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DEVELOPMENT OF SIMPLE PROTOCOLS TO SOLVE THE PROBLEMS OF ENZYME COIMMOBILIZATION.

APPLICATION TO COIMMOBILIZE A LIPASE AND A β-GALACTOSIDASE.

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Abstract

This paper shows the coimmobilization of β-galactosidase from *Aspergillus oryze* (β-gal) and lipase B from *Candida antarctica* (CALB*)*. The combi-biocatalyst was designed in a way that permits an optimal immobilization of CALB on octyl-agarose (OC) and the reuse of this enzyme after β-gal (an enzyme with lower stability and altogether not very stabilized by multipoint covalent attachment) inactivation, both of them serious problems in enzyme co-immobilization. To this goal, OC-CALB was coated with polyethylenimine (PEI) (this treatment did not affect the enzyme activity and even improved enzyme stability, mainly in organic medium). Then, β-gal was immobilized by ion exchange on the PEI coated support. We found that PEI can become weakly adsorbed on OC support, but the adsorption of PEI to CALB was quite strong. The immobilized β-gal can be desorbed by incubation in 300 mM NaCl. Fresh β-gal could be adsorbed afterwards, and this could be repeated for several cycles, but the amount of PEI showed a small decrease that made reincubation of the OC-CALB-PEI composite in PEI preferable in order to keep the amount of polymer. CALB activity remained unaltered under all these treatments. The combi-catalyst was submitted to inactivation at 60 ºC and pH 7, conditions where β-gal was rapidly inactivated while CALB maintained its activity unaltered. All β-gal activity could be removed by incubation in 300 mM NaCl, however, SDS analysis showed that part of the enzyme β-gal molecules remained immobilized on the OC-CALC-PEI composite, as the inactivated enzyme may become more strongly adsorbed on the ion exchanger. Full release of the β-gal after inactivation was achieved using 1 M NaCl and 40 ºC, conditions where CALB remained fully stable. This way, the proposed protocol permitted the reuse of the most stable enzyme after inactivation of the least stable one. It is compatible with any immobilization protocol of the first enzyme that does not involve ion exchange as only reason for enzyme immobilization.

***Key words*:** Enzyme coimmobilization, ion exchange, lipase interfacial activation, enzyme reuse, combi-biocatalysts, cascade reactions, PEI coating.

1. Introduction

Enzymes are powerful tools in green organic chemistry due to their high activity under environmentally mild conditions coupled to a high selectivity and specificity.[1] Among the many uses of enzymes as biocatalysts, the so-called cascade or domino reactions have got a relevant role because they permit to carry out very complex reactions (e.g., copying metabolism).[2] These reactions mean that the product (or side product) of the first reaction is the substrate of the second one and the product of this one is the substrate of the third one and this continues in a successive manner. One of the first and most remarkable examples of multiple reactions catalyzed by several enzymes to get one high added value product was reported by Wong in the production of sialyl Lewis X.[3] However, most examples of cascade reactions are more modest. For example, the relatively simple sequential hydrolysis of an oil, a protein or a polysaccharide may be considered a cascade reaction, even though the order of the modifications is not fully determined in all cases and may depend on the enzyme mixture used (e.g., carboxypeptidase A should be used always after chymotrypsin in a selective hydrolytic process of proteins, but trypsin and chymotrypsin may act in a more free order). [4] In other cases, the objective is to perform several modifications with a strict order, like in the transformation of benzaldehyde into mandelic acid by sequential HCN addition and hydrolysis catalyzed by oxynitrilase and nitrilase.[5] In other cases, the second enzyme function is to regenerate a cofactor used by the main enzyme (NAD(P)H or NAD(P)+,[6] or ATP or a phosphorylated compound[7]). In some instances, the side product of one enzyme is used to perform a modification of the target substrate by the other enzyme, like using oxidases that produce hydrogen peroxide that is utilized by lipases to produce peracids,[8] or by peroxidases or laccases to oxidize the desired compound.[9] Some examples involve the use of a cascade reaction just to destroy one side product with a second enzyme that may affect the main product or the main enzyme (e.g., to destroy hydrogen peroxide by catalase in reactions catalyzed by oxidases).[10] All these reactions are just

some examples of the huge variety of cascade reactions, keeping in mind that the casuistic is very broad. Moreover, cascade reactions may involve the same or different enzymes. For example, in some cases the cofactor recycling using dehydrogenases may be achieved using the same enzyme and two different substrates[11] and in many instances full hydrolysis of oils or production of biodiesel are performed using just one lipase. However, a more general case is that each reaction is catalyzed by a different enzyme, as this has some advantages.[2]

Immobilization is a requirement for most industrial uses to facilitate the recovery of the enzymes and their reuse provided that they are stable enough.[11] However, nowadays the objective of immobilization must be far more than a simple enzyme reuse; the improvement of many enzyme features (stability, but also activity, selectivity or specificity) may be accomplished by a proper immobilization, transforming this step in a powerful instrument in the biocatalyst design.[12]

From an industrial point of view, cascade reactions are better performed in one pot.[2] In fact, in some instances such as in the regeneration of cofactors, there are no alternatives to the one pot configuration. This makes finding conditions where all involved enzymes are active and stable compulsory, and this may produce additional difficulties in the design of the process[2] and enhance the interest of having as improved a biocatalyst as possible (e.g., via immobilization).[12] Moreover, the enzymes co-immobilized on a same particle are usually preferred, because that way the second enzyme may act on a higher concentration of their substrate from the beginning of the reaction time.[2] This avoids the lag-time usually observed in these reactions, permitting the second enzyme to act from the beginning and may shorten the full reaction course depending on the kinetic properties of the enzymes and the concentration of substrate.[2] In other instances, like when the product of the first enzyme is unstable (production of alpha-keto acids using d-aminoacid oxidases and catalase,[13]

or mandelic acid from benzaldehyde[5]) or if this product is able to render the first enzyme inactive (oxidases and catalases),[14] the coimmobilization is fully required.

However, coimmobilization of enzymes has several problems which are usually overlooked.[15] The first one is that when the least stable enzyme is inactivated, both enzymes need to be discarded. The second one refers to the necessity of immobilizing all enzymes on the same support, and usually using the same protocol, that may not be optimal for both enzymes. Recently, a brilliant solution has been reported: the use of heterofunctional supports, where one enzyme is immobilized on one kind of support group and the second enzyme is immobilized on the other kind of group.[16] However, this nice strategy has some problems yet. Both groups will be under the enzyme surface of both enzymes, and that may produce some problems in the intensity of the desired enzyme-support interactions and the existence of some undesired ones, and this may reduce the final stabilization for both enzymes achieved via immobilization.[17]

Our group is trying to advance on the solution of these problems concerning coimmobilization. In this first approach, we have focused on a situation where one of the enzymes may be just marginally stabilized via multipoint immobilization and it is less stable that the other enzyme. The strategy is simple: an optimal immobilization protocol may be applied for the more stable enzyme, and this enzyme is later coated with an ionic polymer. This treatment with ionic polymers generally does not alter the enzyme activity and has been even used to stabilize the enzymes versus diverse inactivating causes (subunit dissociation, oxygen, solvents, etc.)[18] or even to improve enzyme properties.[19] Then, the labile and hard to stabilize enzyme may be immobilized via ion exchange on the already immobilized one. If the first enzyme remains active and immobilized at high ionic strength, after the labile enzyme inactivation, this enzyme may be desorbed while the support immobilized one is reused. That way, it is possible to have an optimal

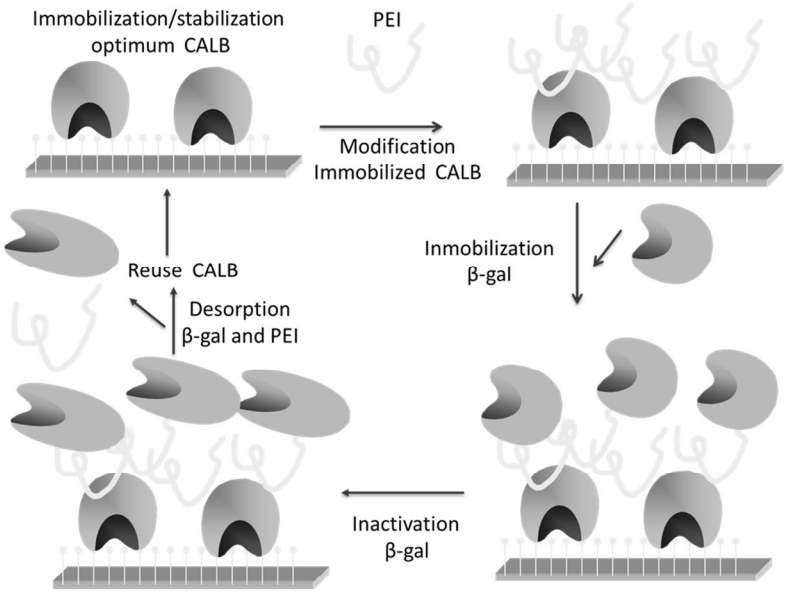
biocatalyst for the most stable enzyme that can be reused many times to immobilize the labile enzyme, and some cycles of inactivation, desorption and reloading of the second enzyme may be accomplished reusing the most stable enzyme. This is not a fully general situation, but many enzymes couples may fulfill these requirements.

For example, in this proof of concept paper we have employed two very widely used enzymes. The lipase B from *Candida antarctica* is among the most used ones in biocatalysis,[20] it is very stable and may be further stabilized via immobilization. For example, CALB has been greatly stabilized by immobilization on octyl-agarose supports via interfacial activation on the hydrophobic surface of the support. The final stability thus achieved by even gives a higher stabilization than the same biocatalyst prepared via multipoint covalent attachment.[21] This immobilization is reversible[22] and may be useful to study the molar relation of both enzymes via SDS-PAGE. Therefore, we have selected this immobilization strategy.

The CALB modification with PEI produced a further enzyme stabilization, mainly in organic medium, without affecting the enzyme activity.[23] As a second model enzyme to get the combi-biocatalyst via this new strategy, we have selected the β-galactosidase from *Aspergillus oryze*, an enzyme employed in many reactions and which has a high transglycosylation activity.[24] This enzyme is quite stable, but a maximum of 12 folds using epoxy-amino supports (best results reported for this enzyme) can be stabilized,[25] and immobilization via ion exchange gave good results.[26] This has been explained by its high glycosylation (this reduces the exposition of the protein structure of the enzyme) and the low stability at alkaline pH value (reducing the possibilities of forcing the enzyme-support reactions)[27] making their covalent immobilization not recommendable as that way support and enzyme should be discarded.[15] Thus, this enzyme may be valid for the proposed strategy: it is difficult to stabilize the enzyme via multipoint covalent attachment in a support but the immobilization via ion exchange produced reasonable good results

in terms of activity and stability. Both enzymes might be used to produce galactose modified in the position 1 with 1,2-diacetin via a glycosidic bond, using triacetin and lactose as substrates. 1,2-diacetin is produced by CALB in hydrolysis of triacetin, but it is unstable tending to isomerize.[28] Moreover, in a kinetically controlled process like the proposed, the concentration of the nucleophile may be a key point to reach good yields,[29] therefore coimmobilization could have a double justification in this reaction.

In this paper, we just studied if both enzymes may be coimmobilized on the same particle but using different immobilization strategies, permitting an individual support surface optimization for each of them, and we have analyzed the activity/stability features of the biocatalyst compared to that of the individual ones. Finally, we have checked the actual possibility of reusing the immobilized CALB after the β-gal inactivation, a main problem in the standard design of coimmobilized biocatalysts. Scheme 1 resumes the strategy and objectives.



Scheme 1

2. Materials and methods

2.1. Materials

Solution of lipase B from *C. antarctica* (CALB) (6.9 mg of protein /mL) was a kind gift from Novozymes (Spain). β-galactosidase from *Aspergillus oryzae* (20 Units oNPG/mg of protein), *o*-nitrophenyl-β-galactopyranoside (ONPG), polyethylenimine (PEI) (MW 25,000), dextran sulfate (DS) (9-20,000 MW), triton X100, cetyltrimethylammonium bromide (CTAB), sodium dodecylsulfate (SDS), 2,4,6-trinitrobenzensulfonic acid (TNBS), diethyl p-nitrophenylphosphate (D-*p*NPP) and *p*-nitrophenyl butyrate (p-NPB) were purchased from Sigma–Aldrich (St. Louis, USA). Octyl Sepharose CL-4B beads and 4% CL agarose beads were from GE Healthcare. PEI and DS supports were prepared as previously described.[26a,30] Electrophoresis reagents were obtained from Bio-Rad (Hercules, USA). All other reagents were of analytical grade. Protein concentration was estimated by the Bradford dye binding method[31] at 595 nm using bovine serum albumin as a standard.

2.2 Standard determination of enzyme activity

2.2.1. β-galactosidase. This assay was performed by measuring the increase in absorbance at 380 nm produced by the release of *o*-nitrophenol in the hydrolysis of 10 mM ONPG in 25 mM sodium acetate buffer at pH 5 and 25 ºC (ε was 10493 M^{-1} cm^{-1} under these conditions),[26b] using a spectrophotometer with a thermostatized cell and with continuous magnetic stirring. To start the reaction, 100 μL of the enzyme solution or suspension were added to 2.5 mL of substrate solution. One unit of activity (U) was defined as the amount of enzyme that hydrolyzes 1 μmoL of ONPG per minute under the conditions described previously.

2.2.2. Lipase. This assay was performed by measuring the increase in absorbance at 348 nm produced by the released p-nitrophenol in the hydrolysis of 0.4 mM p-NPB in 25 mM sodium phosphate buffer at pH 7.0 and 25 °C (ε under these conditions is 5150 M^{-1} cm^{-1}). 50–100 μL of lipase solution or suspension were added to 2.5 mL of substrate solution to start the reaction. One international unit of activity (U) was defined as the amount of enzyme that hydrolyzes 1 μmol of p-NPB per minute under the conditions described.

2.3. Immobilization of CALB on octyl (OC) supports

The standard immobilization was performed using 10 units of lipase per g of wet support. In some instances, like those to perform SDS-PAGEs or to determine maximum loading of the enzymes, the amount of offered CALB was increased up to 80 mg/g of support. CALB solution was diluted in the corresponding volume of 5 mM sodium phosphate buffer at pH 7 at 25ºC. Then, OC support was added to reach the desired loading.[22] The activity of both supernatant and suspension was followed using p-NPB assay. After immobilization the suspension was filtered and the immobilized biocatalyst enzyme was exhaustively washed with distilled water.

2.4. Modification of OCCALB with PEI

A 50 mL solution of 10% PEI (w/v) was prepared and the pH was adjusted at pH 7. Then, 5 g of OCCALB was suspended and submitted to gentle stirring for 2 h. Afterwards, the modified enzyme was washed with an excess of distilled water to eliminate the free PEI.[23] The enzyme activity was maintained unaltered and the stability improved, mainly in the presence of organic solvents.[23]

2.5. Immobilization of β-galactosidase via ion exchange

The standard immobilization was performed using 20 ONPG units of free beta-galactosidase activity per g of wet support (1 mg of enzyme per gram of support), although in some cases maximum enzyme loading was utilized (4 mg). The support could be PEI, DS or OCCALB-PEI. This low loading was used to prevent diffusional limitations that could make the understanding of the results on molecular enzyme properties more complex. In some instances, the amount of enzyme was increased (e.g. to determine maximum loading of the support, or to perform SDS-PAGE analysis). The commercial sample of the enzymes was dissolved in the corresponding volume of sodium acetate at pH 5, sodium phosphate at pH 7 or sodium bicarbonate buffer at pH 9 at 25ºC, and then the support was added to reach the desired enzyme loading.

2.6. Thermal stability of the enzyme preparations

Immobilized or coimmobilized enzymes were incubated at different pH values (5, 7 and 9) and different ionic strengths (25 or 500 mM of the buffers indicated in the above section). Periodically, samples were withdrawn and the enzyme activity was measured using oNPG and p-NPB, depending on the enzyme analyzed. Half-lives were calculated from the observed inactivation courses.

2.7 Desorption of β-galactosidase from OCCALB-PEI

The coimmobilized derivatives were suspended in 5 mM sodium phosphate and incubated in growing concentrations of NaCl at pH 7 and the activities of both supernatant and suspension were followed using o-NPG and p-NPB.

2.8. Primary amino titration of the different preparations using TNBS.

0.5 g of the enzyme preparation were suspended in 5 ml of 100 mM sodium phosphate at pH 8, and then 0.5 mL of TNBS commercial solution were added.[32] After 30 minutes of gentle stirring, the colored support was exhaustively washed with sodium phosphate at pH 8. Finally, 200 mg of the treated support were suspended in 5 mL of sodium phosphate at pH 8 in a cuvette (1 cm) and submitted to continuous stirring. Spectrum acquisition was performed from 350 to 600 nm of the different supports compared to the non TNBS-treated supports, and the wavelength that permitted an absorption of 425 nm was selected for the comparisons.

2.9. SDS-PAGE experiments

SDS-polyacrylamide gel electrophoresis was performed according to Laemmli[33] using a Miniprotean tetra-cell (Bio-Rad), 14% running gel in a separation zone of 9 cm × 6 cm, and a concentration zone of 5% polyacrylamide. One hundred milligrams of the immobilized enzyme samples was re-suspended in 1 mL of rupture buffer (2% SDS and 10% mercaptoethanol), boiled for 8 min and a 10 μL aliquot of the supernatant was used in the experiments. This treatment released all enzyme which was just interfacially activated on the support.[34] Gels were stained with Coomassie brilliant blue. A low molecular weight calibration kit for SDS electrophoresis (GE Healthcare) was used as a molecular weight marker (14.4 – 97 kDa).

3. Results and discussion

3.1 Immobilization of CALB on octyl support

The immobilization course of CALB on octyl support is shown in Figure 1Sa. In less than 30 minutes, using a ratio of 1 g of support and 10 ml of enzyme suspension, CALB was immobilized and the activity remained almost unaltered. Immobilization yield is over 95% and the activity is maintained at 100%. This result agreed with previous reports in literature using this support and enzyme[34]. Although the immobilization involves the open form of the lipase and stabilizes it,[35] the CALB lid is so small that the enzyme did not experiment a real activation after immobilization.[36] Figure 1Sb shows that the immobilized CALB is far more stable than the free enzyme, maintaining 70% of activity when the free enzyme retained less than 10% of the initial activity. This stabilization of lipases immobilized on octyl supports has been explained by the high stability of the adsorbed open form of the lipases when compared to lipases in the standard conformational equilibrium.[37] The coating with PEI under the conditions used in this paper has been described to present no effect on enzyme activity (activity remained at 100%) and improved stability (mainly in organic solvents).[23] Therefore, we have decided to use this biocatalyst as a method to prepare he coimmobilized biocatalyst. Thus, the OCCALB-PEI seems a very adequate system to be used as "support" to immobilize other enzymes.

3.2 Immobilization of β-gal on PEI and DS supports at different pHs values

Figure 2S shows the immobilization of the β-gal on supports activated with DS or PEI at pH 5 and 7. While using PEI the enzyme immobilization is complete after only 20 minutes at pH 5 and 7, the enzyme is only partially immobilized on DS at pH 5 and negligible at pH 7. Therefore, PEI was selected for all further studies. Immobilization yield was 100% and activity recovery over 90%.

The stability of this enzyme preparations is shown in Figure 3S, showing that the immobilization has a marginal effect on the stability of this enzyme, similar to that found using standard ion exchangers[26b]. Although the enzyme could be immobilized at pH 9 on PEI coated supports, this pH offered a lower stability of the enzyme[26b]: For this reason we discarded the immobilization under this pH condition.

3.3 Immobilization of β-gal on octyl-CALB-PEI

Figure 1 shows the immobilization of (0.5 mg, 10 U/g) β-gal on the composite OC-CALB (2 mg/g)-PEI. Immobilization proceeds very rapidly at both pH values (5 and 7) and the activity of the enzyme remained unaltered. The stability and activity of the CALB of this composite was identical to that of the lipase immobilized on octyl and coated with PEI (results not shown) and the β-gal stability also was identical to that of the enzyme immobilized on the support coated with PEI (results not shown). The difference in stabilities of CALB and β-gal enzymes was very significant, being the CALB much more stable than the β-gal.

3.4 Desorption of β-gal immobilized on octyl-CALB-PEI

We performed cycles of adsorption/desorption of the β-gal on the PEI-lipase composite. That way, OCCALB could be reused after β-gal inactivation. Figure 2 shows that all β-gal activity could be released to the medium using 300 mM of NaCl at pH 7, without affecting the CALB activity that remained fully immobilized and active, and this operation could be repeated several cycles. After enzyme desorption, new β-gal could be immobilized on the OCCALB-PEI. While in the first cycles 100% of the β-GAL was immobilized, it was found that after 6 cycles, the amount of β-gal immobilized decreased to 60%. This result suggested that the PEI could be released from the OCCALB at 300 mM of NaCl, reducing the amount of PEI and that way decreasing the amount of

immobilized enzyme. Therefore, we decided to prepare biocatalysts with maximum loading of β-gal at different CALB amounts to analyze in a more precise manner the intensity of the problem. Surprisingly, we found that we could immobilize a maximum of 4 mg of β-gal per g of OCCALB independently of the amount of the CALB on the support (results not shown). Figure 3 shows the SDS-PAGE analysis of these preparations, showing that although the amount of CALB increased, the maximum amount of β-gal remained constant. β-gal presented two bands, one at 60 kDa and the other at 72 kDa, both have been previously described.[38] This could be caused by the closing of the pores of the agarose with the β-gal and the PEI , thus we did not reach the maximum values of loading with the β-gal, or maybe because β-gal can be immobilized on the support surface and not only in the CALB. Figure 4 shows that while β-gal did not immobilize on OC support, it immobilized very rapidly on OC-PEI. This occurred although agarose is supposed to be an inert matrix, and suggests that some sulfate from agarose remains or that the chemical treatment of the agarose to introduce the octyl groups has produced some oxidations in the agarose hydroxyl groups. PEI is a poly-cation that requires a very low amount of anion groups in the support to establish multiple ionic bridges.

To confirm that PEI was adsorbed on OC, TNBS assay was utilized. Table 1 offers the results, which confirmed that PEI could be adsorbed on OC agarose beads. The incubation of this composite in 300 mM NaCl released almost completely the PEI. As a comparison, OC-PEI and OC-CALB (maximum loading)-PEI were used, and this showed that PEI was only marginally desorbed from the support having maximum CALB loading when incubated in 300 mM NaCl, while a significant percentage of the PEI was released when using OC-PEI preparations (Table 1). Thus, PEI was more strongly attached to CALB than to the OC support.

The stability of the β-gal (0.5 mg to prevent diffusion problems) was rechecked using OCCALB-PEI with maximum CALB loading and the results in terms of activity recovery and stability were identical to the ones previously presented in this paper.

The fact that the commercial OC support could be coated with PEI may become an unexpected advantage, as we can immobilize (while keeping the activity and stability of both enzymes) the desired amounts of both enzymes, e.g. an excess of β-gal regarding the CALB. If the support cannot be modified with PEI, to have an excess of β-gal (or other second enzyme, this paper is just a proof of concept using a model bienzymatic system) could be a complex problem, and this may be a requirement on the design of some reactions.

3.5. Inactivation, desorption/reimmobilization of β-gal immobilized on octyl-CALB-PEI

Next, the combi-biocatalyst prepared using 0.5 mg of β-gal and 2 mg of CALB was incubated at 60ºC and pH 7 (Figure 5). Under these conditions, β-gal activity decreased rapidly while the activity of CALB remained unaltered. When the activity of the β-gal was lower than 40%, the combi-biocatalyst was incubated in 300 mM NaCl to release all β-gal and fresh enzyme was immobilized. This protocol was repeated for 5 cycles: the activity of CALB was unaltered after the last desorption/adsorption experiment, while the amount of immobilized β-gal decreased only after the sixth cycle, very likely due to the loss of PEI. To check if this problem also existed using the PEI adsorbed on the CALB, we used a support with maximal loading of CALB and just 0.5 mg of β-gal. In this case, we can immobilize 100% of the β-gal for 6 cycles. Using the maximum loading of β-gal (in this case the preparations were submitted to the same inactivation conditions but the activity was not followed, due to the diffusion problems), results could be repeated for 6 cycles. However, when the amount of PEI was determined in the OCCALB-PEI biocatalysts after each

cycle by TNBS titration (Table 2), a decrease in the amount of PEI attached to the support was appreciated. Apparently this PEI loss was not enough to prevent β-gal adsorption, but it was significant. To prevent this, the OCCALB preparations were incubated in a solution of 10% PEI after each desorption step of β-gal. This permitted to maintain the amount of PEI on the composite for 6 cycles (results not shown). In case that another enzyme was used and that this was able to immobilize on PEI stronger than CALB the reloading of PEI should be a requirement after each enzyme desorption step because all PEI would be released from the OCCALB.

It has been recently shown that the desorption of inactivated enzyme immobilized on PEI support may be more difficult that the desorption of the active enzyme.[39] Figure 2 shows that 300 mM NaCl was enough to release all β-gal following β-gal activity. Figure 6 shows the SDS-PAGE analysis of the combi-catalysts. While the non-inactivated enzyme showed no enzyme on the support after desorption using 300 mM NaCl, the inactivated preparations after desorption under those conditions showed both bands of the β-GAL, the smaller one with a relative higher intensity. As the objective was to release all β-GAL molecules, the desorption of the β-gal was assayed at different salts concentrations before and after β-gal inactivation using maximum loading of both enzymes (including a step of PEI incubation between cycles). Figure 6 shows that using 1 M NaCl, all β-gal molecules were desorbed from the support (and also almost all PEI). The activity of CALB remained unaltered even under these conditions, but the incubation of the OCCALB preparation with PEI was fully necessary. The established protocol was β-gal immobilization, inactivation, desorption, PEI incubation, and a new β-gal immobilization. And after 6 cycles, OCCALB exhibited more than 90% of the initial activity.

Conclusions

The protocol proposed in this paper overcomes some of the problems associated to coimmobilization of two enzymes: it is possible to optimize the immobilization of one of them, and it is possible to reuse this enzyme after the inactivation of less stable enzyme. The requirement for this strategy is that the immobilization of the first enzyme is not only based on ion exchange (otherwise we can desorb the enzyme when desorbing the other enzyme). The example used in this paper is interfacial activation on hydrophobic support, a method reported as very adequate for lipase immobilization. The strategy is mainly useful if one of the enzymes is not stabilized via multipoint covalent attachment, and it is the least stable enzyme among those involved in the combi-biocatalyst. The coating with PEI (but other ionic polymers may be used) produced even some positive effects on CALB stability[23], and it has been used for stabilizing many other enzymes, with low to null effect on activity due to the random coil structure. The strategy permitted to reuse CALB after several cycles of β-gal inactivation. However, the enzyme inactivation produces a stronger adsorption of the inactivated enzyme on the PEI and makes it harder to regain a CALB-PEI composite free of inactivated enzyme molecules. This is possible to achieve using higher salt concentration and temperatures[39]. These conditions did not affect CALB activity, but make re-incubation of the OCCALB-PEI composite with PEI in each desorption/ adsorption cycle compulsory. This re-incubation in PEI is not a problem at laboratory scale, but may be an inconvenient at industrial level and strategies to avoid this necessity should be explored.

The proposed strategy has fulfilled the initial objectives and may be extrapolated to many other enzyme couples involved in cascade reactions. However, to prepare a real combi-biocatalyst, an adequate relation between the catalytic activity of CALB and β-gal will be required to maximize the product conversion. The optimization of the reaction and preparation of the specific biocatalyst is under way in our laboratory.

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525 **Figures captions**

526

527 **Fig. 1** Immobilization courses of β-galactosidase using 1 mg of enzyme (20 U) at pH 5 (panel A)
528 and 7 (panel B) on octyl-CALB-PEI. Experiments were performed as described in Section 2. *Close*
529 *circles*: suspension; *triangle,* dashed line: supernatant; *open circles*: reference.

530

531 **Fig. 2** Relative activity profiles of the supernatants, with respect to the initial value, during the
532 desorption tests of β-galactosidase and CALB immobilized on octyl-CALB-PEI, at different NaCl
533 concentrations. The β-galactosidase activity was 20 U/g. Experiments were performed as described
534 in Section 2. *Triangles*, dashed line: CALB activity; *close circles*: β-galactosidase; *open circles*:
535 reference.

536

537 **Fig. 3** SDS-PAGE analysis of different biocatalyst preparation and free enzymes used in this study.
538 Lane 1: low molecular weight protein standard from GE Healthcare. Lane 2: commercial free β-
539 galactosidase. Lane 3: commercial free CALB. Lane 4: octyl-CALB (CALB 5 mg/g of support).
540 Lane 5: octyl-CALB-PEI (CALB 5 mg/g of support). Lane 6: β-galactosidase on octyl-CALB-PEI
541 (CALB 2 mg/g of support). Lane 7: β-galactosidase on octyl-CALB-PEI (CALB 4 mg/g of
542 support). Lane 8: β-galactosidase on octyl-CALB-PEI (CALB 5 mg/g of support).

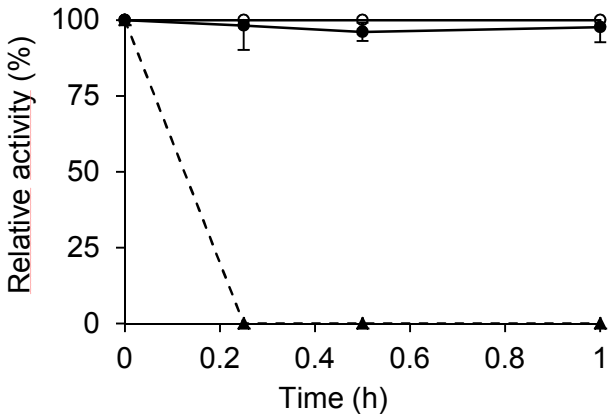
543

544 **Fig. 4** Immobilization courses of β-galactosidase on octyl (panel a) and octyl-PEI (panel b) supports
545 at pH 7. Experiments were performed as described in Section 2. *Circles*: suspension; *triangles*:
546 supernatant.

547

548 **Fig. 5** Cycles of β-galactosidase thermal inactivation- desorption- ionic binding from octyl-CALB-
549 PEI composite. Experiments were performed as described in Section 2. *Circles*: lipase activity,
550 *rhombus*: galactosidase activity.
551
552 **Fig. 6** SDS-PAGE analysis of β-galactosidase desorption from octyl-CALB-PEI composite. Lane 1:
553 low molecular weight protein standard from GE Healthcare. Lane 2: octyl-CALB-PEI-β-gal. Lane
554 3: desorption of β-gal with 0.3M NaCl after thermal inactivation. Lane 4: desorption of β-gal with
555 0.3M NaCl without previous thermal inactivation. Lanes 5 and 6: desorption of β-gal with 1 M
556 NaCl with- and without previous thermal inactivation, respectively.
557

(a)



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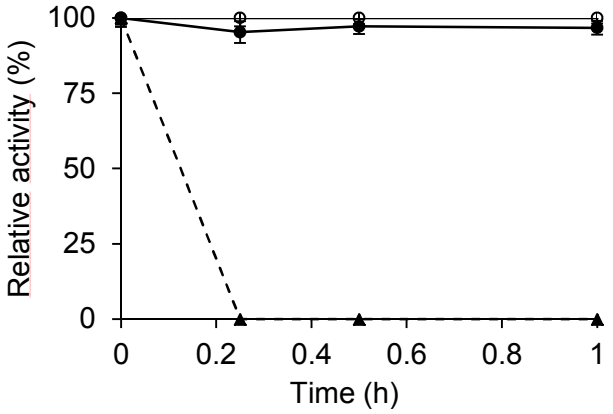
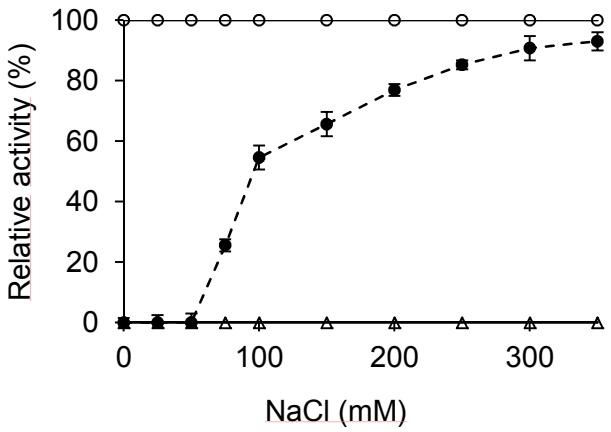


Fig. 1



566 **Fig. 2**

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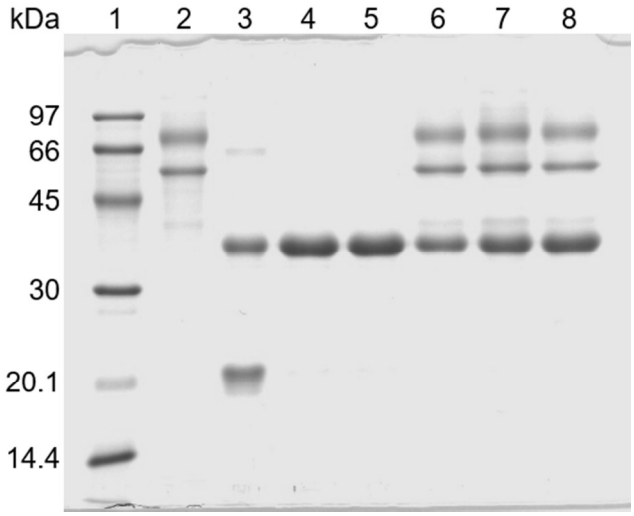

570 **Fig. 3**

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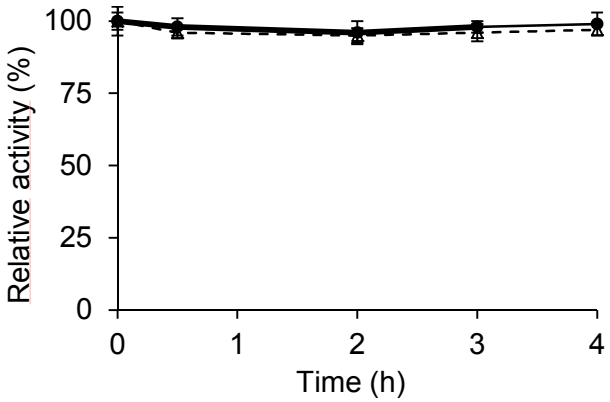
(a)



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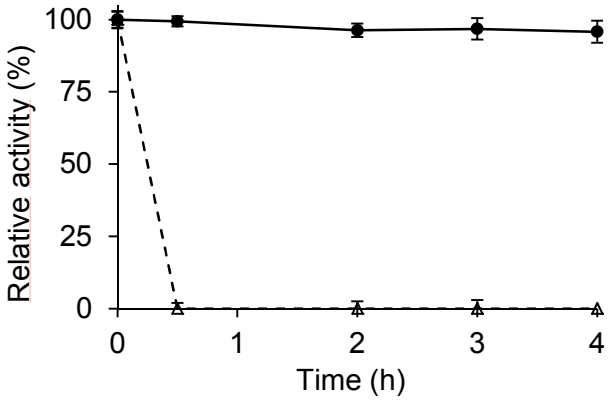
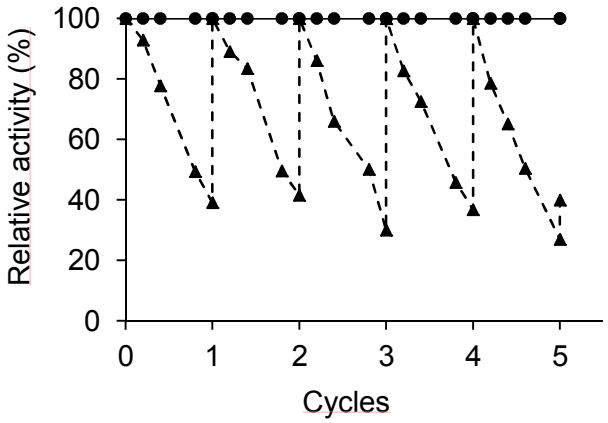


Fig. 4



583

584 **Fig. 5**

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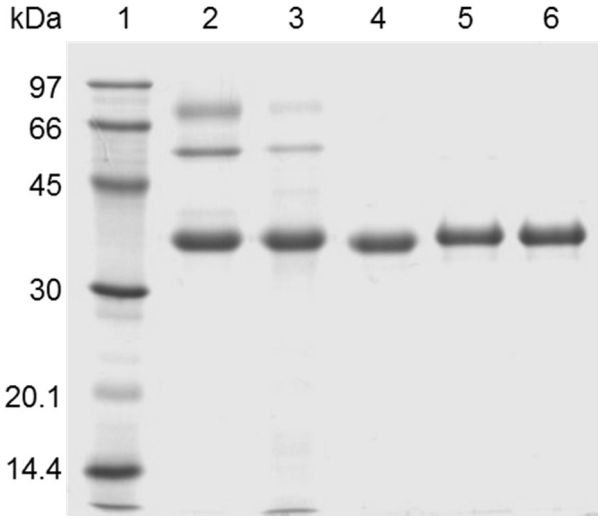
587



588

589 **Fig. 6**

590

591 **Table 1.** Adsorption of polyethyleneimine on octyl-PEI and octyl-CALB-PEI composites before
592 and after treatment with sodium chloride. The PEI content was determined by the TNBS assay and
593 is expressed in absorbance units at 425 nm.
594

Condition	Octyl-PEI	Octyl-CALB-PEI
Without treatment	0.49±0.07	0.84±0.06
After added 300 mM NaCl	0.15±0.03	0.81±0.05

595
596
597
598 **Table 2**. Residual polyethyleneimine on octyl-CALB-PEI after various cycles of union-thermal
599 inactivation-detachment of β- galactosidase.
600

Cycle	Residual PEI (%)
2	82±3
4	72±2
6	55±1

601
602