RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

VERSATILITY OF DIVINYLSULFONE SUPPORTS PERMITS THE TUNING OF CALB PROPERTIES DURING ITS IMMOBILIZATION

Jose C. S. dos Santos[a,b], Nazzoly Rueda[a,c], Alfredo Sanchez[d], Reynaldo Villalonga[d], Luciana R.B. Gonçalves[b] and Roberto Fernandez-Lafuente[a*].

[a]: ICP-CSIC. Campus UAM-CSIC. Cantoblanco. 28049 Madrid. Spain.

[b]: Departamento de Engenharia Química, Universidade Federal do Ceará, Campus do Pici, CEP 60455-760, Fortaleza, CE, Brazil.

[c] Escuela de Química, Grupo de investigación en Bioquímica y Microbiología (GIBIM), Edificio Camilo Torres 210, Universidad Industrial de Santander, Bucaramanga, Colombia.

[d]: Department of Analytical Chemistry, Faculty of Chemistry, Complutense University of Madrid, 28040 Madrid, Spain

* Corresponding author.
Prof. Dr Roberto Fernández-Lafuente
Departamento de Biocatálisis. Instituto de Catálisis-CSIC.
C/ Marie Curie 2. Campus UAM-CSIC. Cantoblanco.
28049 Madrid (Spain).
e-mail: rfl@icp.csic.es

Abstract

The lipase B from *C. antarctica* (CALB) has been immobilized on divinylsulfone (DVS) activated agarose beads under different conditions (pH 5-10). In the presence of 0.3% of Triton X-100, immobilization rate was rapid at pH 10 and the slowest one was that at pH 5. Incubation at pH 10 for 72 h of the immobilized enzymes before blocking of the support with ethylenediamine permitted to improve enzyme stability. Enzyme features (activity, stability, specificity versus different substrates, effect of the pH on enzyme properties) were quite different on the different CALB preparations, suggesting the different orientation of the enzyme. The alkaline incubation produced an increase in enzyme activity with some substrates, and some of the DVS-CALB preparations exhibited a higher specific activity than the octyl- preparations. The indirect fluorescence spectrum of the different immobilized preparations confirmed that different structures of the CALB molecules were generated after immobilization.

Keywords: lipase properties tuning, enzyme immobilization, enzyme stabilization, lipase hyperactivation, interfacial activation, divinylsulfone activated supports

1. Introduction

Lipases are the most utilized enzymes in biocatalysis[1-6] due to their wide substrate specificity, high stability under a broad range of conditions and reaction media (aqueous, organic solvent, neoteric solvents)[7,11] and broad range of reactions (e.g., hydrolysis, esterifications, aminations, acydolysis, transesterifications[1,6] , and also other promiscuous reactions like perhydrolysis or C-C bond synthesis)[12,14] that they are able to catalyze.

Moreover, lipase properties, including selectivity, specificity and activity are very easily modulated by almost any change in the enzyme or in the reaction media (including genetic manipulation[15,16] , medium engineering[17], physico-chemical modification of the enzyme surface by polymers or small reagents[18-20] , or via immobilization[21,24]) . This is due to the flexibility of their active center, which is a consequence of the conformational changes that the lipases suffer during catalysis, involving the movement of an oligopeptide chain (lid or flat) that usually isolates the active center of lipases from the medium[25-28]. The open form of the lipases becomes strongly adsorbed to their natural substrates (drops of oils) or any other hydrophobic surface, becoming stabilized[4,29,30].

Enzyme immobilization is a prerequisite for most industrial processes, as a way to easily recover and reuse these relatively expensive biocatalysts and to avoid product contamination[31,36]. Thus, the coupling of enzyme immobilization to the improvement of other enzyme features seems to be a very adequate goal in biocatalyst design, and in fact it has been reported improvement in enzyme stability, activity, selectivity, etc. upon immobilization[22,23,37,40].

The tuning of lipase catalytic properties via immobilization is based on involving different regions of the enzyme on the interaction with the support and on the control of the

support-enzyme interaction degree[22,24]; this may generate different nano-environments on the enzyme surroundings, may distort the regions involved in the immobilization, or may just avoid some movements during the opening/closing conformational changes. This has been achieved by using different immobilization protocols, which involve different enzyme moieties in the immobilization[22,24]. However, in some cases a versatile support may permit to immobilize an enzyme by different orientations by controlling the immobilization conditions[24]. This is the case of heterofunctional supports, such as glutaraldehyde. This support has been used to give 4 different preparations of a lipase just by altering the ionic strength or adding detergents during immobilization[41,42].

Divinylsulfone activated supports have been used for the successful immobilization of some proteins.[43,51] Recently, activated divinylsulfone agarose beads have been described as a suitable support to stabilize enzymes via multipoint covalent attachment [52]. The reactive group is very stable in a broad range of pH values (from 5 to 10), capable of reacting with primary and secondary amines, hydroxyl, phenyl, thiol and imidazol groups [52] . However, the reactivity of each enzyme group versus the vinylsulfone support differed greatly, and also was greatly influenced by the pH value [52]. At pH 10, the Lys residues are only slightly less reactive that Cys or His (the most reactive ones), while at pH 5 event he reactivity of the Tyr overpassed the reactivity of Lys residues [52]. Thus, altering the immobilization conditions, it is possible to immobilize an enzyme via different orientations on supports activated with divinylsulfone[52]. The further long time incubation at alkaline pH value permitted to increase the number of enzyme-support linkages, increasing the enzyme rigidity[52].

In this paper, we show the results obtained in the immobilization under different conditions of the most popular lipase, the lipase B from *Candida antarctica*,[53,54] on agarose beads activated with divinylsulfone with the objective of checking the possibility of using the

features of this support to alter the catalytic properties of lipases. To this goal, the hydrolytic activity versus structurally different substrates and the stability of the immobilized enzyme under different conditions will be studied. Finally, we will try to correlate the changes in enzyme function after immobilization on the same support but following different protocols with changes in the lipase structure for the first time in the literature.

2. Materials and methods

2.1. Materials

Lipase B from *Candida antarctica* (CALB) was kindly donated by Novozymes (Spain), p-nitrophenyl butyrate (p-NPB), divinylsulfone (DVS), triton X-100, ethylenediamine (EDA), 8-anilino-1-naphthalenesulfonic acid (ANS), 2-mercaptoethanol, methyl mandelate, methyl phenylacetate and ethyl hexanoate were from Sigma Chemical Co. (St. Louis, MO, USA). Octyl sepharose beads 4BCL and cyanogen bromide Sepharose beads 4BCL (CNBr) were from GE Healthcare. All reagents and solvents were of analytical grade.

All experiments were performed by triplicate and the results are reported as the mean of this value and the standard deviation (usually under 10%).

2.2. Standard determination of enzyme activity

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 50 mM sodium phosphate at pH 7.0 and 25^{o}C (ε= 5150 $M^{-1}cm^{-1}$under these conditions). To start the reaction, 50–100 µL of lipase solution or suspension were added to 2.50 mL of substrate solution. One unit of activity (U) was defined as the amount of enzyme that hydrolyzes 1 µmol of *p*-NPB per minute under the conditions previously described. Protein concentration was determined using Bradford's method [55] , bovine serum albumin was used as the reference.

In the studies of the effects of pH on the enzyme activity, the protocol was similar but the buffer in the measurements was changed according to the pH value: sodium acetate at pH

5, sodium phosphate at pH 6-8 and sodium borate at pH 9-10. At 25ºC, all the preparations remained fully active after incubation for several hours at any of these pH values.

2.3. Immobilization of CALB on octyl Sepharose beads

Lipase CALB was immobilized on octyl Sepharose beads at low ionic strength as previously described [56]. A volume of 1.6 mL of commercial enzyme (containing 6.9 mg/mL of protein) was diluted in 88.4 mL of 5 mM sodium phosphate at pH 7, maintaining a 1/10 support–enzyme solution ratio, (w/v) for 60 min. Suspension and supernatant samples were withdrawn for evaluation of immobilization through enzymatic activity measurement as described above. This immobilization strategy also permitted the purification of lipases from contaminant esterases [56].

2.4. Immobilization of CALB on CNBr-agarose beads

Immobilization of CALB on CNBr-agarose beads was performed following a protocol previously described for this enzyme [57] . A volume of 1 mL of commercial CALB was diluted in 99 mL of 5 mM sodium phosphate at pH 7. Then, 6 g of wet CNBr support was added. After 90 min at 4^{o}C under stirring at 250 rpm, around 56% of lipase became immobilized on the support. The enzyme-support reaction was ended by incubating the support with 1 M ethanolamine at pH 8 for 2 h. Finally, the immobilized preparation was washed with abundant distilled water.

2.5. Immobilization of CALB on DVS-agarose beads

2.5.1. Preparation of DVS-agarose beads. 1.5 mL divinylsulfone was added to 40 mL of 333 mM sodium carbonate at pH 12.5 and stirred until the mixture becomes homogeneous, then 2 g of agarose beads was added and left under gentle agitation for 35 minutes [52]. Finally, the activated support was washed with an excess of distilled water and stored at 4°C.

2.5.2 Immobilization of CALB on DVS-agarose beads. A 10 g portion of support was suspended in 100 mL of solutions of CALB (maximum protein concentration was 1 mg/mL) at 25°C using 10 mM of different buffers (sodium acetate at pH 5, sodium phosphate at pH 7 or sodium carbonate at pH 10). In some instances, triton X-100 was added. In some cases, the immobilized lipase preparations were filtered and a portion of the derivatives was incubated in 100 mL of 100 mM bicarbonate at pH 10.0 and 25°C for 72 h. As an enzyme-support reaction end-point, all the immobilized biocatalysts were incubated in 1M EDA at pH 10 and 25ºC for 24 h to block the remaining reactive groups on the support (this was the optimal blocking reagent using chymotrypsin and this support) [52]. Finally, the immobilized preparation was washed with an excess of distilled water and stored at 4°C.

2.6. Thermal inactivation of different CALB immobilized preparations

To check the stability of the different enzyme derivatives, 1 g of immobilized enzyme was suspended in 5 mL of 50 mM sodium acetate at pH 5, sodium phosphate at pH 7 or sodium carbonate at pH 9 and at different temperatures. Periodically, samples were withdrawn and the activity was measured using pNPB. Half-lives were calculated from the observed inactivation courses.

2.7. Stability assays in the presence of dioxane

Enzyme preparations were incubated in mixtures of 70 % dioxane/30% 100 mM Tris buffer at pH 7 and at different temperatures to proceed with their inactivation. Periodically, samples were withdrawn and the activity was measured using p-NPB as described above. Half-lives were calculated from the observed inactivation courses. The acetonitrile presented in the measurement samples had no significant effect on enzyme activity determination experiments.

2.8 Hydrolysis of methyl mandelate

200 mg of the immobilized preparations was added to 2 mL of 50 mM substrate in 100 mM sodium acetate at pH 5, 100 mM sodium phosphate at pH 7 or 100 mM sodium carbonate at pH 8.5 and 25^{o}C under continuous stirring. The conversion degree was analyzed by RP-HPLC (Spectra Physic SP 100 coupled with an UV detector SpectraPhysic SP 8450) using a Kromasil C18 (15 cm × 0.46 cm) column. Samples (20 μL) were injected and eluted at a flow rate of 1.0 mL/min using acetonitrile/10 mM ammonium acetate (35:65, v/v) at pH 2.8 as mobile phase and UV detection was performed at 230 nm. The acid has a retention time of 2.5 minutes while the ester has a retention time of 10 minutes. One unit of enzyme activity was defined as the amount of enzyme necessary to produce 1 μmol of mandelic acid per minute under the conditions described above. Activity was determined by triplicate with a conversion ranging 20–30%, and data are given as average values.

2.9. Hydrolysis of methyl phenylacetate

200 mg of the immobilized preparations were added to 2 mL of 5 mM substrate in 100 mM buffer containing 50% CH_3CN. The buffers were sodium acetate at pH 5, sodium phosphate at pH 7 and sodium bicarbonate at pH 8.5. All experiments were carried out at 25^{o}C under continuous stirring. The conversion degrees were analyzed by RP-HPLC (Spectra PhysicSP 100 coupled with an UV detector Spectra Physic SP 8450) using a Kromasil C18 (15 cm × 0.46 cm) column. Samples (20 µL) were injected and eluted at a flow rate of 1.0 mL/min using a mixture of acetonitrile: 10 mM ammonium acetate aqueous solution (35:65,v/v) and pH 2.8, as mobile phase and UV detection was performed at 230 nm. The acid has a retention time of 3 minutes while the ester has a retention time of 12 minutes. One unit of enzyme activity was defined as the amount of enzyme necessary to produce 1 µmol of phenyl acetic acid per minute under the conditions described above. The activity was determined by triplicate with a maximum conversion of 20–30%, and data are given as average values.

2.10. Hydrolysis of ethyl hexanoate

Enzyme activity was determined by using ethyl hexanoate; 200 mg of the immobilized preparations were added to 2 mL of 25 mM substrate in 50 mM buffer containing 50 % CH_3CN. The buffer was sodium acetate at pH 5, sodium phosphate at pH 7 and sodium bicarbonate at pH 8.5. All experiments were carried out at 25 ºC under continuous stirring. The conversion degree was analyzed by RP-HPLC (Spectra Physic SP 100 coupled with an UV detector Spectra Physic SP 8450) using a Kromasil C18 (15 cm x 0.46 cm) column. Samples (20 µL) were injected and eluted at a flow rate of 1.0 mL/min using acetonitrile /10 mM ammonium acetate aqueous solution (50:50, v/v) and pH 3.2 as mobile phase and UV detection

was performed at 208 nm. Hexanoic acid has a retention time of 3.4 minutes while the ester has a retention time of 14.2 minutes. One unit of enzyme activity was defined as the amount of enzyme necessary to produce 1 μmol of hexanoic acid per minute under the conditions described above. Activity was determined by triplicate with a maximum conversion of 20-30%, and data are given as average values.

2.11. Fluorescence studies of the different immobilized enzyme preparations

The immobilized enzyme preparations (150 mg) were mixed with 15 mL of 13.5 μM 8-anilino-1-naphthalenesulfonic acid (ANS) solution in 10 mM Tris·HCl buffer, pH 7.0. The mixtures were incubated at 25ºC during 1 h under magnetic stirring. The samples were centrifuged and the emission fluorescence spectra of the supernatant solutions were recorded after excitation at 360 nm by using a Cary Eclipse Spectrophotometer (Varian)[58].

3. Results

3.1. Immobilization of CALB on divinylsulfone activated agarose beads at different pH values

Figure 1 shows the immobilization courses of CALB at pH 5, 7 and 10. It should be remarked that free CALB remained fully active under all assayed conditions (not shown results). Surprisingly, the immobilization was very rapid in all cases, even though at pH 5 the reactivity of most nucleophilic groups of a protein versus vinylsulfone should be quite reduced [52]. Furthermore, an increase in enzyme activity after immobilization was appreciated, approximately 50% in the 3 cases.

These facts could be explained if the enzyme was immobilized via another mechanism, such as physical adsorption. This could be, for example, the interfacial activation of the lipase in the fairly hydrophobic divinylsulfone layer on the agarose surface [56]. This hydrophobicity feature of the support was not detected using chymotrypsin [52]. Figure 2 shows the structure of the activating group [45,51].This group is moderately hydrophobic, so that a dense layer of this group may enable interfacial activation of the enzyme [56]. To check if any physical adsorption could be the cause of the immobilization of CALB, the reactive groups in the support were blocked by incubation with 2-mercaptoethanol or destroyed by incubation at pH 12 and 50ºC [52]. These unreactive supports were incubated in the presence of CALB and even though the effects on enzyme activity were not identical, the immobilization rates remained pH independent and were very similar to those of the activated support (results not shown). After these treatments, it has been described that aminoacids cannot immobilize on the support, because their chemical reactivity has been destroyed, and the immobilization of the enzyme confirmed that the covalent attachment was not the first step in the immobilization of CALB on DVS activated agarose in the previous experiments.

3.2 Effect of Triton X-100 on the immobilization of CALB on divinylsulfone support beads

A detergent is able to desorb the enzyme from a hydrophobic support, even a very hydrophobic one, and may be used to prevent the lipase immobilization via interfacial activation [59,60]. By progressively adding Triton X-100 to the DVS-support and the lipase suspension, it was possible to reduce the adsorption of the enzyme on the inactivated support (Figure 3). Using 0.3% detergent, CALB did not immobilize on any of the inactivated supports. These results confirmed that the immobilization on this support could be founded on the interfacial activation of CALB on the fairly hydrophobic surface of the support. In fact, if the enzymes adsorbed on the reactive (neither blocked nor incubated at pH 12) DVS support at pH 5 or 7 were incubated in the presence of detergent just after immobilization, more than 80% of the enzyme released from the support. When this experiment was performed on the preparation at pH 10, less than 10% of the immobilized enzyme was released, showing that most of the enzyme was covalently attached to the support (although it is not clear which one is the first step of the immobilization; covalent attachment or interfacial activation; at least a 10% of the enzyme molecules is not covalently immobilized after 3 hours but it is already immobilized).

Thus, a new batch of CALB immobilizations on DVS-agarose was carried out at pH 5, 7 and 10, but in the presence of enough detergent to prevent lipase adsorption on the inactive DVS support (0.3% Triton X-100) (Figure 4). Immobilization was relatively rapid at pH 10 (full immobilization after 3 h). At pH 7, immobilization was slower (70% after 24 h) and even slower still at pH 5 (under 30% after 24 h). These results fitted better with the expected

chemical reactivity of the enzyme groups at different pH values versus the DVS activated support[52].

Looking at the activity, the immobilization at pH 10 produced an increase in enzyme activity (around 30%) while at the other pH values, the activity slightly decreased after immobilization. This higher activity at pH 10 is curious, as it may not be due to a lower intensity of the enzyme-support reaction [52].

To enhance immobilization yields, a ratio of 1 g of support to 3 mL of enzyme suspension was used. Under these conditions CALB immobilization was almost complete even at pH 5 after 24 h (results not shown).

3.3. Effect of the long term incubation at alkaline pH value on enzyme activity

After immobilization, and in order to favor the multipoint covalent immobilization, the three immobilized CALB biocatalysts (immobilized at pH 5, 7 or 10) were incubated at pH 10 for 72 h, after washing the detergent. Results are shown in Figure 5.

The preparation immobilized at pH 10 increased the activity for 48 h, and later kept that value constant (near 170%).

The enzyme immobilized at pH 7 suffered an increase in the activity during the alkaline incubation (around 220%), and this effect was even more relevant if the enzyme had been immobilized at pH 5 (over 250%). The most active preparations were those incubated at pH 10 in all cases (Table 1), even though under these conditions a higher enzyme-support chemical reaction should occur. This increase in enzyme activity upon incubation at alkaline pH values

could be explained as a function of enzyme distortions caused by the enzyme/support reaction that, in this case, presented positive effects on enzyme activity.

In order to compare the enzyme properties after immobilization, CALB was also immobilized on octyl agarose and CNBr agarose. The enzyme immobilized on octyl agarose (results not shown) presented less than 60% of the activity of the enzyme immobilized on DVS support and incubated at pH 10. It should be considered that the small lid of CALB makes that the activity of the enzyme is not significantly increased after immobilization on octyl agarose (around a 10%). The enzyme immobilized on CNBr agarose did not significantly alter its activity (Table 1).

3.4. Characterization of the immobilized biocatalysts

The 6 new covalent preparations have been compared against each other and also with the two standard immobilization protocols, CALB immobilized on CNBr- and octyl-Sepharose.

3. 4.1. Activity/pH versus pNPB

Table 1 shows the activities of the 8 preparations under standard conditions after blockage. The hyperactivation caused by the alkaline incubation at pH 10 is clearly visualized, the enzyme immobilized at pH 5 started with 2 fold less activity than the enzyme immobilized at pH 10, but after alkaline incubation, the higher increase on enzyme activity permitted to almost equilibrate the observed activities. All of them (except the enzyme just immobilized at pH 5) are more active than the octyl preparation, which is also slightly more active than the CNBr preparation.

306 Figure 6 shows the enzyme activity/pH profile using the different immobilized samples.
307 The main difference is found when comparing the enzymes immobilized on different supports.
308 The enzyme immobilized on CNBr- Sepharose presented the maximum of activity at pH 7,
309 with a sharp decrease at either alkaline or acidic pH values (activity was around 40% at pH 5
310 and 10). Using octyl agarose as support, the maximum activity was found at pH 8, and the
311 decrease in activity at acidic and alkaline pH values is milder (55% at pH 5 and 70% at pH 10).
312 The enzyme immobilized on DVS support under different conditions presented the maximum
313 activity at the highest pH used in the study (pH 10), and only slight differences were found on
314 the immobilization pH or long term incubation at alkaline pH value. The enzyme immobilized
315 at pH 5 showed an 18% or 25% of the maximum activity at pH 5, for the non-incubated or long
316 term incubated enzyme preparations respectively. Both enzyme preparations immobilized at
317 pH 7 exhibited 40% of the maximum activity at pH 5, while the preparations immobilized at
318 pH 10 showed around 30% of this activity.

319

320 **3.4.2. Thermal stability under different conditions at different pH values.**

321 Table 2 shows the half-lives of the different CALB preparations under different
322 inactivation conditions. We only show the results obtained in the temperature where the
323 inactivations have a rate that permitted to obtain reliable results in a reasonable time.

324 The most stable preparation was that obtained using octyl-agarose when the
325 inactivations were performed at pH 5 or 7. The just immobilized DVS preparations were far
326 less stable, but their stabilities improved after long-term incubation to favor multipoint
327 covalent attachment, becoming more stable than the CNBr-CALB in all cases.

If the inactivation was performed at pH 5, the alkaline incubation increased the half live from 4.5 to 35 minutes for the enzyme immobilized at pH 5, if the immobilization was performed at pH 7, the stability increased to a lower extent, from 33 to 60 minutes. The value of the half live of the enzyme immobilized at pH 10 went from 32 to 46 minutes after the long term incubation, a value lower than that obtained with the immobilization at pH 7 and incubated at pH 10.

The pattern was somehow similar looking at the inactivations carried out at pH 7, the enzyme immobilized at pH 7 and incubated at alkaline conditions was the most stable one, followed by the enzyme immobilized at pH 10 and the enzyme immobilized at pH 5.

At pH 9, the situation varied. The enzyme immobilized at pH 7 presented a stability similar to that of the octyl, and the alkaline incubation of this preparation permitted to double the half-life. The stabilities of the enzymes immobilized at pH 5 or 10 were quite similar, both after immobilization and after long term alkaline incubation before blocking. In both cases, the stability became similar to that of the octyl-CALB after the alkaline incubation. It may be likely that at pH 9 the cause of the inactivation is a conformational change in another area of the enzyme or just a chemical modification of some groups, this can explain the significant qualitative change in the stability of the different preparations.

Considering that in all cases the support was the same for the DVS immobilized enzymes, and that the long term incubation of 3 days should permit a similar reaction between the enzyme and the support, the differences on enzyme stability must be related to populations of enzyme molecules having different orientations, with different relevance for enzyme stability [61,62] or different density of groups able to react with the support, giving differences in the final intensity of the multipoint covalent attachment[22].

The high thermostability of the lipases adsorbed on hydrophobic supports has been previously described [63]. These preparations are much more stable than the glyoxyl agarose-CALB, and this was explained by the very stable conformation that the open form of the adsorbed lipases presented [64], and the moderate amount of nucleophilic groups that many lipases presented in its surface make complex a very intense multipoint covalent attachment (e.g., CALB has 9 Lys plus the Leu 1, all of them exposed to the medium).[65,57]

3.4.3. Solvent stability

In opposition to the results obtained during thermal inactivations, Table 2 shows that in all cases the DVS preparations were by far more stable that the octyl or CNBr-Sepharose immobilized enzymes when they were incubated in the presence of 70% dioxane. Analyzing the DVS preparations blocked just after immobilization, the most stable biocatalyst was that prepared at pH 5 (half live of 5 minutes), being the stability of the enzymes immobilized at pH 7 and 10 very similar (1.5-1.7 minutes). However, after the long term incubation the enzyme immobilized at pH 7 greatly improved the stability (to more than 7 minutes), while the enzyme immobilized at pH 5 maintained its stability practically unaltered after alkaline incubation and the enzyme immobilized at pH 10 improved its the stability by only 50%.

The low stability of CALB immobilized on octyl-agarose in the presence of dioxane may be related to the enzyme desorption caused by the presence of this very high cosolvent concentration, the free enzyme is rapidly inactivated under these drastic conditions. [52,66]

The different stability of the enzymes immobilized at different pH value on DVS activated supports, where after long term alkaline incubation the only difference may be the enzyme orientation, suggests that the inactivation of CALB follows a different route on different inactivation conditions, Some protein regions are more relevant on the stability at

certain conditions, while some other areas may be more relevant on other experimental conditions [61,62,67].

3.4.4. Activity versus different esters

Immobilization has been reported to alter enzyme specificity and the influence of the pH on the activity, if enzyme orientation on the support or the intensity of the enzyme/support interaction is different [22,24]. Thus, differences in enzyme specificity or influence on activity/pH curve upon different immobilization protocols can reinforce the idea on a different enzyme orientation on the support surface. Three different substrates have been used at 3 different pH values: esters formed by an aliphatic acid (ethyl hexanoate), one aromatic acid (phenylacetate) or one aromatic and chiral one (mandelic acid) and the results are resumed on Table 3.

Using ethyl hexanoate, results are quite diverse depending on the biocatalyst. The highest activity was usually found at pH 5, except for the preparation immobilized at pH 5 and then incubated at alkaline pH, where the maximum activity was found at pH 7. The enzyme just immobilized at pH 7 on DVS was the most active one at pH 5 and pH 7 while at pH 8.5 the most active one was the octyl-Sepharose preparation. The long term incubation at alkaline pH of the DVS preparations usually decreased the enzyme activity, mainly at pH 5. The enzyme immobilized at pH 5 is the one with the most drastic change after alkaline incubation, with a shift in the maximum activity at pH 7 (becoming more active than the enzyme just immobilized at pH 5 under these conditions, that is, alkaline incubation produced an hyper-activation at pH 7). On the other hand, the enzyme immobilized at pH 5 and at pH 10 improved the activity after alkaline incubation if the activity was determined at pH 8.5. In general, the effect of the change of the pH in the activity determination presented a more drastic effect on

DVS preparations without long term alkaline incubation (e.g., from 425 U/mg to 24 U/mg using the enzyme immobilized at pH 10) than in octyl or CNBr preparations (activity at pH 8.5 was around 60% and 30% than that at pH 5, respectively). Long term incubation at alkaline pH reduced this effect of the pH on DVS-CALB activity.

Using methyl phenylacetate, at pH 5 the most active preparations are two DVS preparations, those just immobilized at pH 7 (22.5 U/mg) and pH 10 (18.7 U/mg). At pH 8.5, octyl and CNBr CALB preparations presented the highest activity, while at pH 7 the most active preparations were CNBr and DVS immobilized at pH 7. The lowest activity for all preparations immobilized on DVS was that found at pH 8.5, except for the enzyme immobilized at pH 5 on DVS and submitted to alkaline incubation that have the minimum activity at pH 7. The highest activity depended on the immobilization protocol. The DVS preparations immobilized at pH 5 had a clear maximum at pH 5; while both preparations immobilized at the other two pH values have not a clear maximum (similar activities are detected at pH 5 and 7). Octyl and CNBr CALB had a clear maximum at pH 7. Long term alkaline incubation decreased enzyme activity in all cases, but the intensity of this effect depended on the immobilization pH and activity determination pH.

Using mandelic ester, new changes were found. The most active preparations at pH 5 were both preparations immobilized at pH 7 on DVS, at pH 7 the most active preparations were the CNBr preparation and the enzyme immobilized at pH 5 or pH 7 and long term submitted to alkaline incubation before blocking. At pH 8.5, the most active biocatalysts were those immobilized at pH 7 and long term incubated and the CNBr preparation. There are examples where the highest activity was found at pH 7 (octyl, CNBr, both DVS immobilized at pH 5 and both immobilized at pH 10). The enzyme immobilized at pH 7 has not a clear maximum activity, and after incubation this optimum is clearly at pH 8.5. The long term

422 incubation of the DVS preparations used to have a positive effect on enzyme activity, except
423 when the enzyme was immobilized at pH 10, where the alkaline incubation decreased the
424 enzyme activity when measured at pH 5 or 8.5, while having almost no effect at pH 7.

425 Thus, CALB immobilized following different protocols on DVS-activated supports
426 (different immobilization pH values, long term incubation or not under alkaline conditions)
427 presented very different enzyme specificity and very different response to changes on
428 environmental conditions, confirming that the different preparations have different orientation
429 and/or degree of enzyme/support interaction[68] .

430

431 **3.4.5 Evaluation of the structure of different CALB immobilized preparations**

432 The influence of the different immobilization strategies on the 3D conformation of the
433 enzyme was determined by using the ANS-binding fluorescence assays. ANS is a hydrophobic
434 fluorescent dye that strongly binds the clusters from hydrophobic amino acid side chains in β-
435 sheet conformations of proteins [59]. Usually, a great density of those hydrophobic clusters is
436 well protected from the solvent in native enzymes due to the rigid packing of the globular
437 protein conformation. Accordingly, a decrease in the fluorescence intensity of the ANS dye can
438 be attributed to its binding to the exposed hydrophobic regions in partially unfolded proteins.

439 Figure 7 shows the fluorescence emission spectra of the biocatalysts prepared through
440 different immobilization protocols. In comparison with the raw support (line a), the
441 fluorescence intensity of ANS decreased after incubation with all immobilized lipase
442 preparations. This fact can be ascribed to the presence of the enzyme molecules on the support
443 surface, and thus, to the binding of ANS molecules to the exposed hydrophobic clusters in
444 these proteins. On the other hand, the results obtained using the biocatalyst prepared by

immobilization at pH 5 and further incubation at 10 (line c) showed the lowest fluorescence signal, much lower than using the enzyme immobilized at pH 5 (line b). This result suggested that the immobilization approach based on two consecutive incubation steps at pH 5 and 10 leads to protein conformations with partially exposed hydrophobic β-sheet clusters, and accordingly, more prone to bind the hydrophobic ASN molecules, than when the enzyme is just immobilized at pH 5 and them blocked. That is, alkaline incubation produced conformational changes on the enzyme that led to the exposition of more hydrophobic groups to the medium.

When the enzyme is immobilized at pH 10 (line d), the effect of the further alkaline incubation is in the opposite direction (line e), the fluorescence signal increased after the alkaline incubation, less hydrophobic groups are partially exposed suggesting a more rigid and compact structure. Again, the changes in enzyme properties could be correlated to conformational changes.

Moreover, it is clear thatthe difference in the exposition of protein hydrophobic groups of the enzyme immobilized at pH 5 and that immobilized at pH 10, in both cases after 72 h of incubation at pH 10 before support blocking is quite significant, with much higher exposition using the enzyme immobilized at pH 5 and incubated at pH 10. The results may be explained by the implication of different areas of the enzyme in the multipoint covalent attachment. This produced fully different effects on the enzyme structure (making more compact one and more relaxed the other), The effects on the exposition of the hydrophobic groups surrounding the active of the lipase (the small lid and adjacent areas) may be also considered. These differences may explain the drastic changes of enzyme properties when immobilized at different pH values discussed along this paper, and suggest that the areas reacting with the support for those 72 h could be different.

Conclusions

Immobilization of CALB on DVS-supports under different conditions permits to have covalently immobilized preparations exhibiting very different properties. The change in the immobilization pH permits to alter the enzyme specificity, activity and stability, whilefurther incubation under alkaline conditions (described as a way to improve the enzyme support reaction) [52] also produced changes in enzyme features. The indirect determination of the ANS incubated enzyme fluorescence showed that the different enzyme derivatives have different structures.

Thus, DVS activated supports may be a potent way to tuning lipase properties via immobilization. The DVS activation of supports compatible with organic media may increase the range of reactions where the biocatalysts may be used and provide new data on the different behavior of CALB immobilized on different supports-

Acknowledgments

We gratefully recognize the support from the MINECO of Spanish Government, by by the grant CTQ2013-41507-R. The predoctoral fellowships for Ms. Rueda (Colciencias, Colombian Goberment) and Mr dos Santos (CNPq, Brazil) are also recognized. The authors wish to thank Mr. Ramiro Martínez (Novozymes, Spain) for kindly supplying the enzymes used in this research. The help and comments from Dr. Ángel Berenguer (Instituto de Materiales, Universidad de Alicante) are kindly acknowledged.

References

1. K. E. Jaeger and T. Eggert, *Curr. Opin. Biotechnol.*, 2002, 13, 390–397.
2. R. Sharma, Y. Chisti and U. C. Banerjee, *Biotechnol. Adv.*, 2001, **19**, 627–662.
3. K. E. Jaeger and M. T. Reetz, *Trends Biotechnol.*, 1998, 16, 396–403.
4. R. D. Schmid and R. Verger, *Analysis*, 1998, **37**, 1608–1633.
5. A. Pandey, S. Benjamin, C. R. Soccol, P. Nigam, N. Krieger and V. T. Soccol, *Biotechnol. Appl. Biochem.*, 1999, **29 (Pt 2)**, 119–131.
6. J. Zhang, H. Shi, D. Wu, Z. Xing, A. Zhang, Y. Yang and Q. Li, *Process Biochem.*, 2014, 49, 797–806.
7. P. Adlercreutz, *Chem. Soc. Rev.*, 2013, **42**, 6406–36.
8. P. Lozano, J. M. Bernal, G. Sánchez-Gómez, G. López-López and M. Vaultier, *Energy Environ. Sci.*, 2013, **6**, 1328.
9. P. Lozano, J. M. Bernal and A. Navarro, *Green Chem.*, 2012, 14, 3026.
10. P. Lozano, J. M. Bernal and M. Vaultier, *Fuel*, 2011, **90**, 3461–3467.
11. Y. Fan and J. Qian, *J. Mol. Catal. B Enzym.*, 2010, 66, 1–7.
12. C. Li, X.-W. Feng, N. Wang, Y.-J. Zhou and X.-Q. Yu, *Green Chem.*, 2008, 10, 616.
13. E. Busto, V. Gotor-Fernández and V. Gotor, *Chem. Soc. Rev.*, 2010, **39**, 4504–4523.
14. M. Kapoor and M. N. Gupta, *Process Biochem.*, 2012, 47, 555–569.
15. N. J. Turner, *Nat. Chem. Biol.*, 2009, **5**, 567–73.
16. U. T. Bornscheuer and M. Pohl, *Curr. Opin. Chem. Biol.*, 2001, 5, 137–143.
17. J. M. Palomo, G. Fernandez-Lorente, C. Mateo, C. Ortiz, R. Fernandez-Lafuente and J. M. Guisan, *Enzyme Microb. Technol.*, 2002, **31**, 775–783.
18. R. C. Rodrigues, Á. Berenguer-Murcia and R. Fernandez-Lafuente, *Adv. Synth. Catal.*, 2011, **353**, 2216–2238.
19. C. Garcia-Galan, J. C. S. J. C. S. dos Santos, O. Barbosa, R. Torres, E. B. Pereira, V. C. Corberan, L. R. B. Gonçalves, R. Fernandez-Lafuente and L. R. B. Gon??alves, *Process Biochem.*, 2014, 49, 604–616.

20. J. C. S. dos Santos, C. Garcia-Galan, R. C. Rodrigues, H. B. de Sant'Ana, L. R. B. Gonçalves and R. Fernandez-Lafuente, *Enzyme Microb. Technol.*, 2014, **60**, 1–8.

21. C. Garcia-Galan, Á. Berenguer-Murcia, R. Fernandez-Lafuente and R. C. Rodrigues, *Adv. Synth. Catal.*, 2011, **353**, 2885–2904.

22. K. Hernandez and R. Fernandez-Lafuente, *Enzyme Microb. Technol.*, 2011, **48**, 107–22.

23. R. C. Rodrigues, C. Ortiz, Á. Berenguer-Murcia, R. Torres and R. Fernández-Lafuente, *Chem. Soc. Rev.*, 2013, **42**, 6290–307.

24. O. Barbosa, R. Torres, C. Ortiz, A. Berenguer-Murcia, R. C. Rodrigues and R. Fernandez-Lafuente, *Biomacromolecules*, 2013, **14**, 2433–62.

25. A. M. Brzozowski, U. Derewenda, Z. S. Derewenda, G. G. Dodson, D. M. Lawson, J. P. Turkenburg, F. Bjorkling, B. Huge-Jensen, S. A. Patkar and L. Thim, *Nature*, 1991, **351**, 491–494.

26. K. K. Kim, H. K. Song, D. H. Shin, K. Y. Hwang and S. W. Suh, *Structure*, 1997, **5**, 173–185.

27. K. E. Jaeger, S. Ransac, H. B. Koch, F. Ferrato and B. W. Dijkstra, *FEBS Lett.*, 1993, **332**, 143–149.

28. M. Cygler and J. D. Schrag, *Biochim. Biophys. Acta*, 1999, **1441**, 205–14.

29. R. Verger, *Trends Biotechnol.*, 1997, 15, 32–38.

30. C. Cambillau, S. Longhi, A. Nicolas and C. Martinez, *Curr. Opin. Struct. Biol.*, 1996, 6, 449–455.

31. I. Chibata, T. Tosa and T. Sato, *J. Mol. Catal.*, 1986, **37**, 1–24.

32. W. HARTMEIER, *Trends Biotechnol.*, 1985, **3**, 149–153.

33. E. Katchalski-Katzir, *Trends Biotechnol.*, 1993, 11, 471–478.

34. J. F. Kennedy, E. H. M. Melo and K. Jumel, *Chem. Eng. Prog.*, 1990, **86**, 81–89.

35. A. M. Klibanov, *Science*, 1983, **219**, 722–727.

36. D. Brady and J. Jordaan, *Biotechnol. Lett.*, 2009, 31, 1639–1650.

37. R. Fernandez-Lafuente, *Enzyme Microb. Technol.*, 2009, **45**, 405–418.

38. C. Mateo, J. M. Palomo, G. Fernandez-Lorente, J. M. Guisan and R. Fernandez-Lafuente, *Enzyme Microb. Technol.*, 2007, **40**, 1451–1463.

39. M. Petkar, A. Lali, P. Caimi and M. Daminati, *J. Mol. Catal. B Enzym.*, 2006, **39**, 83–90.

40. U. Guzik, K. Hupert-Kocurek and D. Wojcieszyńska, *Molecules*, 2014, **19**, 8995–9018.

41. O. Barbosa, R. Torres, C. Ortiz and R. Fernandez-Lafuente, *Process Biochem.*, 2012, **47**, 1220–1227.

42. O. Barbosa, C. Ortiz, Á. Berenguer-Murcia, R. Torres, R. C. Rodrigues and R. Fernandez-Lafuente, *RSC Adv.*, 2014, **4**, 1583.

43. F. J. Lopez-Jaramillo, M. Ortega-Munõz, A. Megia-Fernandez, F. Hernandez-Mateo and F. Santoyo-Gonzalez, *Bioconjug. Chem.*, 2012, **23**, 846–855.

44. P. Prikryl, J. Lenfeld, D. Horak, M. Ticha and Z. Kucerova, *Appl. Biochem. Biotechnol.*, 2012, **168**, 295–305.

45. M. Ortega-Muñoz, J. Morales-Sanfrutos, A. Megia-Fernandez, F. J. Lopez-Jaramillo, F. Hernandez-Mateo and F. Santoyo-Gonzalez, *J. Mater. Chem.*, 2010, **20**, 7189.

46. J. C. Begara-Morales, F. J. López-Jaramillo, B. Sánchez-Calvo, A. Carreras, M. Ortega-Muñoz, F. Santoyo-González, F. J. Corpas and J. B. Barroso, *BMC Plant Biol.*, 2013, **13**, 61.

47. A. L. Medina-Castillo, J. Morales-Sanfrutos, A. Megia-Fernandez, J. F. Fernandez-Sanchez, F. Santoyo-Gonzalez and A. Fernandez-Gutierrez, *J. Polym. Sci. Part A Polym. Chem.*, 2012, **50**, 3944–3953.

48. K. Labus, A. Turek, J. Liesiene and J. Bryjak, *Biochem. Eng. J.*, 2011, **56**, 232–240.

49. J. Bryjak, J. Liesiene and B. N. Kolarz, *Colloids Surf. B. Biointerfaces*, 2008, **61**, 66–74.

50. M. D. Bale Oenick, S. J. Danielson, J. L. Daiss, M. W. Sunderberg and R. C. Sutton, *Ann. Biol. Clin. (Paris).*, 1990, **48**, 651–654.

51. J. Morales-Sanfrutos, J. Lopez-Jaramillo, M. Ortega-Muñoz, A. Megia-Fernandez, F. Perez-Balderas, F. Hernandez-Mateo and F. Santoyo-Gonzalez, *Org. Biomol. Chem.*, 2010, **8**, 667–675.

52. J. C. S. dos Santos, N. Rueda, O. Barbosa, J. F. Fernández-Sánchez, A. L. Medina-Castillo, T. Ramón-Márquez, M. C. Arias-Martos, M. C. Millán-Linares, J. Pedroche, M. del M. Yust, L. R. B. Gonçalves and R. Fernandez-Lafuente, *RSC Adv.*, 2015, **5**, 20639–20649.

53. E. M. Anderson, K. M. Larsson and O. Kirk, *Biocatal. Biotransformation*, 1998, 16, 181–204.

54. V. Gotor-Fernández, E. Busto and V. Gotor, *Adv. Synth. Catal.*, 2006, 348, 797–812.

55. M. M. Bradford, *Anal. Biochem.*, 1976, **72**, 248–254.

56. R. Fernandez-Lafuente, P. Armisén, P. Sabuquillo, G. Fernández-Lorente and J. M. Guisán, *Chem. Phys. Lipids*, 1998, **93**, 185–197.

57. O. Barbosa, M. Ruiz, C. Ortiz, M. Fernández, R. Torres and R. Fernandez-Lafuente, *Process Biochem.*, 2012, **47**, 867–876.

58. G. V Semisotnov, N. A. Rodionova, O. I. Razgulyaev, V. N. Uversky, A. F. Gripas' and R. I. Gilmanshin, *Biopolymers*, 1991, **31**, 119–128.

59. K. Hernandez, C. Garcia-Galan and R. Fernandez-Lafuente, *Enzyme Microb. Technol.*, 2011, **49**, 72–78.

60. C. Garcia-Galan, O. Barbosa, K. Hernandez, J. C. S. dos Santos, R. C. Rodrigues and R. Fernandez-Lafuente, *Molecules*, 2014, **19**, 7629–45.

61. J. Mansfeld and R. Ulbrich-Hofmann, *Biotechnol. Appl. Biochem.*, 2000, **32 (Pt 3)**, 189–195.

62. J. Mansfeld, G. Vriend, B. Van Den Burg, V. G. H. Eijsink and R. Ulbrich-Hofmann, *Biochemistry*, 1999, **38**, 8240–8245.

63. J. M. Palomo, G. Muñoz, G. Fernández-Lorente, C. Mateo, R. Fernández-Lafuente and J. M. Guisán, *J. Mol. Catal. B Enzym.*, 2002, **19-20**, 279–286.

64. G. H. Peters, O. H. Olsen, A. Svendsen and R. C. Wade, *Biophys. J.*, 1996, **71**, 119–129.

65. M. Galvis, O. Barbosa, M. Ruiz, J. Cruz, C. Ortiz, R. Torres and R. Fernandez-Lafuente, *Process Biochem.*, 2012, **47**, 2373–2378.

66. G. Fernandez-Lorente, M. Filice, D. Lopez-Vela, C. Pizarro, L. Wilson, L. Betancor, Y. Avila and J. M. Guisan, *J. Am. Oil Chem. Soc.*, 2010, **88**, 801–807.

67. V. Grazú, F. López-Gallego, T. Montes, O. Abian, R. González, J. A. Hermoso, J. L. García, C. Mateo and J. M. Guisán, *Process Biochem.*, 2010, **45**, 390–398.

68. J. M. Palomo, G. Fernández-Lorente, C. Mateo, M. Fuentes, R. Fernández-Lafuente and J. M. Guisan, *Tetrahedron Asymmetry*, 2002, **13**, 1337–1345.

610 **Figure legends**

611

612 **Figure 1. Immobilization courses of CALB at pH 5, 7 and 10 on DVS-agarose.**
613 Experimental conditions are detailed in Section 2. Circles, solid black line: suspension pH5;
614 circles, solid dash line: supernatant pH5; Square, solid black line: suspension pH7; Square,
615 dash line: supernatant pH7; Triangles, solid black line: pH10 suspension; Triangles, solid dash
616 line: supernatant pH10.

617

618 **Figure 2. Structure of the activated support**

619

620 **Figure 3. Effect of Tritón X-100 on the immobilization of CALB on inactivated DVS-**
621 **supports.** The support was incubated 24 h in 0.1 M NaOH to destroy the vinylsulfone groups.
622 Experimental conditions are detailed in Section 2. Circles, solid black line: supernatant
623 without Tritón X-100; squares, solid black line: supernatant with 0.05% Tritón X-100;
624 triangles, solid black line: supernatant with 0.15% Tritón X-100; rhombus, solid black line:
625 supernatant with 0.3% Tritón X-100.

626

627 **Figure 4. Immobilization courses of CALB on DVS-supports in presence of 0.3% Triton**
628 **at pH 5, 7 and 10**. Experimental conditions are detailed in Section 2. Circles, solid black
629 line: suspension pH5; circles, solid dash line: supernatant pH5; Square, solid black line:
630 suspension pH7; Square, dash line: supernatant pH7; Triangles, solid black line: pH10
631 suspension; Triangles, solid dash line: supernatant pH10.

Figure 5. Effect of the long term incubation at pH 10 value on enzyme activity on CALB immobilized on DVS agarose at different pH values: Experimental conditions are detailed in Section 2. Circles, solid black line: pH5; Square, solid black line: pH7; Triangles, solid black line: pH10.

Figure 6. Effect of the pH on the activity versus pNPB of the different CALB preparations. Experimental conditions are detailed in Section 2. Circles, solid black line: pH5; Gray circles, solid gray line: pH5-pH10; Squares, solid black line: pH7; Grays squares, solid gray line: pH7-pH10; Triangles, solid black line: pH10; Gray triangles, solid Gray line: pH10-pH10. Stars, solid black line: Octyl; Gray stars, solid Gray line: CNBr.

Figure 7. Spectra of ANS incubated in the presence of different DVS immobilized CALB. Experimental conditions are detailed in Section 2. Line a) Blocked DVS-Support ; Line b) DVS-CALB-pH 5 , Line c) DVS-CALB pH5 + 72 h at pH10, Line d) DVS-CALB pH 10; Line e) DVS-CALB pH 10+ 72 h at pH 10

Biocatalysts	Activity
DVS-pH 5-EDA	7.79±1.7
DVS-pH5 /pH10-EDA	22.3±2.2
DVS-pH7-EDA	20.44±2.9
DVS-pH7 /pH10-EDA	27.15±2.5
DVS-pH10 (2 h) EDA	23.79±1.91
DVS-pH 10 (72 h)-EDA	32.15±1.94
Octyl	16.92 ± 2.16
CNBr	5.90± 1.17

Table 1 Activities of the different CALB preparations versus p-NPB. DVS-CALB was blocked using EDA. Activity was determined at pH 7 and 25^{o}C as indicated in Section 2. Activity is given in μmoles of substrate hydrolyzed per minute and mg of immobilized enzyme. The preparation of the biocatalyst is in Section 2.

CALB preparation	Inactivation conditions			
	pH 5, 55 °C	pH 7, 55 °C	pH 9, 55 °C	70% Dioxane , 25 °C, pH 7
DVS-pH5-EDA	4.5±0.3	3±0.3	4.5±0.3	5±0.3
DVS-pH5-pH10-EDA	35±1.2	10±1.1	33±1.9	5.3±0.7
DVS-pH7-EDA	33±1.0	33±2.2	27±2.1	1.5±0.2
VS-pH7-pH10-EDA	60±2.4	60±3.3	60±3.2	7.3±0.3
DVS-pH10-EDA	32±1.3	4±0.2	4.2±0.4	1.7±0.2
DVS-pH10-pH10-EDA	46±2.2	25±1.2	25±1.2	2.6±0.4
Octyl	240 (100%)*	240(100%)*	30±2.1	0.17±0.02
CNBr	45±3.3	24±2.3	4.6±0.3	0.21±0.02

Table 2 Half-lives (expressed in minutes) of the different CALB preparation under different inactivation conditions. Experiments were performed as described in Section 2. * The enzyme retained full activity during the inactivation assay.

CALB preparations	MM/ pH5	MM/ pH7	MM/ pH8.5	MPA/ pH5	MPA/ pH7	MPA/ pH8.5	EH/ pH5	EH/ pH7	EH/ pH8.5
Octyl	16.45± 0.8	55.00± 2.8	41.07± 2.1	14.02±0.7	24.27±1.2	19.17±1	450.00± 23	300.00±15	273.44±14
CNBr	28.25± 1.1	124.15± 5.0	85.61± 3.4	15.32±0.6	30.54±1.2	19.00±0.8	627.85±25	436.65±17	197.44±8
DVS-pH5	11.61± 0.6	58.78± 2.9	23.28± 1.2	6.97±0.3	3.94±0.2	2.39±0.1	200.89±10	139.18±7	30.97±2
DVS-pH5-pH10	39.6± 0.9	82.32± 1.1	35.08± 1.8	2.86±0.1	1.38±0.1	1.95±0.1	74.40±4	194.20±10	50.22±3
DVS-pH7	52.17± 2.6	57.07± 2.9	52.41± 2.6	22.47±1.1	25.64±1.3	5.43±0.3	760.87±38	456.52±23	188.52±9
DVS-pH7-pH10	56.13± 2	78.80± 3.9	86.43± 1.9	9.34±0.5	9.29±0.5	3.36±0.2	217.39±11	157.07±8	142.66±7
DVS-pH10	12.50± 0.6	67.92± 3.5	51.07± 2.6	18.65±0.9	17.59±0.9	8.13±0.4	425.00±21	191.25±10	24.38±1
DVS-pH10-pH10	8.35± 0.4	69.00± 3.5	29.11± 1.5	6.47±0.3	6.68±0.3	2.71±0.1	62.50±3	41.25±2	38.53±2

Table 3. Activity of different CALB preparations versus different substrates at different pH values. Experimental details may be found in Section 2. MM, methyl mandelate; MPA, methyl phenylacetate; EH, Ethyl hexanoate. The activity is given in μmoles of substrate hydrolyzed per minute and mg of immobilized enzyme.

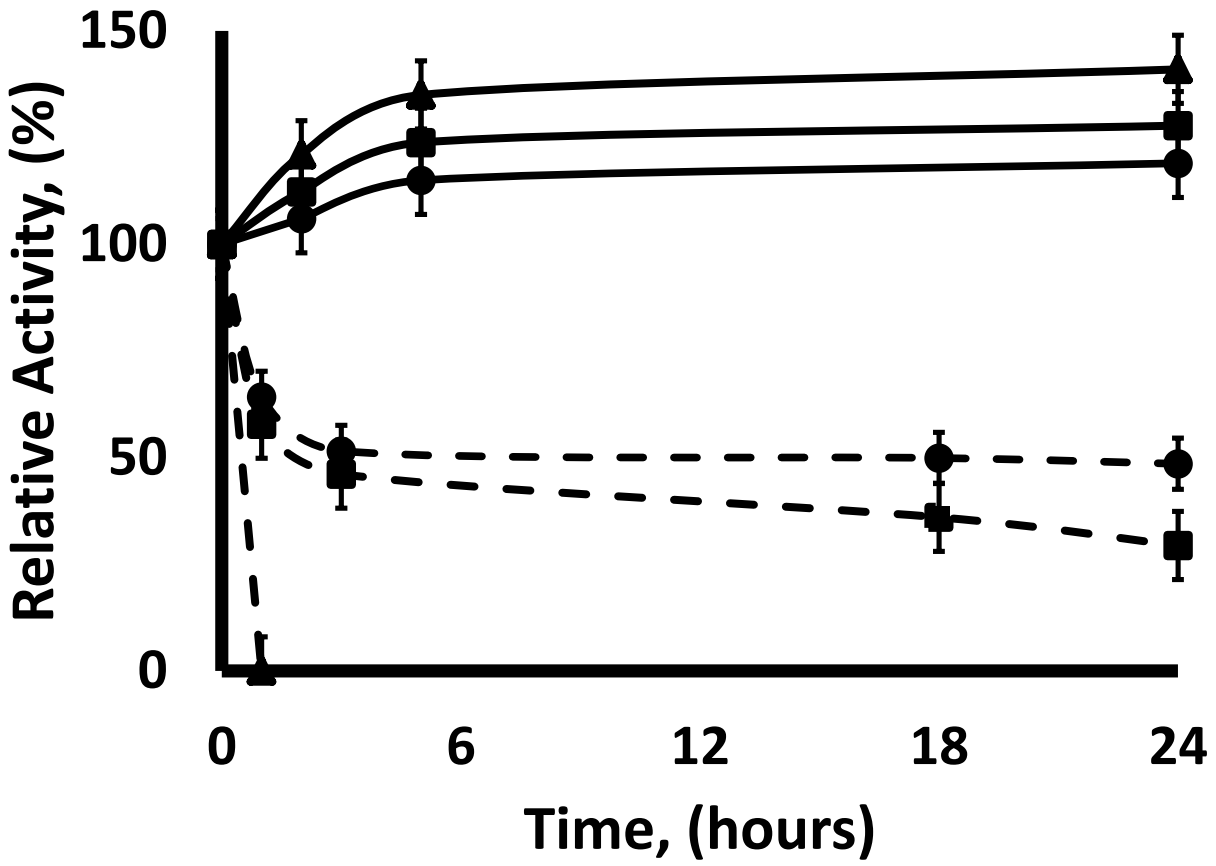

Figure 1.

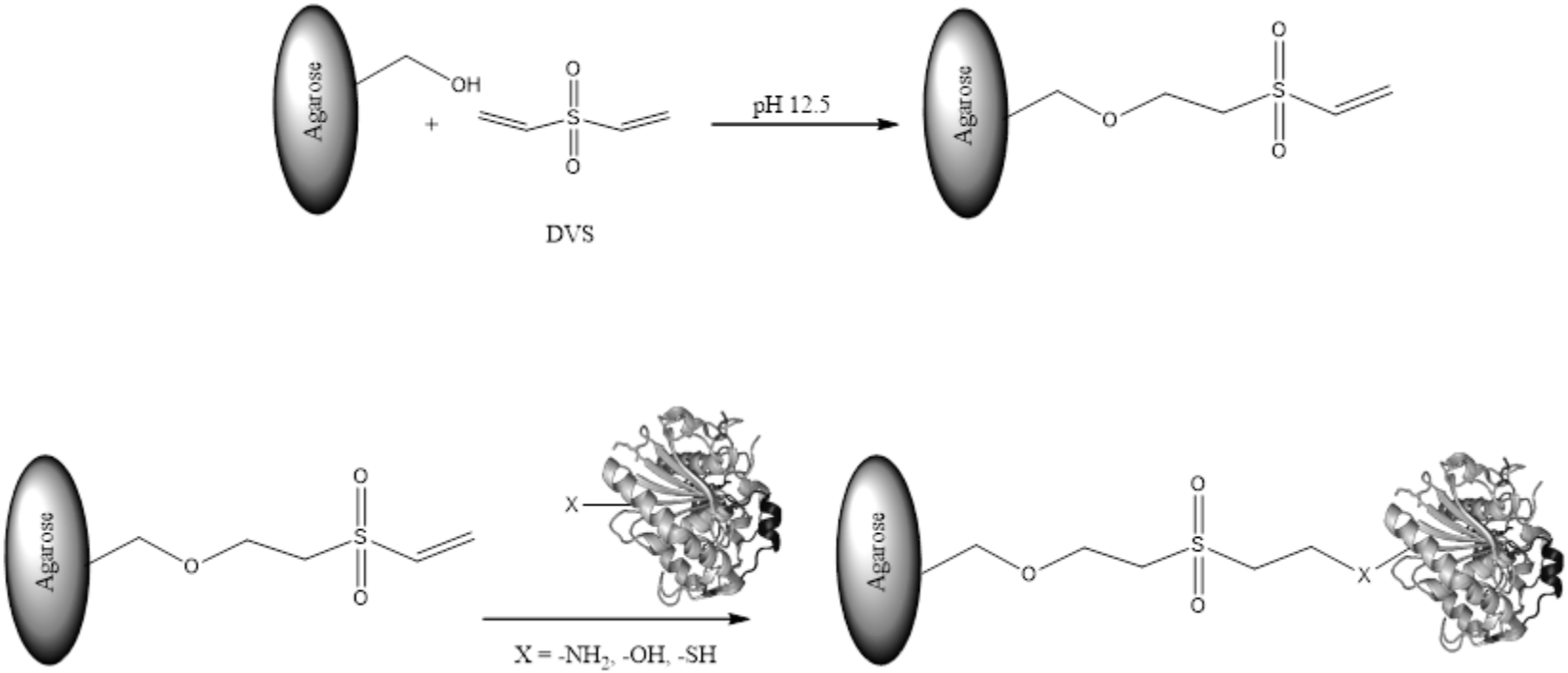

Figure 2

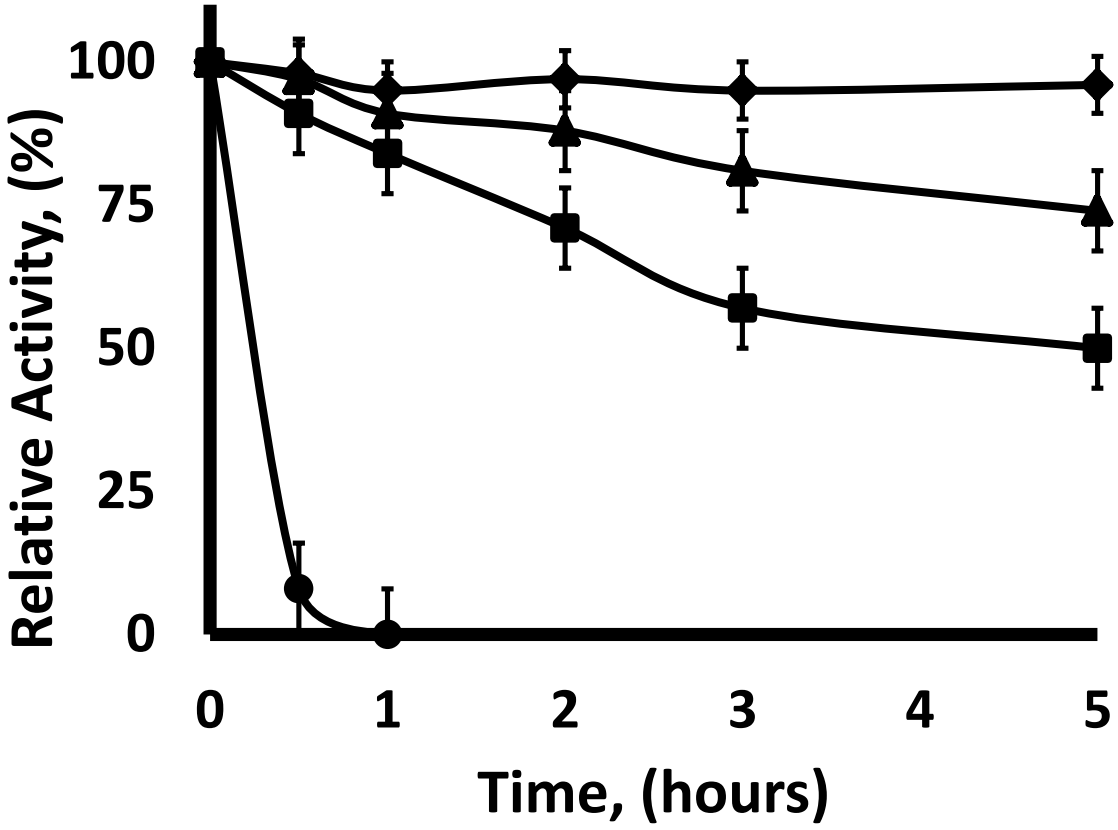

Figure 3.

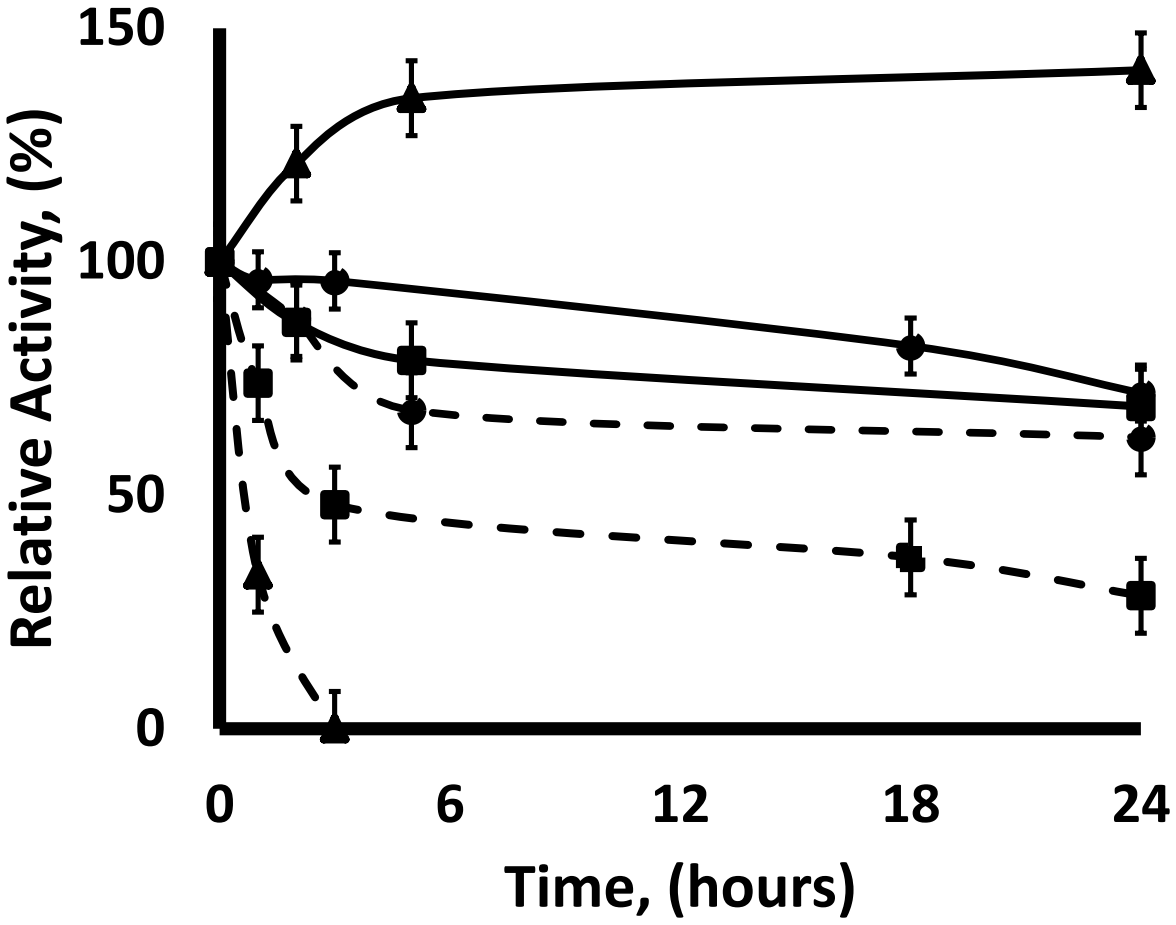

Figure 4..

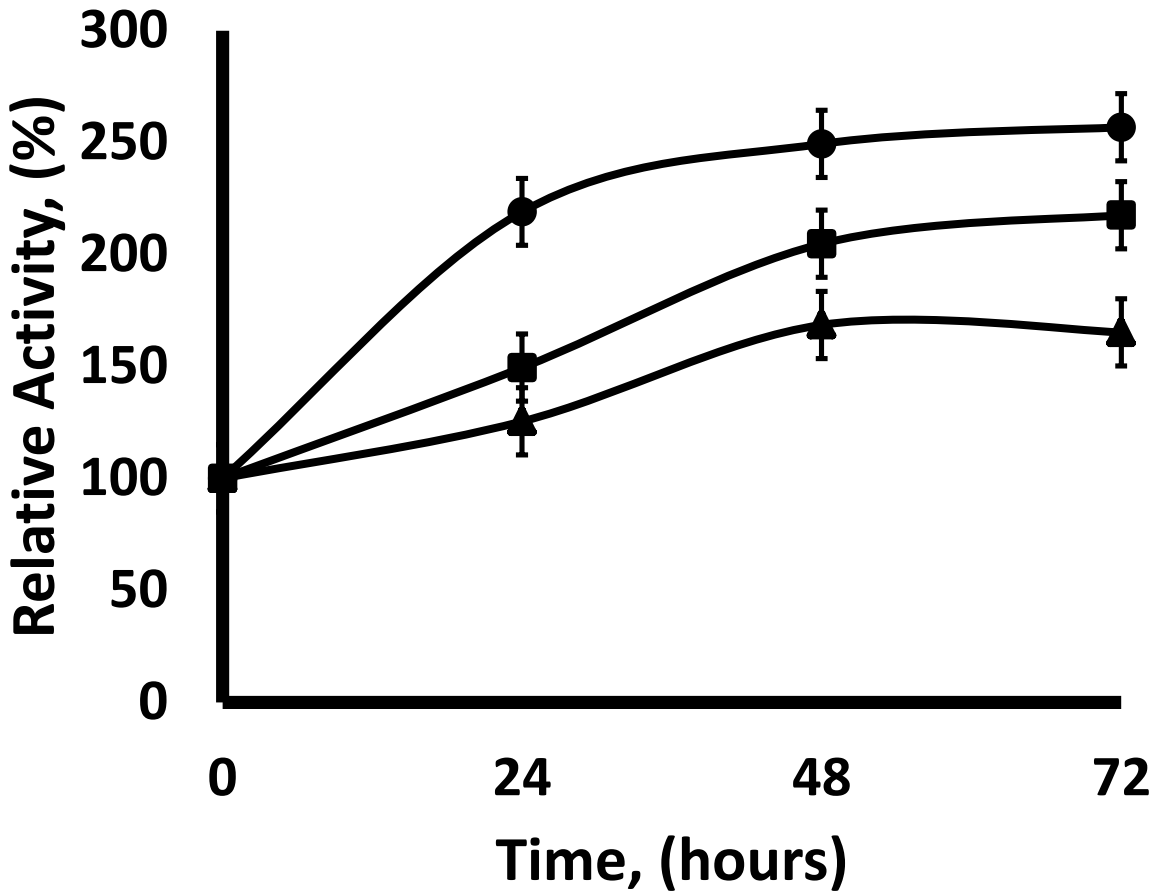

Figure 5.

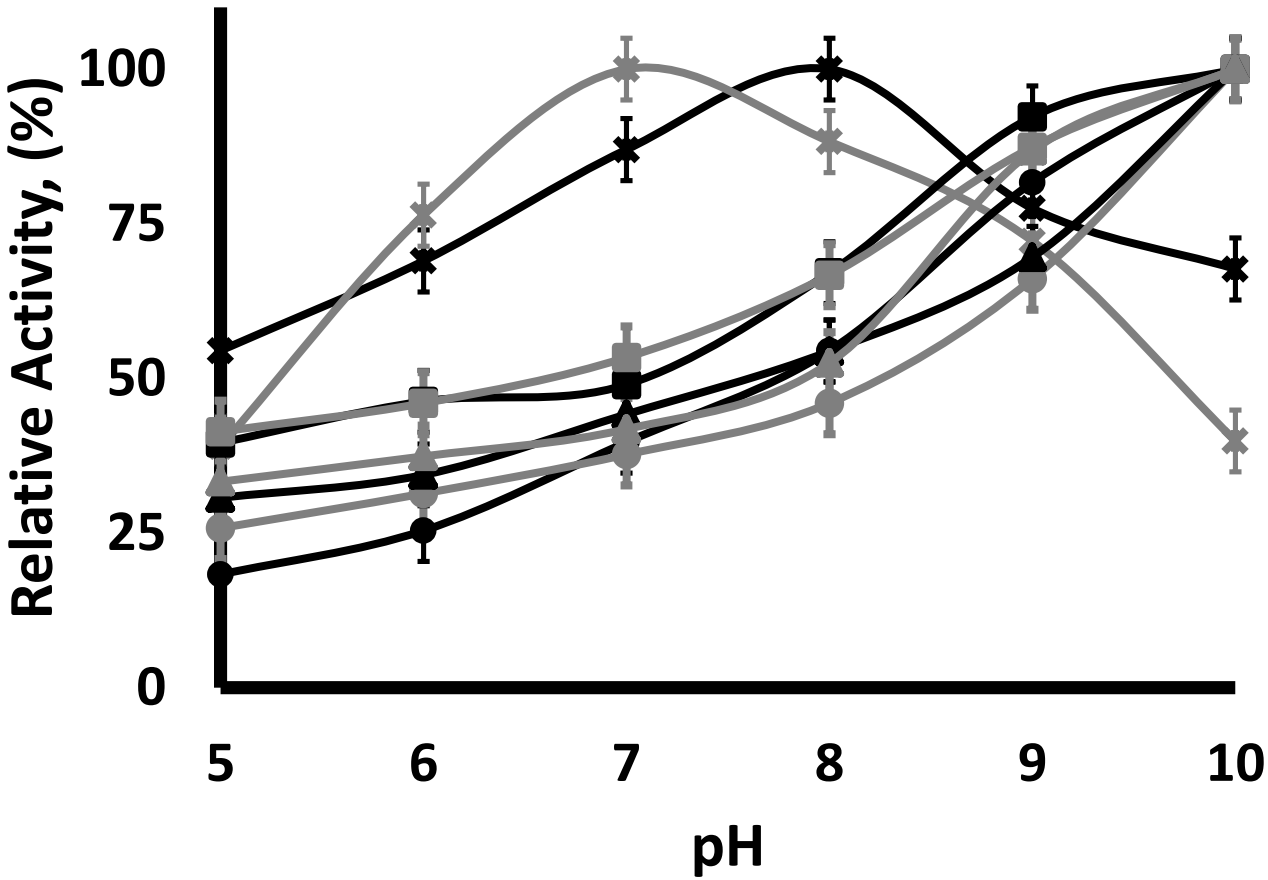

Figure 6.

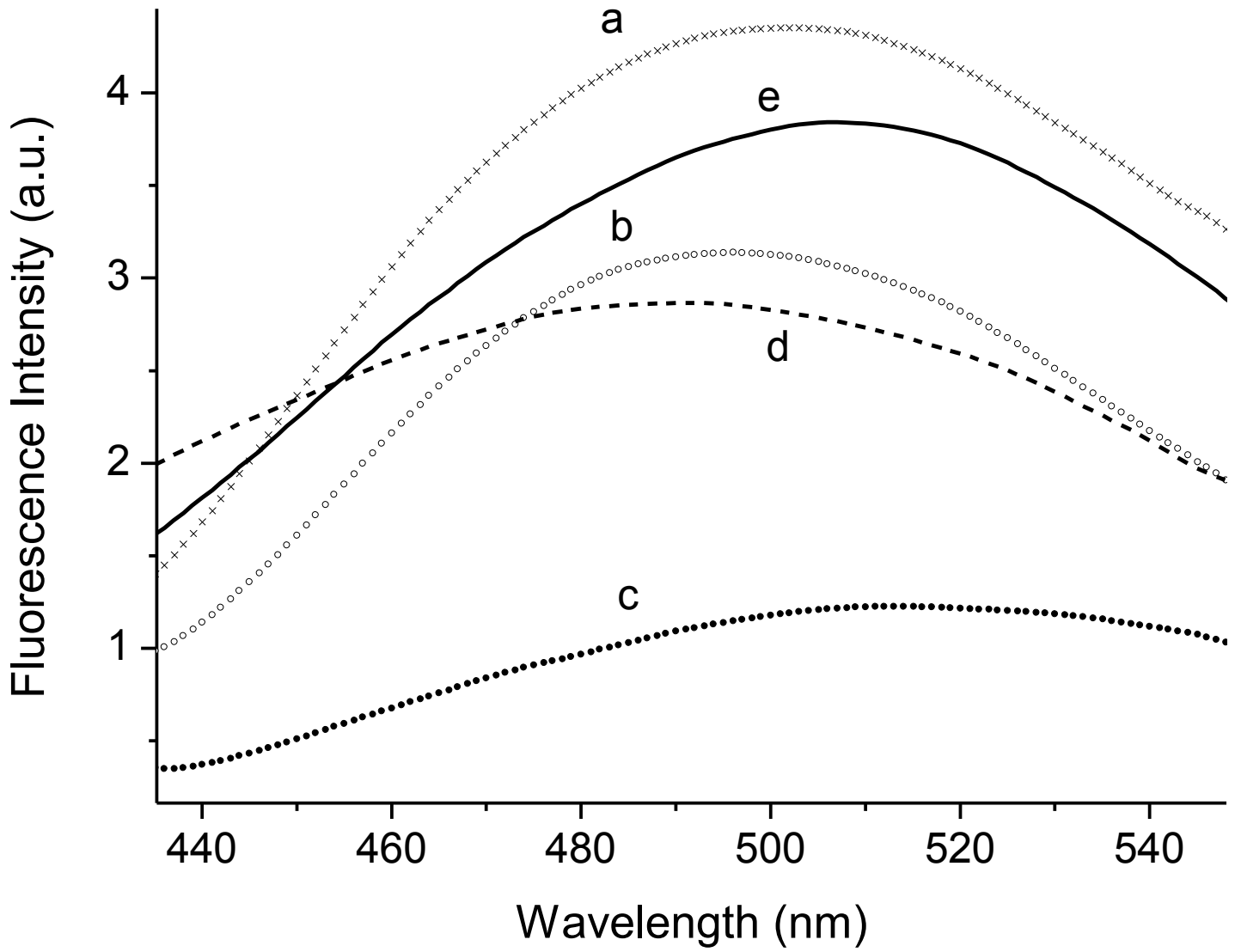

Figure 7