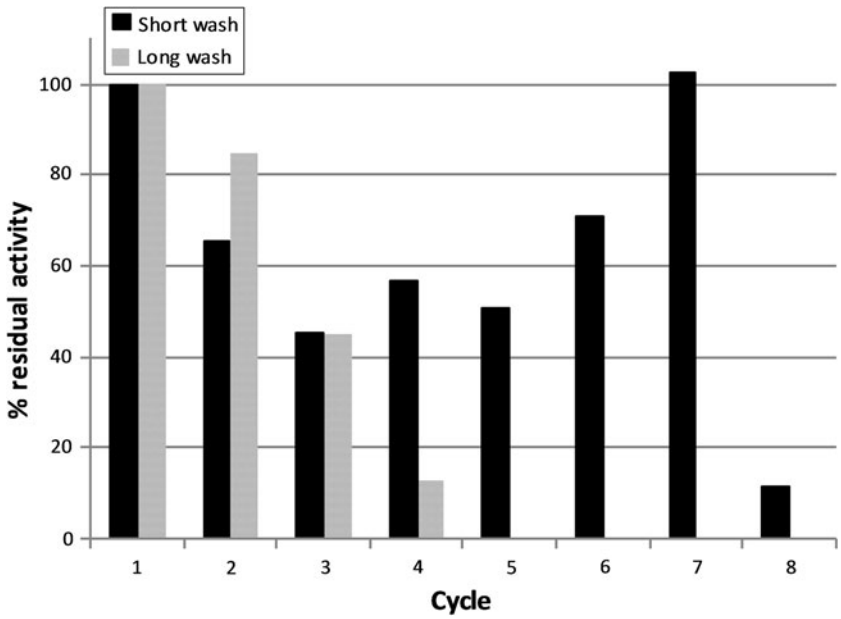


Fig. 5 Residual relative activity of the biocatalyst after *long* or *short* washes

Conclusions

A novel magnetic biocatalyst was designed by simple physical adsorption of CALB on magnetic nanoparticles. Accurate and reproducible methods to estimate the efficiency of immobilization have been proposed. It was demonstrated that this biocatalyst tolerates 4 to 8 cycles depending on the washing conditions. It is necessary to explore lower CALB/support ratios to minimize aggregation, and to test the immobilization employing coupling agents like γ aminopropyl-triethoxysilane or glutaraldheyde.

This investigation demonstrates that modified magnetite nanoparticles are a promising alternative to other supports for lipase immobilization, not only for its proven activity but also its low fabrication cost and easiness of separation, especially for solvent-free systems.

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