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About the role of typical spacer/crosslinker on the design of efficient magnetic biocatalysts based on nanosized magnetite

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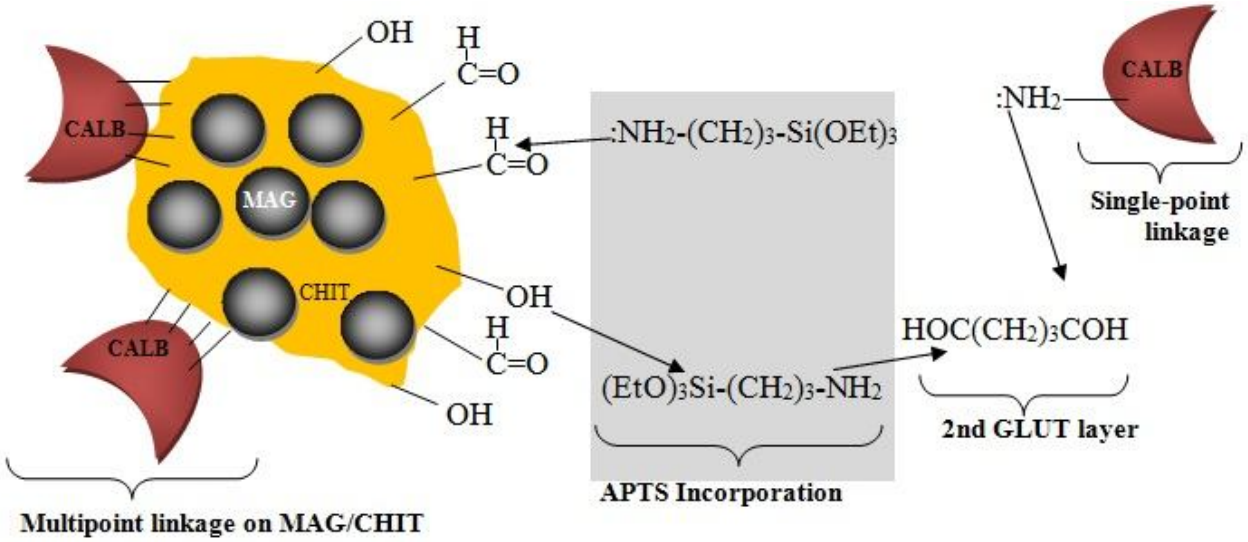
Graphical abstract



Highlights

- Magnetic biocatalysts based on magnetite nanoparticles coated with chitosan and CALB as enzyme.
- Role of glutaraldheyde and 3-aminopropyl-triethoxisilane on efficiency of biocatalyst.
- Interactions between GLUT and/or APTS and magnetic support and/or lipase.
- Practical implementation of biocatalysts: evaluation of thermal and storage stability, reusability and economical feasibility.

Abstract

The immobilization of *Candida antarctica* lipase B (CALB) was carried out using glutaraldehyde (GLUT) and/or 3-aminopropyl-triethoxisilane (APTS). The aim of this work was to elucidate the role of these crosslinkers/functionalizers on the efficiency of the prepared nanosized catalysts in solvent-free oleic acid esterification.

A series of biocatalysts were prepared in presence or absence of GLUT and APTS. The impact of the amount of initial CALB was also explored. An experimental design was utilized to study the variables that maximize biocatalyst activity.

A strong dependence of enzymatic activity with the nominal amount of GLUT as well as the final protein/CALB loading was found. Nominal quantity of APTS did not affect catalyst´s activity when used in combination with GLUT. Additional studies demonstrated that stability during storage was mainly dependent on the enzyme loading. The optimum biocatalyst was reused 6 cycles without mass loss. Biocatalyst´s performance decreased with reuse. Mechanisms justifying these results were proposed.

The role of GLUT and APTS on stability during storage and on differences between initial enzymatic activity and the performance in the reaction after two months was discussed. The problem of mixed interaction of CALB (covalent bonding plus simple adsorption) was carefully addressed to explain leaching of the lipase

Leaching and stability on storage should be included in the analysis of modifiers impact when support´s modifiers are used. The fresh and stored biocatalyst enzymatic activity has to be addressed looking at the practical aspects of implementation in technological settings.

Keywords enzyme immobilization, CALB, magnetic support, biocatalyst, glutaraldehyde, mechanism.

1.Introduction

Several unquestionable advantages may be recognized when using enzymes as biocatalysts. However, a number of problems related to their practical implementation have been detected. To these belong: the high cost of isolation and purification of enzymes, the instability of their structures once they are isolated from their natural environments, and their sensitivity both to process conditions other than the optimal ones, normally narrow-ranged, and to trace levels of substances that can act as inhibitors. The latter two result in enzymes' short operational lifetimes. Also, unlike conventional heterogeneous chemical catalysts, most enzymes operate dissolved in water in homogeneous catalysis systems, which is why they contaminate the product and as a rule cannot be recovered in the active form from reaction mixtures for reuse.In this context the immobilization appears as a feasible solution [1].

Organic polymers like Nylon, agarose, or polymethacrylate; and inorganic compounds such as carbon, gold particles, or titanium oxide have been used as enzyme supports [2-7]. All these materials required filtration or centrifugation to recover and wash the biocatalyst after its utilization. If a magnetic support is used, the final catalyst can be easily isolated by magnetic separation [8-11] .Several biocatalysts with magnetic properties have been prepared including magnetite (Fe_3O_4-MAG) and one or several crosslinkers/adittives [12-15].

Ma *et. al*. have prepared aminosilane coated magnetite nanoparticles by coprecipitation. The coating could significantly improve their ability to protein immobilization [16].

Magnetic Fe_3O_4–chitosan nanoparticles were used by Kuo *et al.* to immobilize lipase using the 1-ethyl 3 (3-dimethylaminopropyl)carbodiimide (EDC) method. They observed that after 5, 10 and 20 repeated uses, the immobilized

lipase retained about 98, 88 and 83% of its original activity, respectively. Besides, the immobilized lipase retained 76%of its activity after incubation at 60°C for 1h while free lipase showed 21% of activity [17].

When the support is aminated, glutaraldehyde (GLUT) is commonly used. Betancor and col. concluded that the activity/stability properties of enzymes immobilized on glutaraldehyde-activated supports depended on the immobilization protocol. The GLUT monomer could give high rigidity whereas GLUT dimmer may allow the reaction with more groups and yield the best results [18].

In a previous work, a magnetic biocatalyst was prepared using magnetite nanoparticles stabilized with oleic acid and chitosan (MAG/CHIT) as support [8, 19]. The simple physical adsorption of the lipase on the nanosupport was achieved. In the present contribution an extended investigation was carried out to optimize CALB immobilization and to obtain an efficient biocatalyst. A spacer (3- aminopropyl triethoxysilane or APTS) and a crosslinker (GLUT) were employed in different proportions and combinations. The role of these additives in stability, leaching and activity after storage have been less explored and /or reported in open literature.

Our motivation was to prepare an efficient biocatalyst with wide implementation chances. In this case, the efficiency was defined in terms of enzymatic activity in the solventless synthesis of ethyl oleate. The main goal was to increase thermal stability, storage resistance and reuse perspectives by using a simple, versatile and low cost protocol. To reach this goal the understanding of mechanistic aspects on immobilization is strongly required.

A systematic study was developed varying the concentration of each additive as well as the lipase´s concentration. The best catalysts, in terms of their

activity in first use were selected to assess thermal stability, stability on storage and reuse capability. Economical aspects were analyzed with the aim to compare the real potential of these biocatalysts with the commercially available ones. To the best of these authors knowledge, the system MAG/CHIT/APTS/GLUT/ CALB and the analysis of the impact of magnetic modified support in the biocatalyst stability after storage have not been reported before in the open literature.

2. Methods

2.1. Materials

APTS was from Avocado Research Chemicals (United Kindom), GLUT was from Fluka (25 % p(p). Free CALB broth (batch LCN02103) was kindly donated by Novozymes (Brazil). Oleic acid (OA, 96.3% carboxylic acids purity) was from Anedra. Analytical grade solvents from Dorwill (Argentina) were used in all the described procedures.The crude lipase solution used in this research contained 300 mg/mL of precipitable proteins (PP, obtained with $(NH_4)_2SO_4$), but only near 17.7 mg/mL are CALB. This information was obtained using an ICP study that quantified sulfur. This technique (as an alternative or complementary one to Bradford´s method) has not yet been reported as a tool to quantify proteins.[ref d econgresos]

2.2. Experimental

The synthesis of the MAG/CHIT nanoparticles (MCNPs) has been reported in previous works [8, 19]. The variants of this methodology are detailed as follows:

2.2.1. Coating of MCNPs with 3-APTS

400 mg of MCNPs support (SUP) was magnetically stirred in 4 mL of ethanol containing 0, 200 or 400 mg of 3-APTS, for 24 hours at room temperature. The supernatant was withdrawn and the solid was washed three times with 3 mL of fresh ethanol to remove unbound silane. The product was dried under vacuum at 40 °C. These formulations will be called SUP_A.

2.2.2. Activation of MCNPs with GLUT

300 mg of 3-APTS-modified particles were magnetically stirred in 5 mL of distilled water containing 0, 100 or 200 mg of GLUT, for 3 h at 45 °C. The supernatant was withdrawn and the solid was washed three times with distilled water to remove unbound GLUT. The solid product was dried under vacuum at 40 °C. These formulations will be called SUP_{AG}.

2.2.3. Immobilization of lipase

25 mL of CALB aqueous solution containing 50, 100 or 150 mg of PP (i.e. 3; 6 or 9 mg CALB) were magnetically stirred for 30 minutes at room temperature to induce enzyme disaggregation. Then, 25 mL of an aqueous dispersion containing 200 mg of SUP_{AG} particles were added after a sonication treatment of about 30 min. The mixture was magnetically stirred during 7h at room temperature. After magnetic decantation, the supernatant was withdrawn and the solid was washed three times with distilled water to remove residual enzyme. The product was dried under vacuum at 37 °C. These formulations will be called CAT. Thirteen CATs were prepared, following the pretreatments and conditions shown in Table 1. The CATs with higher activity were stored at 4°C and used after 2 months for the first time to evaluate storage stability. CAT1 to CAT10 were the ones of the experimental design carried out in this work. CAT0 was the biocatalyst prepared and reported

elsewhere [8]. CAT11 and CAT12 were prepared using a nominal GLUT excess.

2.2.4. Lipase quantification

Enzyme loading was obtained measuring protein in the supernatant and in washing residues after immobilization. Protein concentration in CALB solution was determined by measuring the sulfur content through high resolution Atomic Emission-ICP in combination with a modified Bradford method. The amount of lipase was calculated considering 12 sulfur-containing aminoacids per CALB and the CALB molecular weight (33 kDa) [20]. The results were reproducible.

2.2.5. Solvent-free ethyloleate synthesis

Briefly, 1 g of oleic acid (OA), 200 mg distilled water and 150 µL ethanol was magnetically stirred along with 30 mg of CAT at 24 °C for 3 h [8]

2.2.6. Experimental design and data analysis

Statgraphics Centurion XV.II software (StatPoint Inc.) was used to design and analyze the experiments. The design was a factorial 2^3 with two central points. Independent variables and responses are presented in Table 1.

2.2.7. Thermal and storage stability tests

Esterification reactions were performed using 1 g OA, 150 µL ethanol and 200 µL water using 30, 45, 60 and 80 mg of CAT 7 at 24 and 45 °C.

The CATs with higher activity were stored at 4°C and used after 2.The storage stability was expressed as %of Retained activity (%R) defined as:

$$\%R = \%X_t \, x100 \, / \, \%X_i$$

where $\%X_i$ is the initial conversion and $\%X_t$ is the conversion measured after two months.

2.2.8. Reuse methodology

The reuse capability was explored using 60 mg of CAT. Purification of the catalyst was performed after each cycle of reaction (solventless ethyl oleate synthesis). The washing step was carried out with a heptane-absolute ethanol mixture during five minutes. This mixture was then exposed to a Nd magnet. Finally, the solid was dried in an oven at 37°C overnight. This sequence (esterification reaction, washing and drying) was repeated until no conversion -or at least minimum one- was registered.

2.2.9. Characterization techniques

2.2.9.1. FTIR-DRIFTS spectroscopy

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}. Samples (10–20 mg) were mixed with near 50 mg of KBr and the DRIFTS measurements were done as usual.

2.2.9.2. Electron microscopy

Scanning electron microscopy with energy-dispersive X-rays (SEM-EDX, JEOL 35-CF), and transmission electron microscopy (TEM, JEOL 100 CX II, Tokyo, Japan) The TEM samples were dispersed in ethanol, placed on 200 mesh Cu grids and dried at room temperature. The samples analyzed by SEM-EDX were used as powder and metallized with Carbon.

2.2.9.3. Atomic emission spectroscopy

High-resolution inductively coupled plasma atomic emission spectroscopy (ICP-AES, Shimadzu 9000) was used to determine the composition of SUP_{AG} and CAT in terms of total Fe and Si content. A known mass was dispersed in 10 ml of 15% HCl and sonicated at 40-45 °C. For S determination, the

immobilization supernatants and washing residues were analyzed without pretreatment.

2.2.9.4 Hydrodynamic diameter and Z potential measurements

Particle hydrodynamic diameters were determined by Dynamic Light Scattering (DLS) at 25 °C using a Malvern Zeta Sizer. Aqueous dispersions containing 0,1 mg.of particles per mL were measured using disposable polystyrene cells. Zeta potential was measured through Microelectrophoresis Laser Doppler.

3. Results and discussion

3.1. Characterization of biocatalysts

The regions around 3200-3400 cm^{-1} associated to the stretching of NH (from amide groups) and OH (from ROH of COOH groups) were analyzed using DRIFTS. Signals associated to NH and OH groups of chitosan and OH of the coupling agent (GLUT if present) overlap with the NH bands from the lipase. A shift of this band was observed. A similar situation was appreciated in the C=O absorption region (between 1720-1590 cm^{-1}). The incorporation of CALB was verified by the increase of intensity of bands in NH and C=O regions in the spectra of biocatalysts (Figure 1).

Morphology of magnetic supports was not affected by the lipase incorporation (see Figure 2). Aggregates of variable sizes were found when CATs were examined by TEM.

The support without APTS or GLUT showed hydrodynamic diameters (HD) of 614,5 nm. Whereas HD recorded for CATs were in the range between 500 nm- 1.4 µm (see Figure 3). An increment of the size was evident when only GLUT was employed. The presence of APTS contributed to nanoparticles

stabilization. The concentration of CALB did not increase the HD of biocatalysts.

The magnetic properties of support were measured in terms of magnetization (emu/g) as a function of applied magnetic field (Oe).Satisfactory levels of saturation magnetization was found. These data were included in a previous work devoted to the preparation and characterization of magnetite/chitosan nanoparticles [19].

3.2. Influence of the APTS, GLUT and concentration of CALB on the efficiency of the biocatalyst

3.3. Effect of the incorporation of GLUT

The incorporation of GLUT was carried out: 1-to crosslink the chitosan on the nano-support and 2-to induce covalent interactions support-lipase. In our preliminary work a conversion of oleic acid to ethyl oleate of about 64% was achieved [8]. Conversion varied from 39 to 3 % for CATs 1 to CAT10.

A combined mechanism seems the best explanation to these data. That means enzyme adsorption plus covalent linkage enzyme-support. Physical interactions among the exposed groups of GLUT-CHIT-MAG and the lipase; plus covalent bonds between GLUT with lipase´s lysine groups were the most probable interactions. N-N distances in chitosan surface (NH_2-NH_2) vary from 4.5 to near 7Å and match with the structure of glutaraldehyde. GLUT decreased the surface concentration of NH_2/NH_3^+ groups increasing the rigidity of CALB after covalent linkage [21].The Figure 4 depicts the feasible interactions between CALB and GLUT and CHIT-MAG surfaces.

Bentacor *et. al*. found that when glutaraldehyde was used to activate an aminated support ; remaining amino groups [18] can give some ionic-exchanger features to such support. Then, these supports could be considered as heterofunctional matrices [22-24].

Further GLUT may react with the available formerly added GLUT to produce higher local concentration of carbonyl groups (See figure 4).

This procedure yielded a biocatalyst with a conversion of ethyl oleate of near 34% (nominal 4.5 mg CALB per 100 mg support and 1.5% of immobilized protein-CAT4, see Table 1). We found 58% conversion of ethyl oleate, with nominal 10.4 mg CALB per 100 mg support and near 3.9 % of immobilized protein-CAT0. The conversion may be higher (58% vs 34 %) but the efficiency of the biocatalyst per enzyme (mg) is much lower (67-70%). Probably, the high concentration of CALB in the immobilization media (10.5 vs. 4.5 mg per 100 mg) produced aggregated immobilized enzyme in CAT0.

The conversion of oleic acid (X%) was correlated with the preparation variables (GLUT and EL) through equation 1 (p-value of $8.10^{-4}<0,05$, R^2 =96,4%):

$$X\% = -39.5323 + 33.6604\ EL\% - 6.45151\ EL\%^2 + 2.03938\ GLUT - 0.0267512\ GLUT^2 \quad (1)$$

Using the Fisher test, quadratic fitting was superior than the linear one since the obtained F-ratios were 33.8 and 5.5 respectively. Figure 5 shows the variability of oleic acid conversion with GLUT and EL%.

The best conversion (maximum) was found for CAT containing 2.6 %EL and 38 mg nominal GLUT.

3.4. Effect of the incorporation of APTS

When APTS was incorporated on the nano-magnetic support without extra GLUT a drastic fall of activity was observed even when the protein content was not lower than 2% (X= 3% and X= 4%, EL%= 2.1 and 2.5%). Glutamic or aspartic acid of lipase may react with NH_2/NH_3^+ (from CHIT and APTS) through ionic or covalent bonding. These interactions are depicted in Figure 6.

3.5. Effect of the combination of GLUT, APTS and EL

Harrington suggested the desirability values [25]. The desirability function approach is one of the most used methods for the optimization of multiple response processes. The method finds operating conditions that provide the "most desirable" response values. First, several responses are fitted to the variables and Second these responses are minimized or maximized to obtain the best combination of experimental conditions to optimize responses. It varies between 0 and 1 (maximum).Outcomes at the center of the operational region were given a desirability value of 1.0, whereas outcomes at other points in the operational region were between 0 and 1 [26].

Figure 7 shows that GLUT is a critically significant variable, whereas the responses are independent on the nominal amount of APTS.

This finding does not imply that APTS had no role. It suggests that the selected responses in our design were not affected.

The real amounts of GLUT could not be measured due to interferences of different nature, however the APTS concentration was estimated by ICP. These results have been included as Supplementary material with a short explanation.

3.6. Analysis of the performance of CALB biocatalysts with the goal of their practical implementation

3.6.1-Stability as a function of storage time

The criteria to investigate the role of the additives on the performance on the biocatalyst were the retention of activity as a function of the time and the magnitude of the leaching.

The mixture after ethyl oleate synthesis- once biocatalyst was removed - was tested for catalytic activity. Extra known amount of oleic acid was added and its conversion to ethyl oleate was measured. Conversion of oleic acid achieved 20% after using the biocatalyst containing APTS; whereas 12% was recorded when using the catalyst without APTS. These findings suggest that the APTS played a negative role regarding to the long time stability/leaching of the magnetic biocatalysts. Table 2 presents data on the influence of combination of GLUT/APTS/ on the EL(%), conversion (%X) and activity retention (R%) after two months of storage.

From the Table, it is detected that even when the nominal amount of lipase changed from 1.5 to 4.5mg; the conversion was not improved as expected (compare CAT4 with CAT12 and CAT 7 with CAT 11). The %EL resulted proportional to 1.5 to 4.5 % of CALB, but 10.4 % of CALB did not increase the retained amount of protein. The biocatalyst exhibited lower lipase content (i.e. lower %EL) resulted almost inactive after two months; whereas the corresponding to the higher lipase content retained 100% of its initial activity.

Comparing CAT7 and CAT 11, the %EL was equivalent in spite of the great difference of nominal CALB. Examining the CAT7 and CAT12, the %EL was comparable in both cases and consequently also the %R was similar.

Thermal stability, impact of storage time in residual enzymatic activity, concentration in the test reaction and retained activity in consecutive cycles were studied in CAT 7 and CAT 4.

Fernández Lafuente et al. published several manuscripts in the period 2006 to 2014 on different immobilization protocols [27-29]. In the manuscript of Barbosa et al. the authors reported that at high ionic strength, CALB was adsorbed through interfacial activation. Using a non-ionic detergent like Triton X-100, the enzyme was ionically adsorbed. If detergent and salt were both present in the immobilization media, a covalent attachment to the support was produced. Without detergent or high ionic strength, a mixed immobilization coexisted. The authors presented 5 different CALB biocatalysts prepared with the previous described protocols. In our case no detergent was used and ionic force was low. The mixed adsorption was then expected and we explained our results with that model.

3.6.2-. Reutilization potential

The conversion decreased with reuse (see Figure 8). Reuses were performed through 14 days. Activity loss could be ascribed to partial enzyme desorption ("leaching") and/or inhibition caused by substrates or products [30]. The aggregation of magnetic nanoparticles containing the lipase during magnetic separation and re-dispersion cycles was other problem. This phenomenon resulted in partial loss of the enzymatic activity due to the limited accessibility of substrate molecules (and enzyme blocking) [31] and not necessarily to deactivation/inhibition.

An enhanced performance of our biocatalyst was evident. For instance, being the test reaction performed in a solvent free media the concentration of oleic acid is near 2,4 M and 60 mg of immobilized CALB were used, at 24 °C. In this reaction 1.35 g was the total mass of reaction media with 1g of oleic acid (mass ratio oleic acid / biocatalyst=16,67). Silva et. al, [32] reported a concentration of oleic acid of 0,05M and butyl alcohol 1:1, with 2.5 mL

heptane 20 mg of Novozym435 at 37 °C. No more than 35 mg of oleic acid were used in each reaction cycle , with 20 mg of immobilized CALB (mass ratio oleic acid / biocatalyst=35/20=1.75).In our case, the first use rendered a total conversion of 63 % for CAT7 (or 630 mg of OA converted in 3 h at 24 °C using 60 mg of CAT as much as 3.5 mg OA converted per mg biocatalyst per hour for a ratio oleic acid/biocatalyst of 1000/60=16.67). Silva et al. reported 100 % of conversion (or 35 mg of OA totally converted in 12 h at 37 ºC with 20 mg or 0.1458 mg OA converted per mg biocatalyst per hour) in the first use. Our system CAT7 was then 24 times more productive than the one reported by Silva et al. using a first shorter reaction (3 h vs 12 h) at lower temperature. CAT 7 in 5 reuses was near 5.6 times more productive than the catalyst reported by Silva in 8 reuses with less energy consumption. This is only one example of how the experimental data should be completely reported employing substrates *in quantities (not concentrations).*

Quality of the final reaction media in terms of contamination with enzyme and other compounds should also be considered. The support in Novozym®435 dissolved in ethanol and other alcohols. This is a serious limitation leading to product contamination with support and enzyme [33]. This problem is absent using the magnetic biocatalysts prepared within this work. No mass loss of biocatalyst was found after the 7 uses with CAT7, especially due to the use of magnetic separation with the Nd magnet.

3.6.3-Economical viability

Economy represents one of the main limitations associated to the practical implementation of biocatalysts. The development of a non-expensive, versatile, easy to separate, reusable and stable biocatalyst is a great challenge. Most of reported immobilization studies estimate the biocatalyst viability in

terms of their activity, correlated with the highest enzyme loading on the support which is often expensive [31]. According to the work of Séverac *et al.* [34], some factors have to be considered to estimation of the biocatalyst's cost. First, the funds associated to the support. In our case, these are costs related to the synthesis (biopolymer, Fe precursors, surfactants, etc.). Then, the lipase´s and operational costs have to be evaluated. Taking into account the sources of the formulation, our biocatalyst CAT 7 fees were about 10.18US$/g .This cost is considerably lower than the fees corresponding to commercial catalysts such as Lipase immobilized from Candida Antarctica (73940) USD 354.75/g or Lipase acrylic resin from Candida Antarctica L4777USD 200.42/3g (website Sigma Aldrich Argentina).

4. Concluding Remarks

Our manuscript explored the role of common additives (GLUT and APTS) on the preparation of an efficient magnetic biocatalyst. Evidence was presented related to the mixed nature of the covalent reaction/adsorption of CALB onto modified supports.

Best catalyst included GLUT and APTS and was prepared with 10.5 % nominal CALB (in a per support basis) with formula CALB/GLUT/APTS/GLUT/CHIT/MAG. The exposed surface was complex and heterogeneous. Even when enough GLUT was available for the lipase, not necessarily all the reactions produced covalent bonding lipase-GLUT.

The initial leaching may be related to the loss of weakly adsorbed CALB (onto APTS, oligomeric glutaraldehyde or exposed magnetite) or CALB from immobilized CALB multilayers. Lower than 2 % of immobilized protein from

CALB generated highly active biocatalyst per mg of immobilized protein but without retention of activity on 2 months of storage.

The keys to asses a magnetic biocatalyst with great possibilities of implementation were here presented. This information resulted useful not only when working with similar systems support/lipase but also in selecting the set of variables to analyze in order to achieve an efficient biocatalyst.

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Figure Captions

Figure 1-FTIR-DRIFTS of magnetic supports before and after addition of GLUT and APTS; and CATs. The spectrum of CALB is also included as reference.

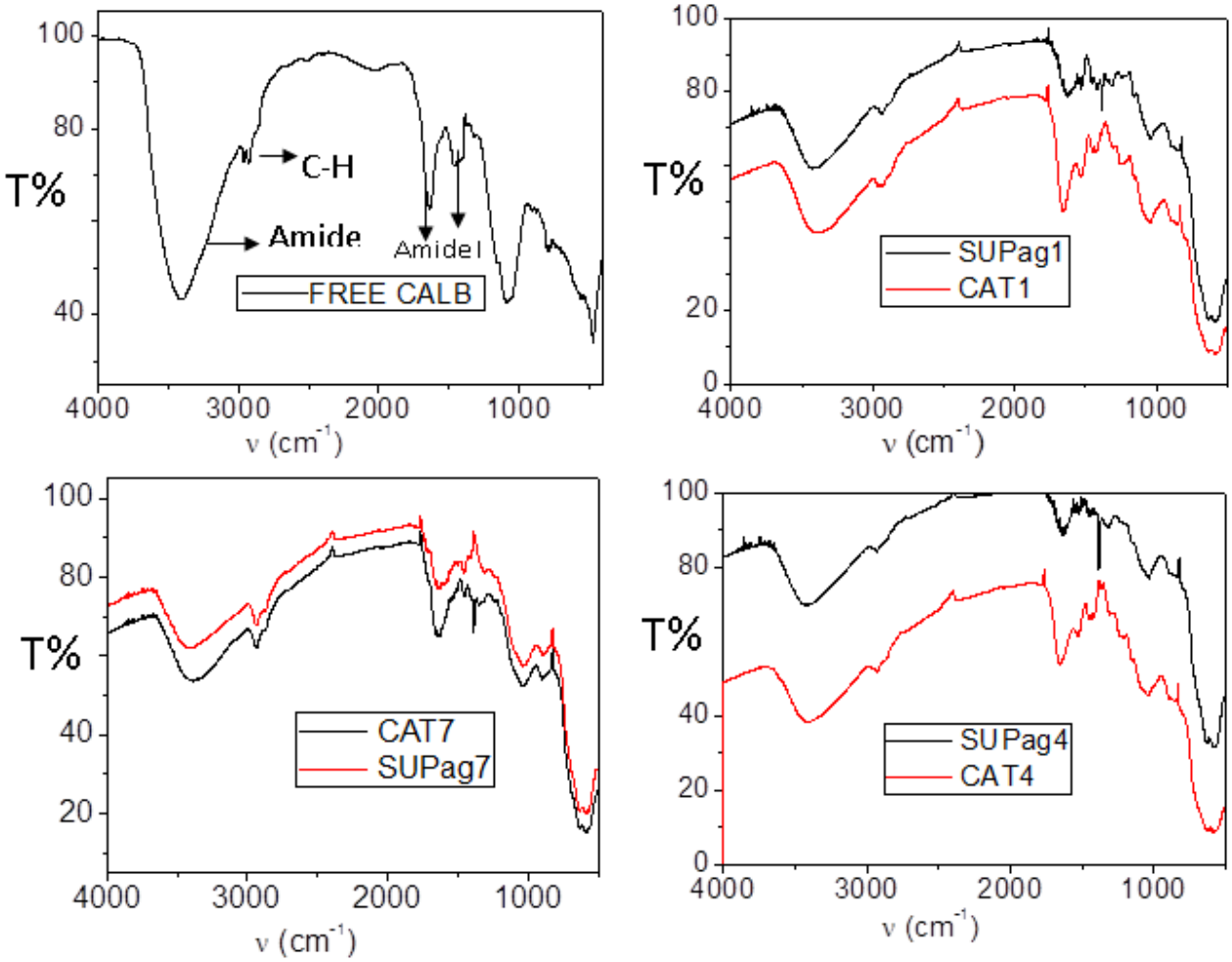


Figure 2-TEM micrographies comparing morphologies of magnetic supports and CATs.

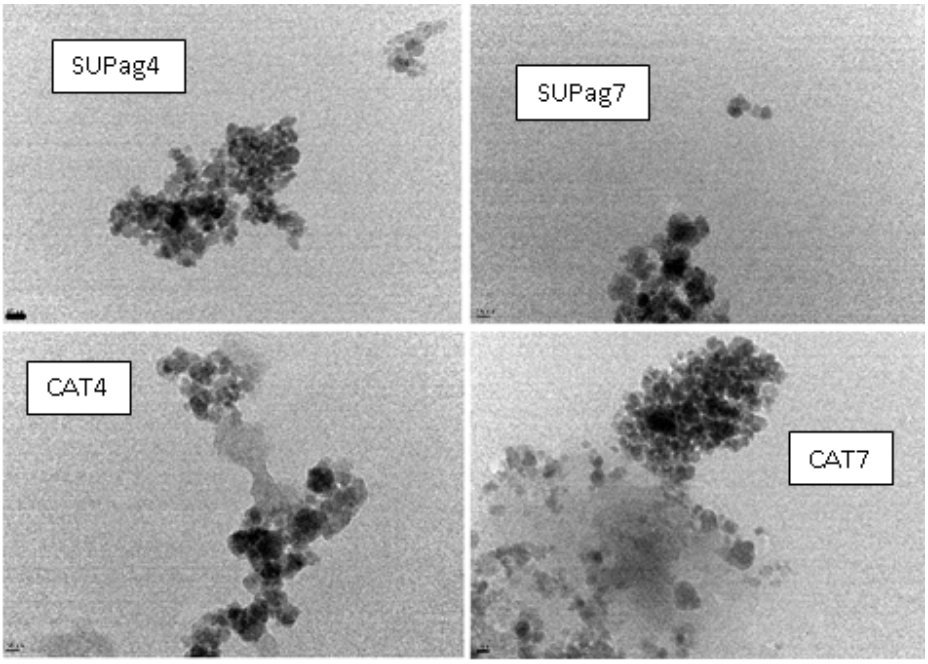


Figure 3-Hydrodinamic diameters (HD) of CATs compared with the HD of raw support (dark line).

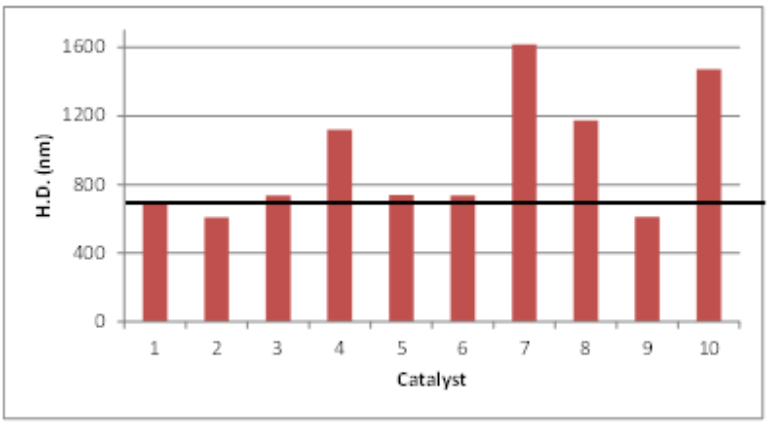
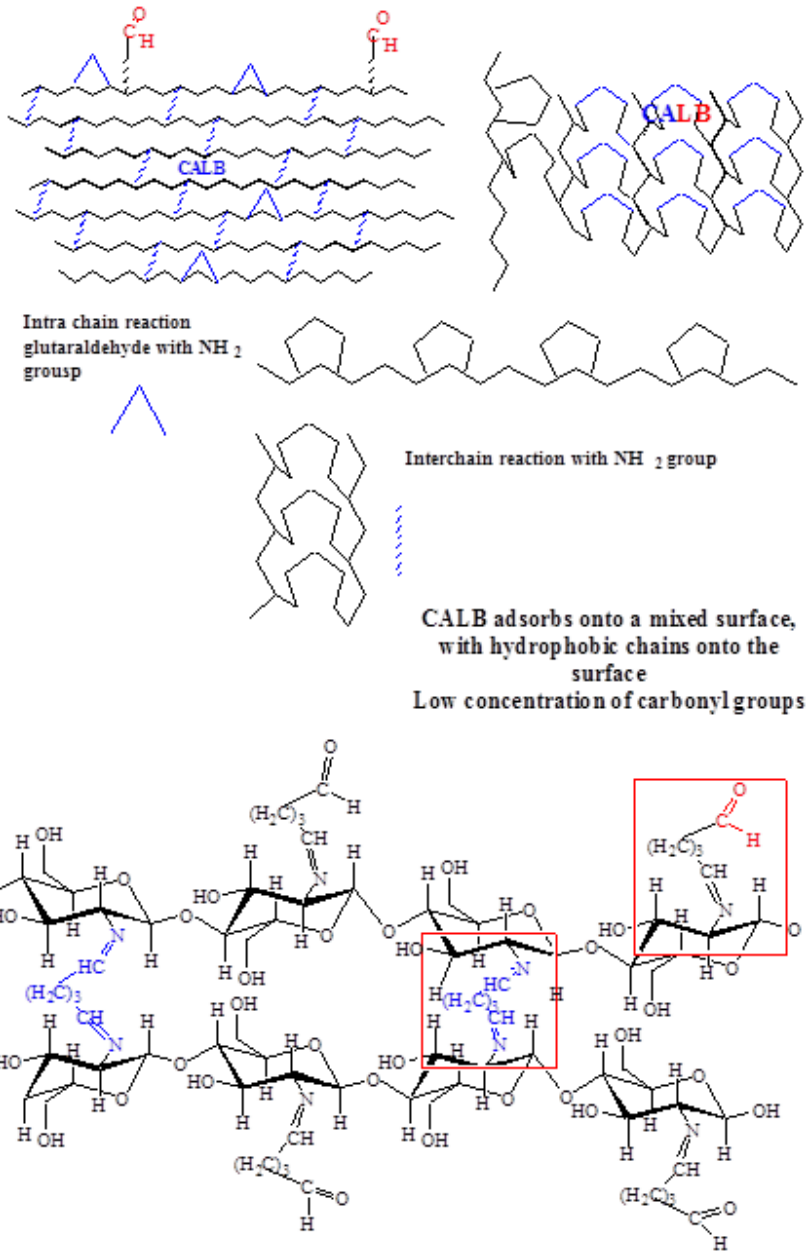
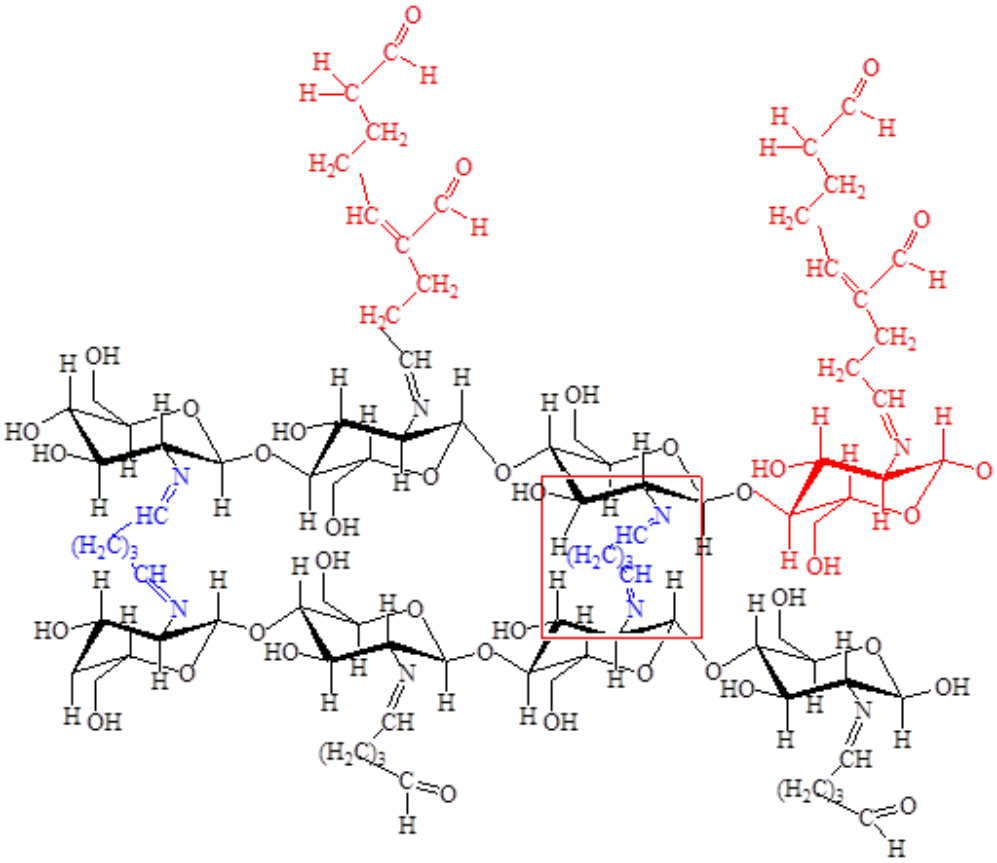


Figure 4a-Interactions CHI-GLUT; b-Representation of adsorption and covalent interactions liase-support; c-Representation of interactions CALB-GLUT.

CHITOSAN-GLUTARALDEHYDE +ENZYME CALB





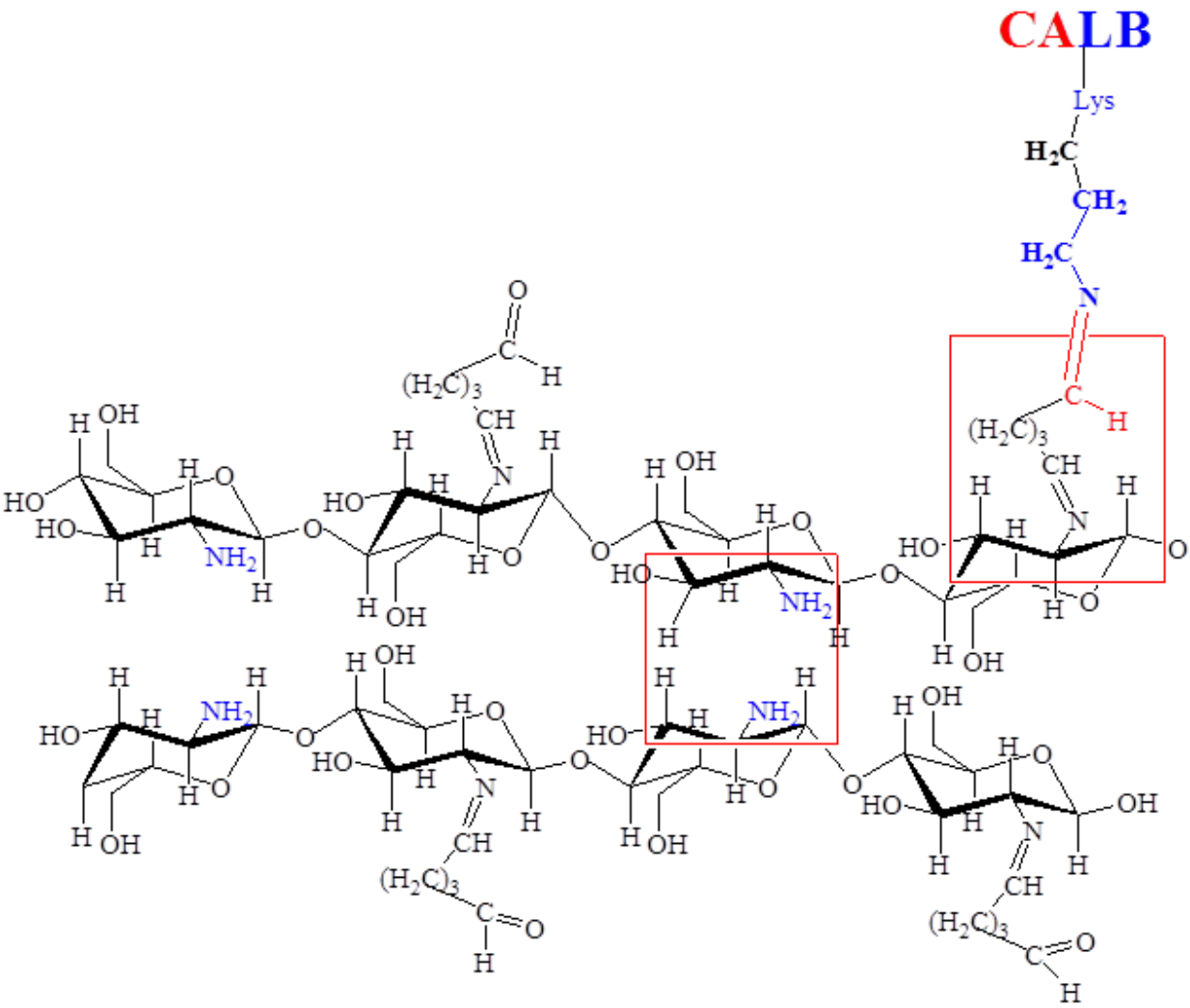


Figure 5- Variability of oleic acid conversion with GLUT and EL% obtained from experimental design.

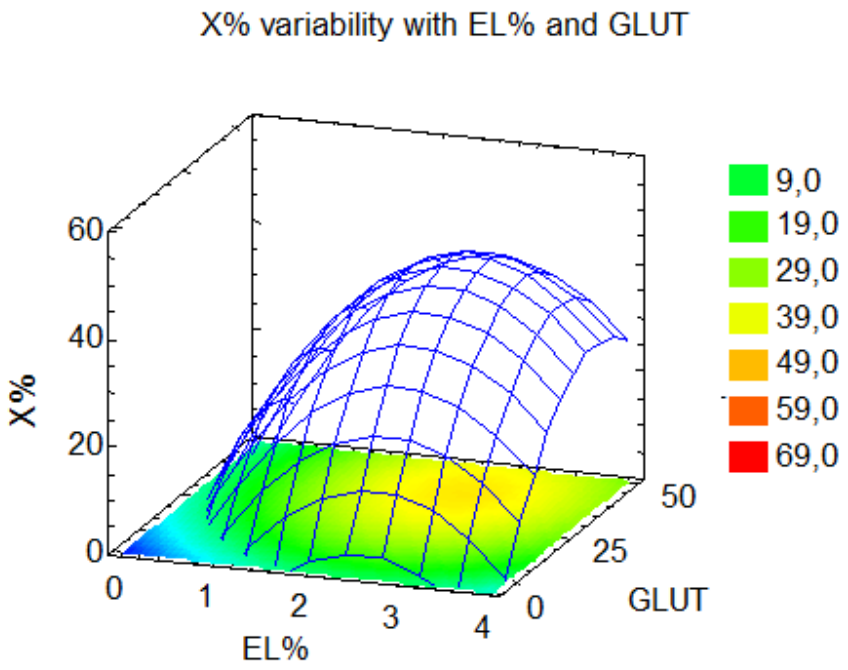


Figure 6-Interactions of CALB with APTS.

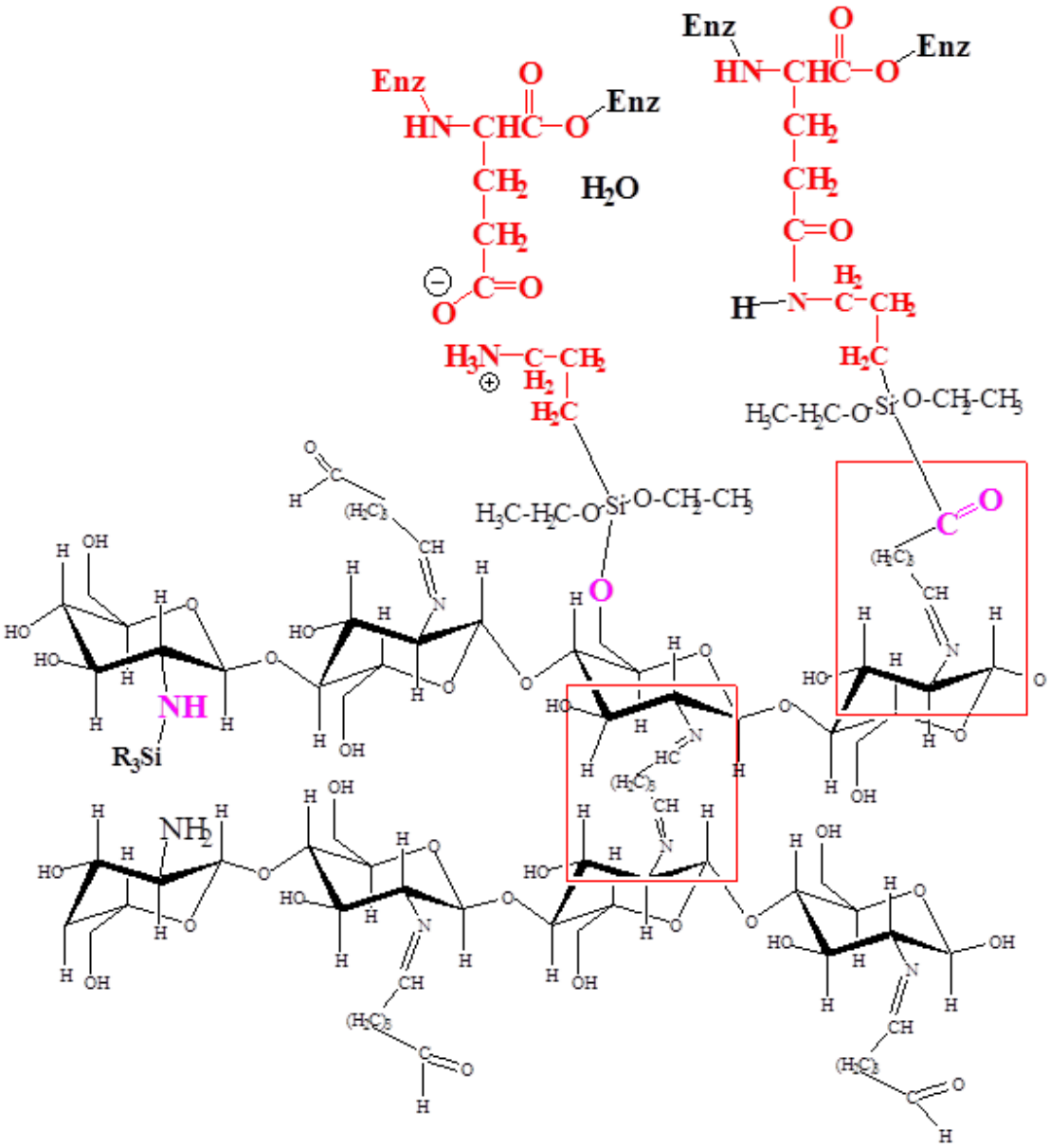


Figure 7-Surface of response on the effect of combination of the three factors APTS, GLUT and %EL.

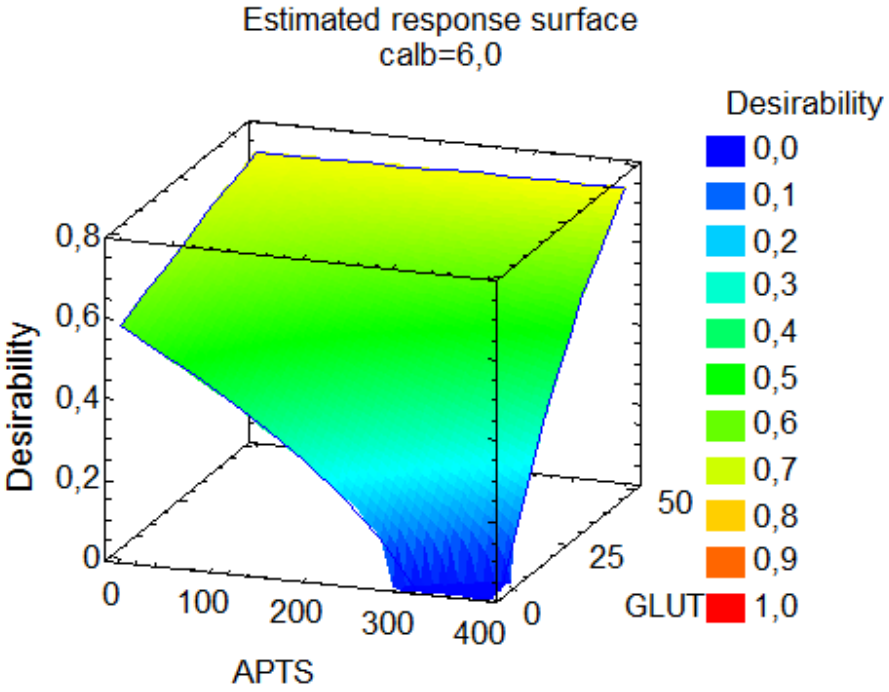
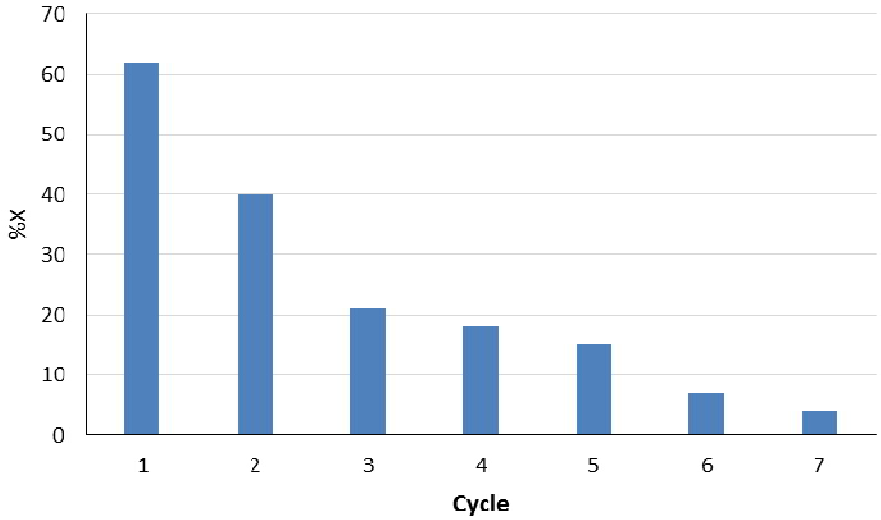


Figure 8-Sucesive cycles of use-purification employing CAT7.



Tables

Table 1. Variables and responses (in terms of conversion (X%) and enzyme loading (%EL)) involved in the experimental design.

	Mass percentage of offered crosslinkers or enzyme				
CAT	APTS	GLUT	CALB	X%	EL%
0	0	0	10,4	56	3,9
1	0	8,3	4,5	39	2,2
2	50	0	3	4	2,1
3	0	16,6	4,5	35	2,6
4	0	8,3	1,5	34	1,5
5	100	8,3	1,5	10	0,7
6	100	16,6	1,5	16	0,6
7	100	16,6	4,5	39	3,0
8	0	16,6	1,5	22	0,9
9	50	0	3	3	2,5
10	100	8,3	4,5	36	3,4
11	100	200	10,4	40*	3,3
12	0	200	4,5	36	3,6

*at 45°C

Table 2. Retention of activity (%R) calculated from the conversion obtained after two months of storage (Xt%) relative to the initial Xi%.

CAT	SUP/APTS/GLUT/CALB	EL%	Xi%	Xt%	% R
0	100/0/0/10,4	3,9	56	50	90
4	100/0/8,3/1,5	1,5	34	0	0
7	100/100/16,6/4,5	3,0	39	27	70
11	100/100/200/10,4	3,3	40*	36	90
12	100/0/200/4,5	3,6	36	36	100

*at 45°C