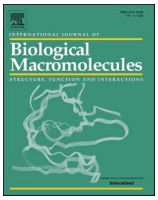
International Journal of Biological Macromolecules 263 (2024) 130403

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac



Adsorption features of reduced aminated supports modified with glutaraldehyde: Understanding the heterofunctional features of these supports

Diandra de Andrades [a,b,1], Pedro Abellanas [a,1], Diego Carballares [a,c], Andres R. Alcantara [d], Maria de Lourdes Teixeira de Moraes Polizeli [b], Javier Rocha-Martin [e], Roberto Fernandez-Lafuente [a,*]

[a] Departamento de Biocatálisis. ICP-CSIC, C/Marie Curie 2, Campus UAM-CSIC Cantoblanco, 28049 Madrid. Spain
[b] Department of Biology, Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo, Ribeirão Preto 14040-901, SP, Brazil
[c] Chemical and Materials Engineering Department, Faculty of Chemical Sciences, Complutense University of Madrid, Complutense Ave., Madrid 28040, Spain
[d] Departamento de Química en Ciencias Farmacéuticas, Facultad de Farmacia, Universidad Complutense de Madrid, Plaza de Ramón y Cajal, s/n, Madrid 28040, Spain
[e] Department of Biochemistry and Molecular Biology, Faculty of Biology, Complutense University of Madrid, José Antonio Novais 12, Madrid 28040, Spain

ARTICLE INFO

Keywords:
Heterofunctional supports
Mixed adsorption
Enzyme release

ABSTRACT

Immobilization of enzymes on aminated supports using the glutaraldehyde chemistry may involve three different interactions, cationic, hydrophobic, and covalent interactions. To try to understand the impact this heterofunctionality, we study the physical adsorption of the beta-galactosidase from *Aspergillus niger*, on aminated supports (MANAE) and aminated supports with one (MANAE-GLU) or two molecules of glutaraldehyde (MANAE-GLU-GLU). To eliminate the chemical reactivity of the glutaraldehyde, the supports were reduced using sodium borohydride. After enzyme adsorption, the release of the enzyme from the supports using different NaCl concentrations, Triton X100, ionic detergents (SDS and CTAB), or different temperatures (4 °C to 55 °C) was studied. Using MANAE support, at 0.3 M NaCl almost all the immobilized enzyme was released. Using MANAE-GLU, 0.3 M, and 0.6 M NaCl similar results were obtained. However, incubation at 1 M or 2 M NaCl, many enzyme molecules were not released from the support. For the MANAE-GLU-GLU support, none of the tested concentrations of NaCl was sufficient to release all enzyme bound to the support. Only using high temperatures, 0.6 M NaCl, and 1 % CTAB or SDS, could the totality of the proteins be released from the support. The results shown in this paper confirm the heterofunctional character of aminated supports modified with glutaraldehyde.

1. Introduction

Enzyme immobilization is a discipline undergoing fast development [1–3], as it has revealed itself as a powerful tool to improve many enzyme features, such as stability (via multipoint or multi-subunit attachment, generation of favorable environments, etc.) [4–7], activity, specificity, or selectivity [8,9]. It can also reduce inhibitions or enzyme inactivation by harmful reagents [10]; or it can be coupled to enzyme purification [11–13].

The final results depend on the support features, the active groups that are present on the support surface and the exact immobilization protocols (including immobilization, incubation and reaction end point) [2,14]. To take full advantage of immobilization, it is necessary to know the phenomena that occur between the enzyme and the support [2,15]. This is not always a simple goal and may be much more complex when using supports bearing several moieties able to interact with the enzyme [16,17]. These heterofunctional supports increase the versatility of the immobilization of enzymes on them, but usually they can generate some unexpected problems to understand the results, mainly when the researcher is not considering this heterofunctionality [2].

Immobilization of enzymes on supports containing primary amino groups via the glutaraldehyde chemistry is one of the most utilized immobilization strategies [18–25]. The support is a heterofunctional one and it is highly versatile [16,26]. First, the researcher can

* Corresponding author.
E-mail address: rfl@icp.csic.es (R. Fernandez-Lafuente).
[1] Both authors have worked evenly in this paper.

https://doi.org/10.1016/j.ijbiomac.2024.130403
Received 7 December 2023; Received in revised form 20 February 2024; Accepted 21 February 2024
Available online 27 February 2024

immobilize the enzyme via ion exchange and then treat the enzyme and the support with glutaraldehyde under conditions where just one glutaraldehyde molecule attaches to each external amino group [16]. The first anionic exchange may be performed under different conditions (pH, ion strength) to alter the enzyme orientation regarding the support surface. Due to the good reactivity between amino-glutaraldehyde moieties, this strategy permits intense multipoint covalent immobilization [27–30]. The final support surface will be formed by amino-glutaraldehyde linkages, giving the surface a cationic character, a little hindered by the moderately hydrophobic glutaraldehyde cycle. Monsan and coworkers showed a long time ago that amino-glutaraldehyde moieties are quite inefficient to react with another amine group [31]. Second, the aminated support can be pre-activated using glutaraldehyde. In this case, Monsan and coworkers showed the convenience of having two molecules of glutaraldehyde per primary amino group in the support [31]. This can react with non-ionized primary groups [31], but it hardly reacts with another amino-glutaraldehyde-glutaraldehyde moiety [32]. The cationic character of the support is maintained, but in this instance the amino group is masked by a larger moiety, that is more hydrophobic than in the previous case.

That way, the immobilization of enzymes on aminated supports using the glutaraldehyde chemistry is an outstanding example of versatility due to the tri-functionality of the support: cationic, hydrophobic, and covalent interactions can be established between the support and the enzyme [27–30,33]. This heterofunctionality is ignored in many papers, and in general, when the researchers use these strategies, if the enzyme is immobilized, it is considered that it does so in a covalent way without further analysis [2,34]. However, this may be far from reality, in some instances, the enzyme may just be physically adsorbed, perhaps via ionic and hydrophobic interactions [35]; and that way, the release of the enzyme may be very difficult to achieve, even if a single covalent bond has not been produced, leading to wrong assumptions by the researcher [2].

For this reason, in this paper we focus our attention on the impact of the modification of aminated supports with one or two molecules of glutaraldehyde on their protein adsorption features (Fig. 1S). This study has not been previously performed, and to eliminate the chemical reactivity of the glutaraldehyde, we have employed sodium borohydride-reduced glutaraldehyde supports [36–40]. As model enzyme, the beta-galactosidase from *Aspergillus niger* has been used [41–46].

This research may also have an impact on the application of glutaraldehyde chemistry using aminated supports to coimmobilize enzymes using different mechanisms, which is critical to solve the problem of coimmobilizing enzymes with very different stabilities where the least stable one can define the global combibiocatalyst stability [47,48]. This is only possible if the least stable enzyme can, after covalent immobilization of the most stable immobilized enzyme, be physically adsorbed on the support and, after its inactivation, be released under conditions that do not affect the stability of the covalently and almost fully active coimmobilized enzyme [49–54].

2. Materials and methods

2.1. Materials

β-galactosidase from *Aspergillus oryzae* (20 Units ONPG/mg of protein), o-nitrophenyl-β-galactopyranoside (*o*-NPG), triton X100, cetyltrimethylammonium bromide (CTAB), sodium dodecylsulfate (SDS), *p*-nitrophenyl butyrate (p-NPB), ethylenediamine (EDA) and glutaraldehyde (GLU) were purchased from Sigma–Aldrich (Madrid, Spain). 4 % CL agarose beads was from GE Healthcare (Uppsala, Sweden). All other compounds used in this work were of analytical grade.

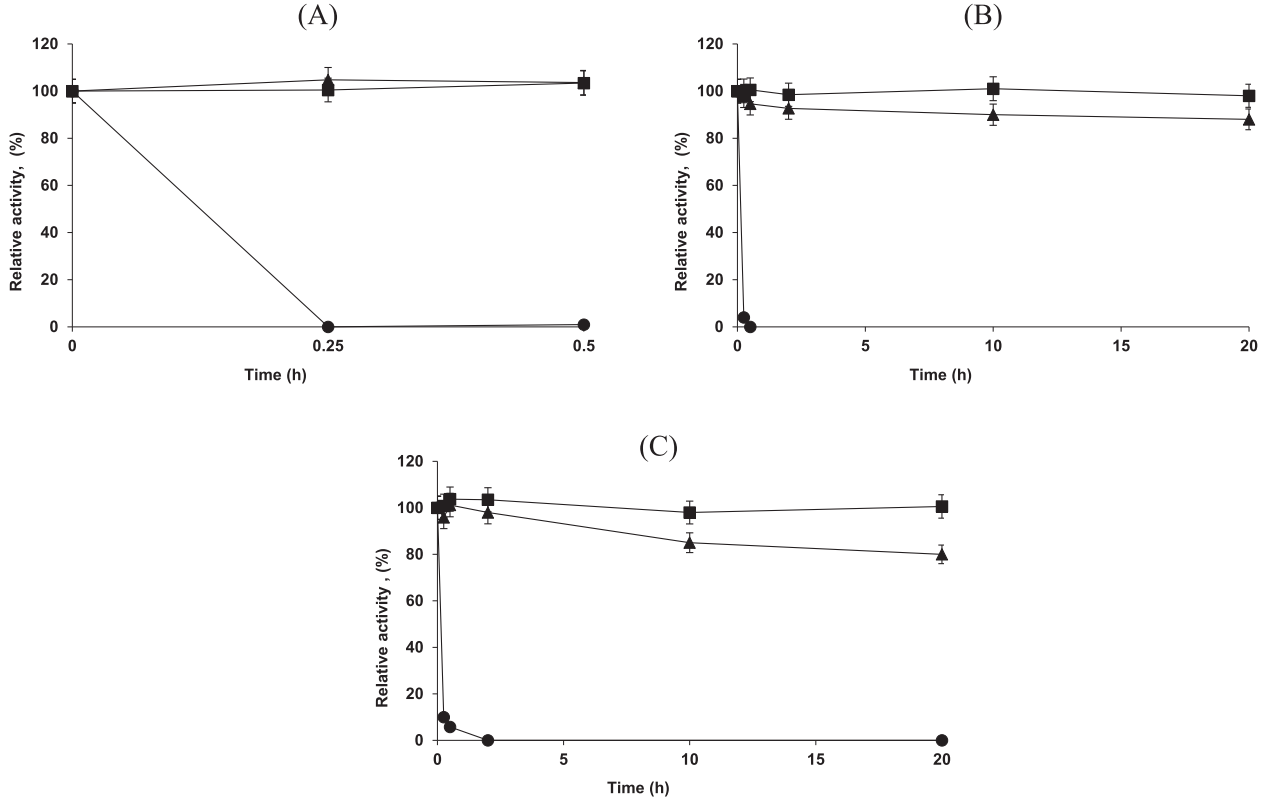


Fig. 1. Immobilization course of β-galactosidase on different supports (A): MANAE (B): MANAE-GLU and (C): MANAE-GLU-GLU. The experiments were performed using 5 mM Tris-HCl at pH 7.0 and 25 °C. Other specifications can be found in Materials and Methods sections. Solid triangles: suspension; solid squares: reference; and solid circles: supernatant.

2.2. Methods

2.2.1. Enzymatic activity and protein concentration

The β-galactosidase activity was determined 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 °C (extinction coefficient was 10,493 M^{-1} cm^{-1} under these conditions), using a spectrophotometer with a thermostatized cell and continuous magnetic stirring. For the reaction, 50 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. Protein concentration was calculated using the method reported by Bradford [55], using bovine serum albumin as a standard.

2.2.2. Preparation of supports

MANAE support was prepared as previously described [56,57]. This aminated support was treated with glutaraldehyde under two different conditions. In the first study, 10 g MANAE-agarose was incubated in 100 mL of 200 mM sodium phosphate pH 7 containing 1 % (*v*/v) glutaraldehyde (MANAE-GLU). Under these conditions, Prof Monsan has described that one molecule of glutaraldehyde reacted with one amino group [31]. In the second study, 10 g MANAE-agarose was incubated under the same conditions, but 10 % (*v*/v) glutaraldehyde was utilized (MANAE-GLU-GLU). Under these conditions, Prof Monsan has described that one molecule of glutaraldehyde reacted with one amino group [31], confirmed in further studies [58]. The systems were stirred for 1 h or 24 h at 25 °C, respectively. Then, were filtered, washed with abundant distilled water, and stored at 4 °C.

Reduced supports were obtained by incubating 10 g of MANAE-GLU and MANAE-GLU-GLU in 100 mL of 25 mM sodium bicarbonate at pH 10, adding 100 mg of solid sodium borohydride [59,60]. The solution was stirred for 30 min at room temperature. Finally, the reduced supports were filtered, washed with abundant distilled water, and stored at 4 °C. The proposed structure of the supports may be found in Fig. 1S [61,62].

2.2.3. Immobilization

β-galactosidase immobilization on both supports was carried using 3 mg of protein per g of support. Enzyme was diluted in a 5 mM Tris-HCl solution at pH 7 and subsequently adding the supports. The enzyme immobilization course was followed by measuring the enzyme activity in the supernatant and in the whole suspension at different time intervals using *o*-NPG as substrate, as described above. After immobilization, the biocatalysts were filtered and washed with abundant distilled water and stored at 4 °C.

2.2.4. Desorption of β-galactosidase from the supports

β-galactosidase immobilized on MANAE, MANAE-GLU or MANAE-GLU-GLU supports were incubated in a solution of 5 mM Tris-HCl containing different concentrations of sodium chloride or different concentrations of Triton X-100. In some instances, NaCl and some detergents (Triton X-100, CTAB, SDS) were simultaneously utilized. The incubations were performed at pH 7.0 and 25 °C for 1 h and the activities of both supernatant and suspension were followed using *o*-NPG. Afterwards, the biocatalysts were washed 5 times with 10 volumes of desorption solution (to eliminate the free enzyme molecules contained in the pores of the support) and 5 times with 10 volumes of distilled water.

2.2.5. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The SDS-PAGE experiments were carried out following the Laemmli protocol [63] with some modifications. The protein samples (including enzyme immobilized on supports) were diluted in 4 % SDS (*w*/*v*) and 10 % mercaptoethanol (*v*/v) to have a protein concentration of 0.3–0.5 mg of protein/ml solution in the samples. The samples were boiled for 8 min, centrifuged, and finally 15 μL of the samples were loaded in 12 % polyacrylamide gels, which was run at 100 V. The proteins were stained with Coomassie brilliant blue dye.

3. Results

3.1. Preparation of the supports

After preparing MANAE agarose beads, they were treated with 1 % or 10 % of glutaraldehyde as detailed in Methods section. This treatment promoted the apparition of a certain brown color in the support, more intense for the support treated with 10 % glutaraldehyde for 16 h (Fig. 2S) and permits to modify the primary amino groups with two glutaraldehyde molecules (MANAE-GLU-GLU) [31]. The modification for 1 h using only 1 % glutaraldehyde at pH 7 permitted to fully modify the support mainly with one glutaraldehyde molecule (MANAE-GLU) [31]. The reduction of the modified supports with glutaraldehyde promoted a decrease in its color, in the case MANEA-GLU the brown color almost disappeared (Fig. 2S). These supports almost fully lost their reactivity versus Schiff reagent.

3.2. Immobilization of beta-galactosidase on the different aminated supports

Fig. 1 shows the immobilization of the enzyme on the 3 supports. In all cases, the enzyme immobilization proceeded very rapidly, although the presence of reduced GLU or GLU-GLU moieties promoted a progressive but slight decrease in the immobilization rate. This can be caused by the increase in the difficulties to establish a multipoint ionic interaction of the enzyme with the support, necessary to fix the enzyme to the support [64].

While the enzyme activity was almost fully maintained using MANAE, a small decrease in activity after immobilization was detected using MANAE-GLU and this reached a 20 % using MANAE-GLU-GLU. The hydrophobic interactions promoted by the hydrophobic reduced glutaraldehyde moieties could be responsible for this negative effect on the enzyme activity (Fig. 1).

The reversible immobilization as confirmed by SDS-PAGE, the amount of enzyme in the gel was similar for the 3 supports (no shown results). Using enzyme adsorbed on MANAE and treated with 1 % glutaraldehyde at pH 7 for 1 h [27–30], and then incubated at pH 8 for 24 h, no enzyme was detected in the supernatant after boiling in SDS (the same treatment as in the samples PAGE preparation), confirming the irreversible immobilization obtained by the glutaraldehyde chemistry (results not shown).

3.3. Release of the enzyme from the different aminated supports in the presence of growing concentrations of NaCl

Fig. 2 shows the desorption of beta-galactosidase from MANAE, MANAE-GLU and MANAE-GLU-GLU supports when incubated at different concentrations of NaCl.

Using MANAE support, the increase in NaCl concentration promoted a progressive release of the enzyme, at 0.3 M almost all enzyme activity was released and further increases in the ionic strength almost did not affect the results. Using MANAE-GLU, at 0.15 M NaCl there was a clearly higher release of enzyme than using MANAE. This can be related to the higher difficulties to establish an intense multipoint ionic exchange between the enzyme and the support, caused by the steric hindrances promoted by the reduced glutaraldehyde molecule located over the amines. However, when using a concentration of NaCl of 1 M or higher, some enzyme molecules were no released from the support. This could be related to the moderate hydrophobicity of the reduced glutaraldehyde moiety, that at this high ionic strength seemed to be able to fix some enzyme molecules to the support. This was confirmed by SDS-PAGE analysis (Fig. 3), while at 0.3 and 0.6 no protein bands could be

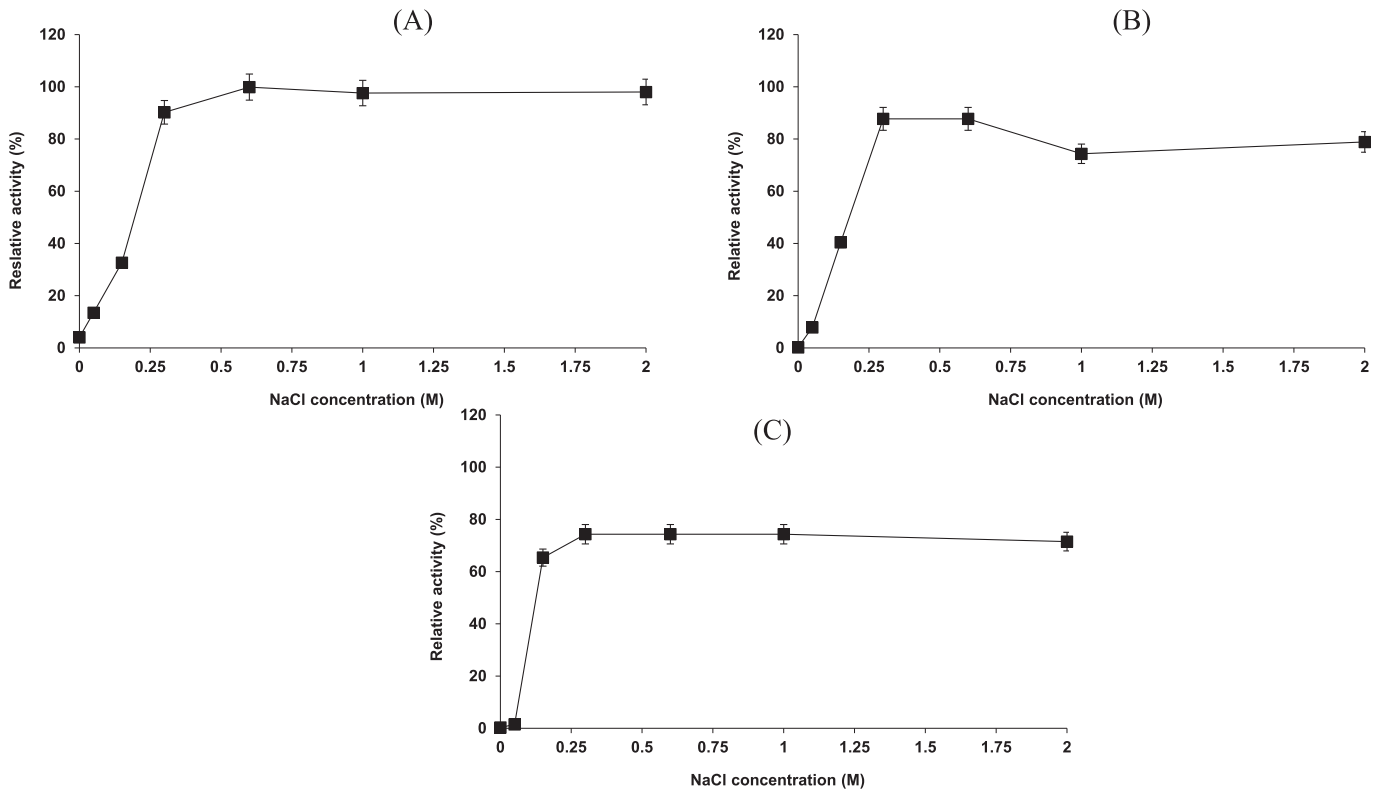


Fig. 2. Relative activity of the supernatants during the desorption tests using different NaCl concentrations of β-galactosidase immobilized on (A): MANAE support. (B): MANAE-GLU and (C): MANAE-GLU-GLU. Experiments were performed as described in Materials and Methods. Relative activity corresponded to the percentage of β-galactosidase activity released from the support. The total enzyme activity on suspension was considered as 100 %. Solid circle: MANAE support; Solid triangles: MANAE-GLU; and solid squares: MANAE-GLU-GLU.

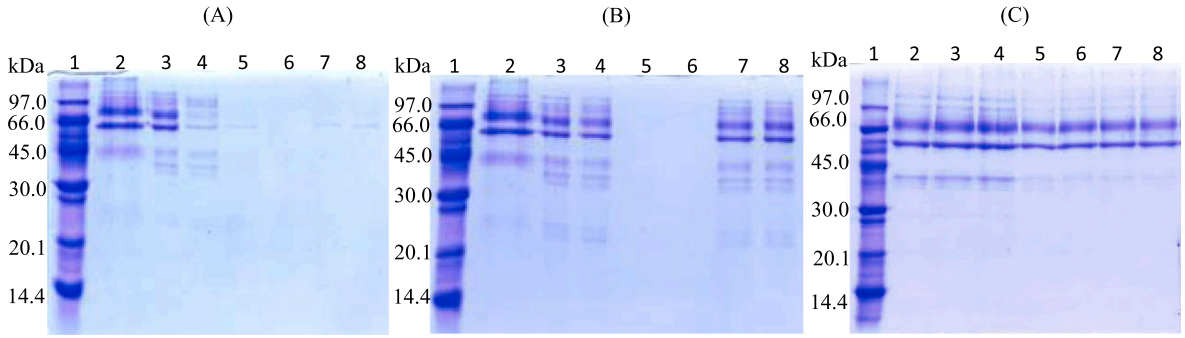


Fig. 3. SDS-PAGE analysis of β-galactosidase preparations submitted to different treatments, immobilized on different supports (A): MANAE, (B): MANAE-GLU support, (C): MANAE-GLU-GLU support. Lane 1: low molecular weight protein standard from GE Healthcare. Lane 2: immobilized β-galactosidase (control). Lane 3: enzyme remaining in the support after desorption of β-gal with 0.05 M NaCl. Lane 4: enzyme remaining in the support after desorption of β-gal with 0.15 M NaCl. Lane 5: enzyme remaining in the support after desorption of β-gal with 0.3 M NaCl. Lane 6: enzyme remaining in the support after desorption of β-gal with 0.6 M NaCl. Lane 7: enzyme remaining in the support after desorption of β-gal with 1 M NaCl. Lane 8: enzyme remaining in the support after desorption of β-gal with 2 M NaCl.

visualized in the gel, at 1 and 2 M NaCl, there are clear protein bands.

Using MANAE-GLU-GLU, at 0.15 M NaCl there was a much higher release of enzyme than using the other supports (Fig. 2), suggesting that the difficulties to establish many ionic interactions between the enzyme and the support were increased by the bulkier GLU-GLU moiety. However, the use of higher concentration of NaCl did not permit to release more galactosidase from the support, maintaining similar values even at 2 M NaCl. This was confirmed by SDS-PAGE (Fig. 3).

3.4. Release of the enzyme from the different aminated supports in the presence of growing concentrations of detergents and solvents

Using organic solvents (e.g., until 50 % ethanol and dioxane) under 5 mM Tris buffer we were not able to release any enzyme from the three supports. This confirms that ionic exchange is a critical point in the enzyme adsorption on the supports.

Figs. 4 and 5 show that using growing concentrations of nonionic detergent (Triton X-100), no significant activity was released from any of the utilized supports, suggesting that the hydrophobic interactions were not the main cause for enzyme adsorption on MANAE-GLU or

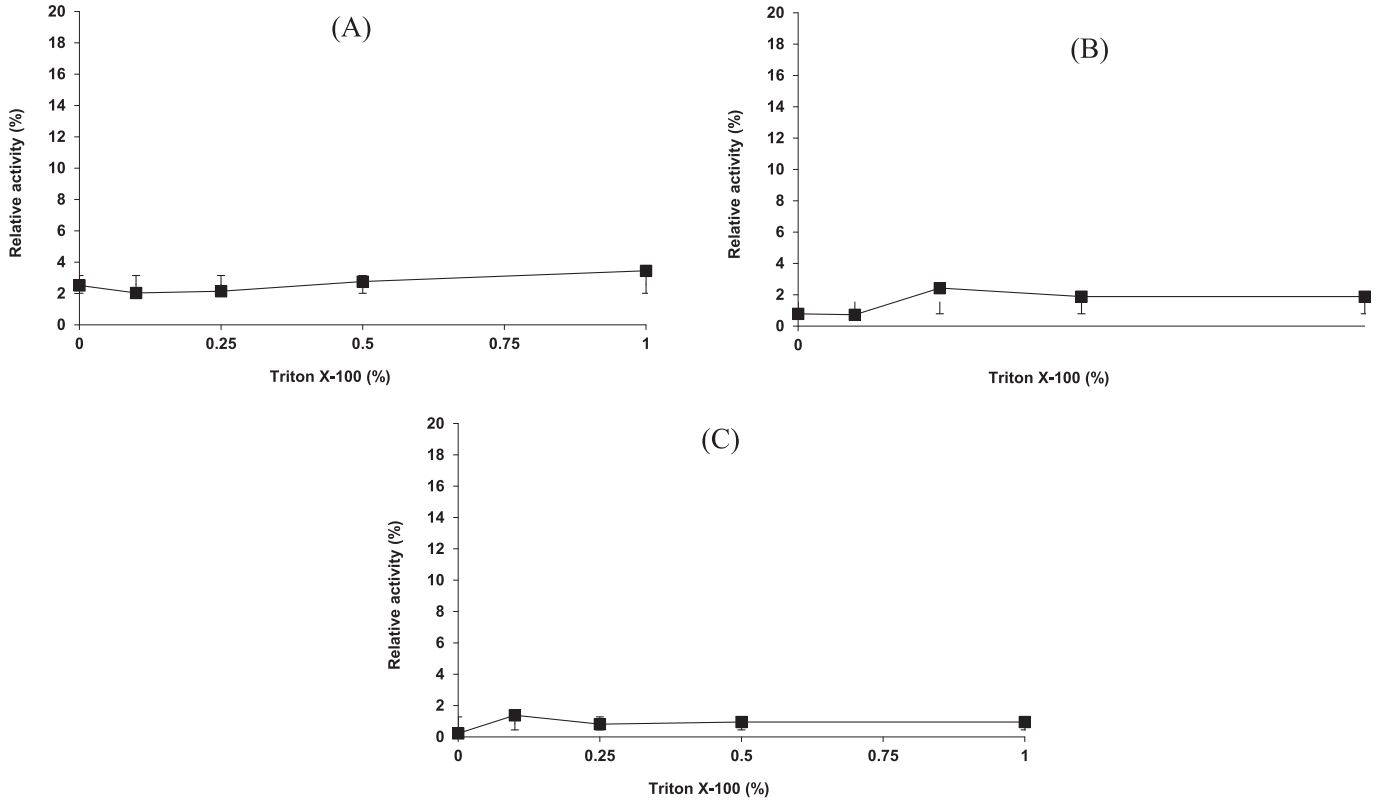


Fig. 4. Relative activity profiles of the supernatants when the β-galactosidase immobilized biocatalysts have been incubated at different triton X-100 concentrations, using enzyme immobilized on different supports (A): MANAE. (B): MANAE-GLU and (C): MANAE-GLU-GLU. Experiments were performed as described in Materials and Methods. Relative activity corresponds to the percentage of β-galactosidase activity released from the support. The total enzyme activity on suspension was considered as 100 %. Solid circle: MANAE support; Solid triangles: MANAE-GLU; and solid squares: MANAE-GLU-GLU.

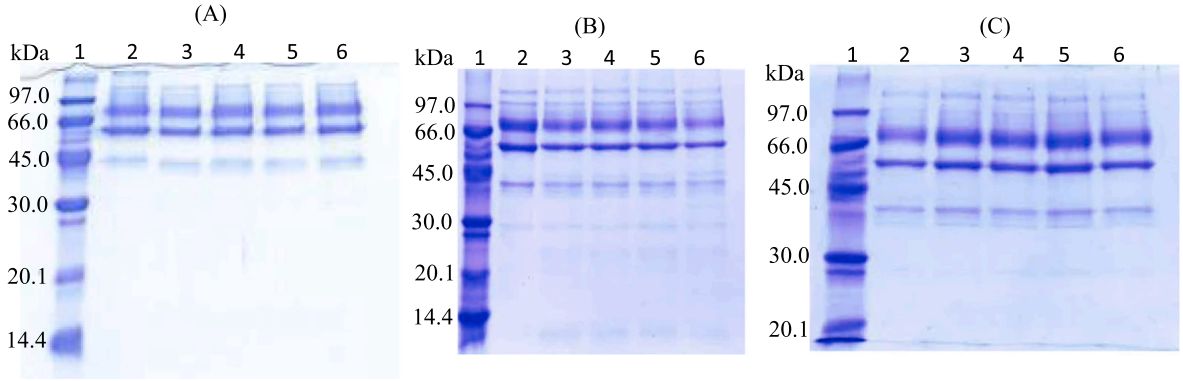


Fig. 5. SDS-PAGE analysis of immobilized β-galactosidase that remains on the support after different treatments, using different supports (A): MANAE support. (B): MANAE-GLU support. (C): MANAE-GLU-GLU support. Lane 1: low molecular weight protein standard from GE Healthcare. Lane 2: β-galactosidase immobilized (control). Lane 3: enzyme remaining in the support after desorption of β-gal with 0.1 % triton X-100. Lane 4: enzyme remaining in the support after desorption of β-gal with 0.25 % triton X-100. Lane 5: enzyme remaining in the support after desorption of β-gal with 0.5 % triton X-100. Lane 6: enzyme remaining in the support after desorption of β-gal with 1 % triton X-100.

MANAE-GLU-GLU. However, the previous results suggested that the adsorption of the enzymes on the MANAE-GLU-GLU could have a mixed character.

For that reason, we utilized simultaneously a 300 or 600 mM NaCl and a growing concentration of Triton X100 to study the release the enzyme from the support. The change in the activity of the enzyme makes the use of these data confusing. Thus, we only present the SDS-PAGE results (Fig. 6). Results showed that still a large percentage of enzyme remained on the support even using these desorption solutions.

Next, we tried to release the enzyme using different ionic detergents (SDS and CTAB) and employed different temperatures (from 4 °C to 55 °C) using 0.3 (Fig. 7) or 0.6 M (Figs. 8 and 9) NaCl. The use of higher temperatures enabled the release of a higher percentage of enzyme, but only using 55 °C, 0.6 M NaCl and 1 % CTAB or SDS all proteins could be released from the support, using Triton X-100 some enzyme molecules still remained in the support (Fig. 8). This shows the very strong adsorption that the MANAE-GLU-GLU was able to promote, a strong mixed ionic-hydrophobic adsorption that permitted establishing many enzyme-support interactions that cannot be easily broken later.

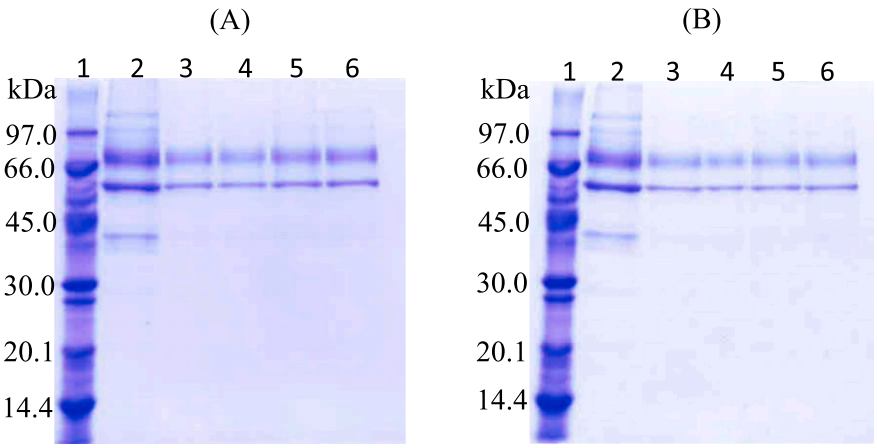


Fig. 6. SDS-PAGE analysis of β-galactosidase immobilized on MANAE-GLU-GLU submitted to different incubations using 0.3 or 0.6 M NaCl and different triton X-100 concentrations at 25 °C. 0.3 M NaCl (A): Lane 1: low molecular weight protein standard from GE Healthcare. Lane 2: β-galactosidase immobilized (control). Lane 3: enzyme remaining in the support after desorption of β-gal adding 0.1 % triton X-100. Lane 4: enzyme remaining in the support after desorption of β-gal adding 0.25 % triton X-100. Lane 5: enzyme remaining in the support after desorption of β-gal adding 0.5 % triton X-100. Lane 6: enzyme remaining in the support after desorption of β-gal adding 1 % triton X-100. 0.6 M NaCl (B): Lane 1: low molecular weight protein standard from GE Healthcare. Lane 2: β-galactosidase immobilized (control). Lane 3: enzyme remaining in the support after desorption of β-gal adding 0.1 % triton X-100. Lane 4: enzyme remaining in the support after desorption of β-gal adding 0.25 % triton X-100. Lane 5: enzyme remaining in the support after desorption of β-gal adding 0.5 % triton X-100.

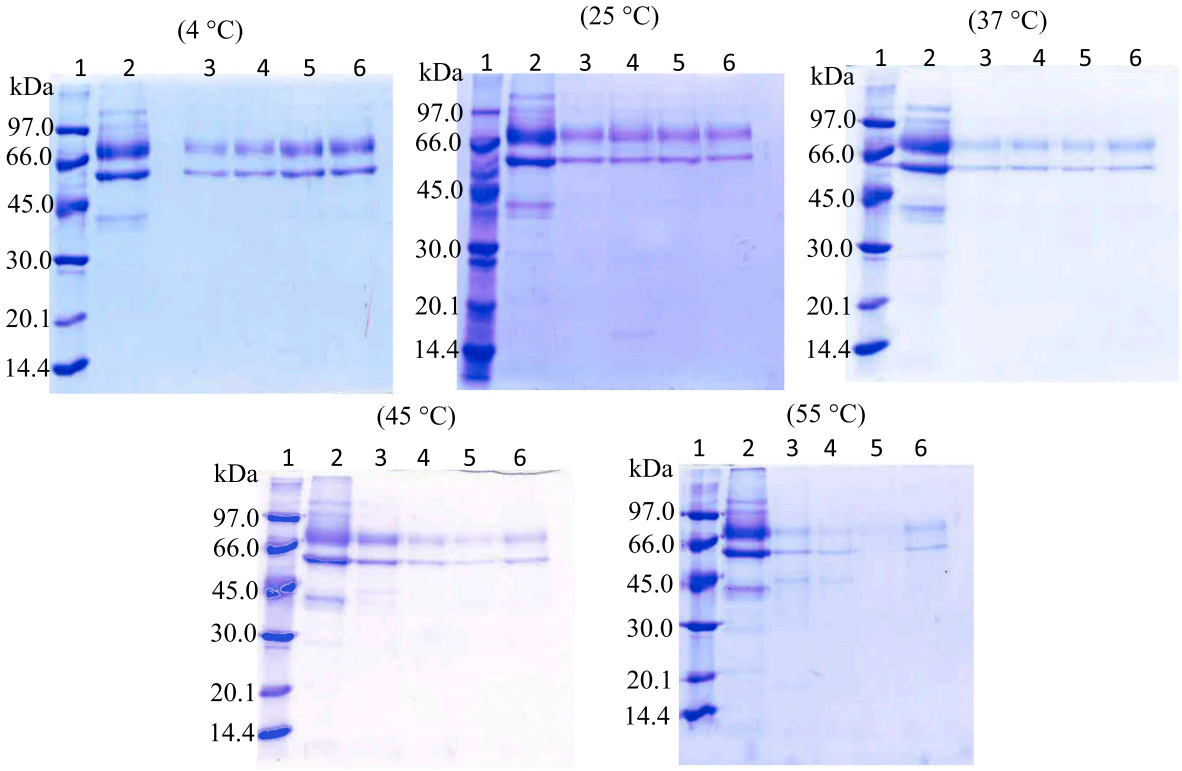


Fig. 7. SDS-PAGE analysis of β-galactosidase immobilized on MANAE-GLU-GLU after incubation in 0.3 M NaCl and different detergents at 4 °C; 37 °C; 45 °C; and 55 °C. Lane 1: low molecular weight protein standard from GE Healthcare. Lane 2: MANAE-GLU-GLU-β-gal. Lane 3: enzyme remaining in the support after desorption of β-gal with 0.3 M NaCl. Lane 4: enzyme remaining in the support after desorption of β-gal with 0.3 M NaCl and 1 % triton X-100. Lane 5: enzyme remaining in the support after desorption of β-gal with 0.3 M NaCl and 1 % CTAB. Lane 6: enzyme remaining in the support after desorption of β-gal with 0.3 M NaCl and 1 % SDS.

3.5. Effect of the ionic strength on the immobilization of beta-galactosidase on different aminated supports

Considering the fact that the galactosidase was able to establish mixed ionic/hydrophobic interactions with the MANAE-GLU and MANAE-GLU-GLU supports, and that this last one was able to immobilize lipases via interfacial activation [65], we assayed the possibility of immobilizing the galactosidase on the 3 supports when increasing the ionic strength. The immobilization via these physical causes requires the simultaneous establishment of several enzyme-support interactions [64]. Fig. 3S shows how MANAE support was only able to immobilize galactosidase in 5 mM Tris, the addition of 0.25 M NaCl already prevented the immobilization. These results were confirmed by SDS-PAGE analysis (Fig. 9).

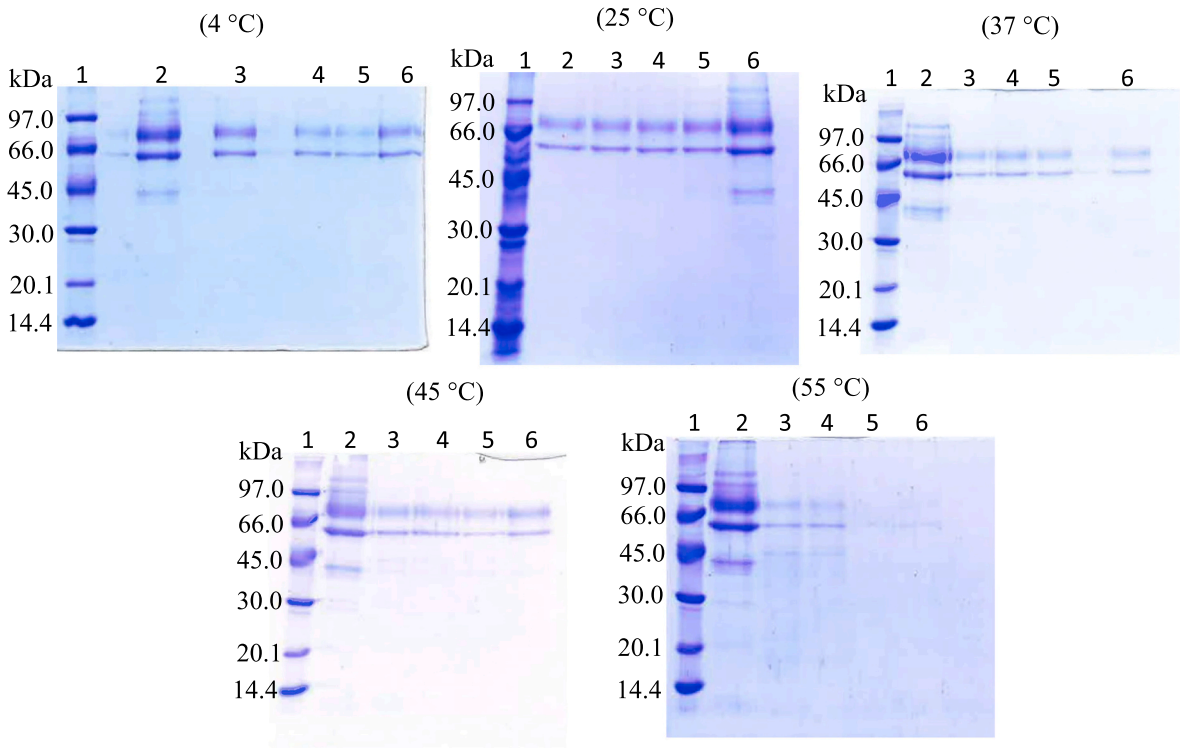


Fig. 8. SDS-PAGE analysis of β-galactosidase desorption with 0.6 M NaCl and different detergents at 4 °C; 37 °C; 45 °C; and 55 °C. Lane 1: low molecular weight protein standard from GE Healthcare. Lane 2: MANAE-GLU-GlU-β-gal. Lane 3: enzyme remaining in the support after desorption of β-gal with 0.6 M NaCl. Lane 4: enzyme remaining in the support after desorption of β-gal with 0.6 M NaCl and 1 % triton. Lane 5: enzyme remaining in the support after desorption of β-gal with 0.6 M NaCl and 1 % CTAB. Lane 6: enzyme remaining in the support after desorption of β-gal with 0.6 M NaCl and 1 % SDS.

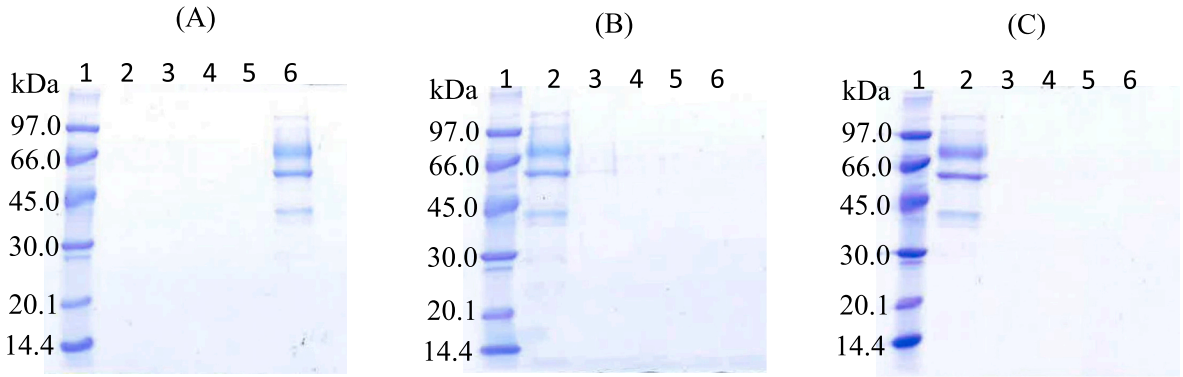


Fig. 9. SDS-PAGE analysis of β-galactosidase immobilized on different supports in 5 mM of Tris-HCl buffer at pH 7.0 adding different NaCl concentrations. (A) ß-Galactosidase immobilized on MANAE support. Lane 1: low molecular weight protein standard from GE Healthcare. Lane 2: adsorption of β-gal adding 0.3 M NaCl. Lane 3: adsorption of β-gal in 0.6.

Using MANAE-GLU (Fig. 4S), results was fairly similar; the enzyme could be immobilized using 5 mM Tris, while this was not possible using 0.25 mM NaCl. Even using 1 or 2 M NaCl, conditions that cannot release all the immobilized enzyme, there was not a significant immobilization of the enzyme (Fig. 9).

Using MANAE-GLU-GLU, results were surprisingly similar to those obtained using the other supports (Fig. 5S). The enzyme was immobilized only at low ionic strength, but the support was unable to hydrophobically immobilize the enzyme at 2 M NaCl, even with the fairly hydrophobic character that can be assumed of the reduced GLU-GLU moiety. This occurred even if the enzyme cannot be released from the support just using ionic strength and also requires adding ionic detergents and using high temperatures.

Using MANAE supports activated with glutaraldehyde (reactive glutaraldehyde, not reduced), it has been shown that using a moderate-to-high ionic strength, the immobilization rate of the enzymes significantly decrease in most instances and it becomes tricky to have, as first event of the immobilization process on these supports, the direct enzyme covalent immobilization [56,57].

4. Conclusion

The results shown in this paper confirm the heterofunctional character of aminated supports modified with glutaraldehyde [18]. Even after eliminating the chemical reactivity of the supports by reduction with borohydride [18–25], the glutaraldehyde moieties were able to establish interactions with the immobilized enzyme, making their further release complex.

The results clearly show that it is easier to prevent protein adsorption that, once the enzyme is immobilized, to release the enzyme. This very likely is caused because the protein immobilization via ion exchange or hydrophobic interactions requires the simultaneous establishment of several enzyme-supports interactions, and this may be not easy to reach [64]. However, when the enzyme is already immobilized on the support, the proximity of other moieties may enable the formation of multiple enzyme-support interactions, making enzyme release under mild conditions highly challenging [66,67]. This should be similar to different reports on enzyme immobilization on heterofunctional supports, where the enzyme is unable to become directly immobilized on a covalent way on the support, while covalent immobilization proceeds very rapidly after a previous enzyme adsorption [68–71].

From an applied point of view, it seems that the use of reduced or inactive amino-glu-glu supports can be a way of preparing reversibly immobilized enzymes reducing the risks of enzyme release during operation and enabling the support reuse after enzyme inactivation [66,67]. As the protein adsorption on these supports is a multipoint process, if the enzyme release is to be facilitated, a possibility may be the use of supports with a lower density of amination. This can decrease the relevance of the adsorption (ionic exchange and hydrophobic interactions) but it will also have a lower immobilization rate and a decrease in the possibility of having multipoint covalent immobilization (a strategy of stabilizing the immobilized enzymes) as counter-effects.

However, these results also show some problems. The first one is academic. Using amino-GLU-GLU, the first immobilization cause uses to be ion exchange, far faster than covalent immobilization. In most cases, the researcher will take for granted that using this support immobilization means covalent immobilization, in the best case scenario some trials of releasing the enzyme using high ionic strength or detergent are presented. The current results show that this can lead to misleading conclusions, as the enzyme may be very strongly adsorbed to the support; only the SDS-PAGE analysis will show if really the all enzyme molecules are covalently immobilized [2].

Moreover, as the release of the enzyme adsorbed on amino-glu-glu requires the simultaneous use of moderate ionic strength, ionic detergents, and 55 °C, it can be assumed that under these conditions any immobilized enzyme may have their stability highly compromised. That way, it does not look feasible to use glutaraldehyde preactivated supports to immobilize a stable enzyme, reduce the support and later coimmobilize an enzyme via ion exchange (that may be far less stable than the first one after immobilization), to establish a strategy to reuse the most stable enzyme. However, the enzyme can be easily released from amino-glutaraldehyde supports. It should be considered that, after the paper by Monsan [31] (and many results from our research group), it is well known that the reactivity of amino-Glu to immobilize enzymes is very limited. However, the previous adsorption of the enzyme on aminated support, and its further treatment with glutaraldehyde is a very good strategy to get multipointly immobilized enzymes, and the final support should have amino-glutaraldehyde moieties that can be reduced [18–25]. Thus, this immobilization strategy using glutaraldehyde may be compatible with the design of combibiocatalysts that permits the reuse of the most stable and covalently attached enzyme [50–54,72–74]. This research is in course in our research group [72].

CRediT authorship contribution statement

Diandra de Andrades: Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Pedro Abellanas:** Writing – review & editing, Writing – original draft, Investigation. **Diego Carballares:** Writing – review & editing, Investigation. **Andres R. Alcantara:** Data curation, Formal analysis, Investigation. **Maria de Lourdes Teixeira de Moraes Polizeli:** Writing – review & editing, Formal analysis. **Javier Rocha-Martin:** Writing – review & editing, Data curation, Conceptualization. **Roberto Fernandez-Lafuente:** Writing – review & editing, Writing – original draft, Supervision, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We gratefully recognize the financial support from Ministerio de Ciencia e Innovación and Agencia Estatal de Investigación (Spanish Government) (PID2022-136535OB-I00). DC is holding a FPI fellowship. The authors gratefully acknowledge FAPESP (São Paulo Research Foundation) by research scholarship to DA (Grant No: 2023/01338-7). The help and suggestions from Dr. Ángel Berenguer (Departamento de Química Inorgánica, Universidad de Alicante) are gratefully recognized.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2024.130403.

References

[1] X. Pei, Z. Luo, L. Qiao, Q. Xiao, P. Zhang, A. Wang, R.A. Sheldon, Putting precision and elegance in enzyme immobilisation with bio-orthogonal chemistry, Chem. Soc. Rev. 51 (2022) 7281–7304, https://doi.org/10.1039/D1CS01004B.
[2] J.M. Bolivar, J.M. Woodley, R. Fernandez-Lafuente, Is enzyme immobilization a mature discipline? Some critical considerations to capitalize on the benefits of immobilization, Chem Soc Rev 51 (2022) 6251–6290, https://doi.org/10.1039/D2CS00083K.
[3] K. Xu, B. Appiah, B.-W. Zhang, Z.-H. Yang, C. Quan, Recent advances in enzyme immobilization based on nanoflowers, J. Catal. 418 (2023) 31–39, https://doi.org/10.1016/j.jcat.2023.01.001.
[4] R.C. Rodrigues, Á. Berenguer-Murcia, D. Carballares, R. Morellon-Sterling, R. Fernandez-Lafuente, Stabilization of enzymes via immobilization: multipoint covalent attachment and other stabilization strategies, Biotechnol. Adv. 52 (2021) 107821, https://doi.org/10.1016/j.biotechadv.2021.107821.
[5] R.A. Sheldon, A. Basso, D. Brady, New frontiers in enzyme immobilisation: robust biocatalysts for a circular bio-based economy, Chem. Soc. Rev. 50 (2021) 5850–5862, https://doi.org/10.1039/D1CS00015B.
[6] D.-M. Liu, C. Dong, Recent advances in nano-carrier immobilized enzymes and their applications, Process Biochem. (2020), https://doi.org/10.1016/j.procbio.2020.02.005.
[7] R.A. Wahab, N. Elias, F. Abdullah, S.K. Ghoshal, On the taught new tricks of enzymes immobilization: an all-inclusive overview, React. Funct. Polym. 152 (2020) 104613, https://doi.org/10.1016/j.reactfunctpolym.2020.104613.
[8] C. Garcia-Galan, Á. Berenguer-Murcia, R. Fernandez-Lafuente, R.C. Rodrigues, Potential of different enzyme immobilization strategies to improve enzyme performance, Adv. Synth. Catal. 353 (2011) 2885–2904, https://doi.org/10.1002/adsc.201100534.
[9] C. Mateo, J.M. Palomo, G. Fernandez-Lorente, J.M. Guisan, R. Fernandez-Lafuente, Improvement of enzyme activity, stability and selectivity via immobilization techniques, Enzyme Microb. Technol. 40 (2007) 1451–1463, https://doi.org/10.1016/j.enzmictec.2007.01.018.
[10] K. Hernandez, A. Berenguer-Murcia, R.C. Rodrigues, R. Fernandez-Lafuente, Hydrogen peroxide in biocatalysis. A dangerous liaison, Curr Org Chem 16 (2012) 2652–2672, https://doi.org/10.2174/138527212804004526.
[11] O. Barbosa, C. Ortiz, Á. Berenguer-Murcia, R. Torres, R.C. Rodrigues, R. Fernandez-Lafuente, Strategies for the one-step immobilization–purification of enzymes as industrial biocatalysts, Biotechnol. Adv. 33 (2015) 435–456, https://doi.org/10.1016/j.biotechadv.2015.03.006.
[12] M.-G. Sánchez-Otero, R. Quintana-Castro, A.-S. Rojas-Vázquez, G.-L. Badillo-Zeferino, K. Mondragón-Vázquez, G. Espinosa-Luna, A. Kumar Nadda, R.M. Oliart-Ros, Polypropylene as a selective support for the immobilization of lipolytic enzymes: hyper-activation, purification and biotechnological applications, J. Chem. Technol. Biotechnol. 97 (2022) 436–445, https://doi.org/10.1002/jctb.6876.
[13] A. Gennari, R. Simon, N.D.M. de Sperotto, C.V. Bizarro, L.A. Basso, P. Machado, E. V. Benvenutti, A. Da Cas Viegas, S. Nicolodi, G. Renard, J.M. Chies, G. Volpato, C.F. Volken de Souza, One-step purification of a recombinant beta-galactosidase using magnetic cellulose as a support: rapid immobilization and high thermal stability, Bioresour. Technol. 345 (2022) 126497, https://doi.org/10.1016/j.biortech.2021.126497.
[14] E.E. Esparza-Flores, L.B. Siquiera, F.D. Cardoso, T.H. Costa, E.V. Benvenutti, I. E. Medina-Ramírez, M. Perullini, P.R. Santagapita, R.C. Rodrigues, P.F. Hertz, Chitosan with modified porosity and crosslinked with genipin: a dynamic system

structurally characterized, Food Hydrocoll. 144 (2023) 109034, https://doi.org/10.1016/j.foodhyd.2023.109034.
[15] M.C.C. Pinto, S.S. Everton, L.C.M. Cirilo, T.N.M. de Null, E.P. Cipolatti, E. A. Manoel, J.C. Pinto, D.M.G. Freire, Effect of hydrophobicity degree of polymer particles on lipase immobilization and on biocatalyst performance, Biocatal. Biotransformation 38 (2020) 304–314, https://doi.org/10.1080/10242422.2020.1739026.
[16] O. Barbosa, R. Torres, C. Ortiz, Á. Berenguer-Murcia, R.C. Rodrigues, R. Fernandez-Lafuente, Heterofunctional supports in enzyme immobilization: from traditional immobilization protocols to opportunities in tuning enzyme properties, Biomacromolecules 14 (2013) 2433–2462, https://doi.org/10.1021/bm400762h.
[17] R.M. Bezerra, R.R.C. Monteiro, D.M.A. Neto, F.F.M. da Silva, R.C.M. de Paula, T.L. G. de Lemos, P.B.A. Fechine, M.A. Correa, F. Bohn, L.R.B. Gonçalves, J.C.S. dos Santos, A new heterofunctional support for enzyme immobilization: PEI functionalized Fe3O4 MNPs activated with divinyl sulfone. Application in the immobilization of lipase from Thermomyces lanuginosus, Enzyme Microb Technol 138 (2020) 109560, https://doi.org/10.1016/j.enzmictec.2020.109560.
[18] O. Barbosa, C. Ortiz, Á. Berenguer-Murcia, R. Torres, R.C. Rodrigues, R. Fernandez-Lafuente, Glutaraldehyde in bio-catalysts design: a useful crosslinker and a versatile tool in enzyme immobilization, RSC Adv. 4 (2014) 1583–1600, https://doi.org/10.1039/c3ra45991h.
[19] I. Migneault, C. Dartiguenave, M.J. Bertrand, K.C. Waldron, Glutaraldehyde: behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking, Biotechniques 37 (2004) 790–802, https://doi.org/10.2144/04375RV01.
[20] X. Fan, P. Zhang, S. Teng, M. Fan, P. Jiang, A. Haryono, Y. Leng, Recent research on lipase immobilization with multipoint covalent treatment by glutaraldehyde, Curr. Org. Chem. 27 (2023) 248–259, https://doi.org/10.2174/1385272827666230417084200.
[21] S.D. Gür, N. İdil, N. Aksöz, Optimization of enzyme co-immobilization with sodium alginate and glutaraldehyde-activated chitosan beads, Appl. Biochem. Biotechnol. 184 (2018) 538–552, https://doi.org/10.1007/s12010-017-2566-5.
[22] K. Li, J. Wang, Y. He, G. Cui, M.A. Abdulrazaq, Y. Yan, Enhancing enzyme activity and enantioselectivity of *Burkholderia cepacia* lipase via immobilization on melamine-glutaraldehyde dendrimer modified magnetic nanoparticles, Chem. Eng. J. 351 (2018) 258–268, https://doi.org/10.1016/j.cej.2018.06.086.
[23] L.C.D. Lima, D.G.C. Peres, A.A. Mendes, Kinetic and thermodynamic studies on the enzymatic synthesis of wax ester catalyzed by lipase immobilized on glutaraldehyde-activated rice husk particles, Bioprocess Biosyst. Eng. 41 (2018) 991–1002, https://doi.org/10.1007/s00449-018-1929-9.
[24] S.V. Pawar, G.D. Yadav, Enantioselective enzymatic hydrolysis of rac-Mandelonitrile to R-Mandelamide by nitrile hydratase immobilized on poly(vinyl alcohol)/chitosan–glutaraldehyde support, Ind. Eng. Chem. Res. 53 (2014) 7986–7991, https://doi.org/10.1021/ie500564b.
[25] T.N. Nwagu, B. Okolo, H. Aoyagi, Immobilization of raw starch saccharifying amylase on glutaraldehyde activated chitin flakes increases the enzyme operation range, Bioresour Technol Rep 13 (2021) 100645, https://doi.org/10.1016/j.biteb.2021.100645.
[26] R.R. de Melo, R.C. Alnoch, A.F.L. Vilela, E.M. de Souza, N. Krieger, R. Ruller, H. H. Sato, C. Mateo, New heterofunctional supports based on glutaraldehyde-activation: a tool for enzyme immobilization at neutral pH, Molecules 22 (2017), https://doi.org/10.3390/molecules22071088.
[27] P.G. Vazquez-Ortega, M.T. Alcaraz-Fructuoso, J.A. Rojas-Contreras, J. López-Miranda, R. Fernandez-Lafuente, Stabilization of dimeric β-glucosidase from *Aspergillus niger* via glutaraldehyde immobilization under different conditions, Enzyme Microb. Technol. 110 (2018) 38–45, https://doi.org/10.1016/j.enzmictec.2017.12.007.
[28] D. de Andrades, N.G. Graebin, M.K. Kadowaki, M.A.Z. Ayub, R. Fernandez-Lafuente, R.C. Rodrigues, Immobilization and stabilization of different β-glucosidases using the glutaraldehyde chemistry: optimal protocol depends on the enzyme, Int. J. Biol. Macromol. 129 (2019) 672–678, https://doi.org/10.1016/j.ijbiomac.2019.02.057.
[29] E.-H. Siar, S. Arana-Peña, O. Barbosa, M. Zidoune, R. Fernandez-Lafuente, Immobilization/stabilization of ficin extract on glutaraldehyde-activated agarose beads. Variables that control the final stability and activity in protein hydrolyses, Catalysts 8 (2018) 149, https://doi.org/10.3390/catal8040149.
[30] L. Dal Magro, J.F. Kornecki, M.P. Klein, R.C. Rodrigues, R. Fernandez-Lafuente, Pectin lyase immobilization using the glutaraldehyde chemistry increases the enzyme operation range, Enzyme Microb. Technol. 132 (2020) 109397, https://doi.org/10.1016/j.enzmictec.2019.109397.
[31] P. Monsan, Optimization of glutaraldehyde activation of a support for enzyme immobilization, J. Mol. Catal. 3 (1978) 371–384, https://doi.org/10.1016/0304-5102(78)80026-1.
[32] R. Fernandez-Lafuente, C.M. Rosell, V. Rodriguez, J.M. Guisan, Strategies for enzyme stabilization by intramolecular crosslinking with bifunctional reagents, Enzyme Microb. Technol. 17 (1995) 517–523, https://doi.org/10.1016/0141-0229(94)00090-E.
[33] H. He, Y. Wei, H. Luo, X. Li, X. Wang, C. Liang, Y. Chang, H. Yu, Z. Shen, Immobilization and stabilization of cephalosporin C acylase on aminated support by crosslinking with glutaraldehyde and further modifying with aminated macromolecules, Biotechnol. Prog. 31 (2015) 387–395, https://doi.org/10.1002/btpr.2044.
[34] J. Boudrant, J.M. Woodley, R. Fernandez-Lafuente, Parameters necessary to define an immobilized enzyme preparation, Process Biochem. 90 (2020) 66–80, https://doi.org/10.1016/j.procbio.2019.11.026.
[35] T. Jesionowski, J. Zdarta, B. Krajewska, Enzyme immobilization by adsorption: a review, Adsorption 20 (2014) 801–821, https://doi.org/10.1007/s10450-014-9623-y.
[36] E. Skoronski, D.H. Souza, C. Ely, F. Broilo, M. Fernandes, A. Fúrigo, M.G. Ghislandi, Immobilization of laccase from Aspergillus oryzae on graphene nanosheets, Int. J. Biol. Macromol. 99 (2017) 121–127, https://doi.org/10.1016/j.ijbiomac.2017.02.076.
[37] J.H. Eike, A.F. Palmer, Effect of NaBH4 concentration and reaction time on physical properties of glutaraldehyde-polymerized hemoglobin, Biotechnol. Prog. 20 (2004) 946–952, https://doi.org/10.1021/bp0343155.
[38] M. Bartolini, V. Cavrini, V. Andrisano, Monolithic micro-immobilized-enzyme reactor with human recombinant acetylcholinesterase for on-line inhibition studies, J. Chromatogr. A 1031 (2004) 27–34, https://doi.org/10.1016/j.chroma.2003.11.080.
[39] J.M.C.S. Magalhães, A.A.S.C. Machado, Urea potentiometric biosensor based on urease immobilized on chitosan membranes, Talanta 47 (1998) 183–191, https://doi.org/10.1016/S0039-9140(98)00066-6.
[40] G. Spagna, F. Andreani, E. Salatelli, D. Romagnoli, D. Casarini, P.G. Pifferi, Immobilization of the glycosidases: α-l-arabinofuranosidase and β-d-glucopyranosidase from Aspergillus niger on a chitosan derivative to increase the aroma of wine. Part II, Enzyme Microb Technol 23 (1998) 413–421, https://doi.org/10.1016/S0141-0229(97)00249-4.
[41] O.P. Bahl, K.M.L. Agrawal, Glycosidases of *Aspergillus niger*: I. Purification and characterization of α- and β-galactosidases and β-N-acetylglucosaminidase, Journal of Biological Chemistry 244 (1969) 2970–2978, https://doi.org/10.1016/S0021-9258(18)91719-9.
[42] J.R. Xavier, K.V. Ramana, R.K. Sharma, β-Galactosidase: biotechnological applications in food processing, J. Food Biochem. 42 (2018) e12564, https://doi.org/10.1111/jfbc.12564.
[43] C. Oliveira, P.M.R. Guimarães, L. Domingues, Recombinant microbial systems for improved β-galactosidase production and biotechnological applications, Biotechnol. Adv. 29 (2011) 600–609, https://doi.org/10.1016/j.biotechadv.2011.03.008.
[44] S. Saqib, A. Akram, S.A. Halim, R. Tassaduq, Sources of β-galactosidase and its applications in food industry, 3 Biotech 7 (2017) 79, https://doi.org/10.1007/s13205-017-0645-5.
[45] P.S. Panesar, S. Kumari, R. Panesar, Potential applications of immobilized β-galactosidase in food processing industries, Enzyme Res 2010 (2010) 473137, https://doi.org/10.4061/2010/473137.
[46] M. Dutra Rosolen, A. Gennari, G. Volpato, C.F. Volken de Souza, Lactose hydrolysis in milk and dairy whey using microbial β-galactosidases, Enzyme Res. 2015 (2015) 806240, https://doi.org/10.1155/2015/806240.
[47] S. Arana-Peña, D. Carballares, R. Morellon-Sterlling, Á. Berenguer-Murcia, A. R. Alcántara, R.C. Rodrigues, R. Fernandez-Lafuente, Enzyme co-immobilization: always the biocatalyst designers' choice…or not? Biotechnol. Adv. 51 (2021) 107584 https://doi.org/10.1016/j.biotechadv.2020.107584.
[48] A.V. Høst, R. Morellon-Sterling, D. Carballares, J.M. Woodley, R. Fernandez-Lafuente, Co-enzymes with dissimilar stabilities: a discussion of the likely biocatalyst performance problems and some potential solutions, Catalysts 12 (2022) 1570, https://doi.org/10.3390/catal12121570.
[49] D. Carballares, J. Rocha-Martin, R. Fernandez-Lafuente, Preparation of a six-enzyme multilayer combi-biocatalyst: reuse of the most stable enzymes after inactivation of the least stable one, ACS Sustain Chem Eng 10 (2022) 3920–3934, https://doi.org/10.1021/acssuschemeng.1c08180.
[50] D. Carballares, J. Rocha-Martin, R. Fernandez-Lafuente, Chemical amination of immobilized enzymes for enzyme coimmobilization: reuse of the most stable immobilized and modified enzyme, Int. J. Biol. Macromol. 208 (2022) 688–697, https://doi.org/10.1016/j.ijbiomac.2022.03.151.
[51] D. Carballares, J. Rocha-Martin, R. Fernandez-Lafuente, Coimmobilization of lipases exhibiting three very different stability ranges. Reuse of the active enzymes and selective discarding of the inactivated ones, Int J Biol Macromol 206 (2022) 580–590, https://doi.org/10.1016/j.ijbiomac.2022.02.084.
[52] R. Morellon-Sterling, D. Carballares, S. Arana-Peña, E.-H. Siar, S.A. Braham, R. Fernandez-Lafuente, Advantages of supports activated with divinyl sulfone in enzyme coimmobilization: possibility of multipoint covalent immobilization of the most stable enzyme and immobilization via ion exchange of the least stable enzyme, ACS Sustain. Chem. Eng. 9 (2021) 7508–7518, https://doi.org/10.1021/acssuschemeng.1c01065.
[53] S. Arana-Peña, D. Carballares, V. Cortés Corberan, R. Fernandez-Lafuente, Multi-combilipases: co-immobilizing lipases with very different stabilities combining immobilization via interfacial activation and ion exchange. The reuse of the most stable co-immobilized enzymes after inactivation of the least stable ones, Catalysts 10 (2020) 1207, https://doi.org/10.3390/catal10101207.
[54] S. Arana-Peña, D. Carballares, R. Morellon-Sterling, J. Rocha-Martin, R. Fernandez-Lafuente, The combination of covalent and ionic exchange immobilizations enables the coimmobilization on vinyl sulfone activated supports and the reuse of the most stable immobilized enzyme, Int. J. Biol. Macromol. 199 (2022) 51–60, https://doi.org/10.1016/j.ijbiomac.2021.12.148.
[55] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254, https://doi.org/10.1016/0003-2697(76)90527-3.
[56] R. Fernandez-Lafuente, C.M. Rosell, V. Rodriguez, C. Santana, G. Soler, A. Bastida, J.M. Guisan, Preparation of activated supports containing low pK amino groups. A new tool for protein immobilization via the carboxyl coupling method, Enzyme Microb Technol 15 (1993) 546–550, https://doi.org/10.1016/0141-0229(93)90016-U.

[57] D. de Andrades, N.G. Graebin, M.K. Kadowaki, M.A.Z. Ayub, R. Fernandez-Lafuente, R.C. Rodrigues, Immobilization and stabilization of different β-glucosidases using the glutaraldehyde chemistry: optimal protocol depends on the enzyme, Int. J. Biol. Macromol. 129 (2019) 672–678, https://doi.org/10.1016/j.ijbiomac.2019.02.057.

[58] A. Corma, V. Fornés, J.L. Jordá, F. Rey, R. Fernandez-Lafuente, J.M. Guisan, C. Mateo, Electrostatic and covalent immobilisation of enzymes on ITQ-6 delaminated zeolitic materials, Chem. Commun. (2001) 419–420, https://doi.org/10.1039/B009232K.

[59] C. Mateo, O. Abian, M. Bernedo, E. Cuenca, M. Fuentes, G. Fernández-Lorente, J. M. Palomo, V. Grazu, B. Pessela, C. Giacomini, G. Irazoqui, A. Villarino, K. Ovsejevi, F. Batista-Viera, R. Fernandez-Lafuente, J. Guisan, Some Special Features of Glyoxyl Supports to Immobilize Proteins, 2005, https://doi.org/10.1016/j.enzmictec.2005.03.020.

[60] C. Mateo, J.M. Palomo, M. Fuentes, L. Betancor, V. Grazu, F. López-Gallego, B. Pessela, A. Hidalgo, G. Fernández-Lorente, R. Fernández-Lafuente, J. Guisan, Glyoxyl Agarose: A Fully Inert and Hydrophilic Support for Immobilization and High Stabilization of Proteins, 2006, https://doi.org/10.1016/j.enzmictec.2005.10.014.

[61] A. Jarosz, O. Kapusta, D. Gugała-Fekner, M. Barczak, Synthesis and characterization of agarose hydrogels for release of diclofenac sodium, Materials 16 (2023) 6042, https://doi.org/10.3390/ma16176042.

[62] Y. Wang, X. Zhang, N. Han, Y. Wu, D. Wei, Oriented covalent immobilization of recombinant protein a on the glutaraldehyde activated agarose support, Int. J. Biol. Macromol. 120 (2018) 100–108, https://doi.org/10.1016/j.ijbiomac.2018.08.074.

[63] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685, https://doi.org/10.1038/227680a0.

[64] B.C.C. Pessela, R. Munilla, L. Betancor, M. Fuentes, A.V. Carrascosa, A. Vian, R. Fernandez-Lafuente, J.M. Guisán, Ion exchange using poorly activated supports, an easy way for purification of large proteins, J. Chromatogr. A 1034 (2004) 155–159, https://doi.org/10.1016/j.chroma.2004.01.061.

[65] O. Barbosa, R. Torres, C. Ortiz, R. Fernandez-Lafuente, Versatility of glutaraldehyde to immobilize lipases: effect of the immobilization protocol on the properties of lipase B from *Candida antarctica*, Process Biochem. 47 (2012) 1220–1227, https://doi.org/10.1016/j.procbio.2012.04.019.

[66] J.P. Miguez, R. Fernandez-Lafuente, O.L. Tavano, A.A. Mendes, The immobilization and stabilization of trypsin from the porcine pancreas on chitosan and its catalytic performance in protein hydrolysis, Catalysts 13 (2023), https://doi.org/10.3390/catal13101344.

[67] N.S. Okura, G.J. Sabi, M.C. Crivellenti, R.A.B. Gomes, R. Fernandez-Lafuente, A. A. Mendes, Improved immobilization of lipase from *Thermomyces lanuginosus* on a new chitosan-based heterofunctional support: mixed ion exchange plus hydrophobic interactions, Int. J. Biol. Macromol. 163 (2020) 550–561, https://doi.org/10.1016/j.ijbiomac.2020.07.021.

[68] T.L.D. Albuquerque, N. Rueda, J.C.S. Dos Santos, O. Barbosa, C. Ortiz, B. Binay, E. Özdemir, L.R.B. Gonçalves, R. Fernandez-Lafuente, Easy stabilization of interfacially activated lipases using heterofunctional divinyl sulfone activated-octyl agarose beads. Modulation of the immobilized enzymes by altering their nanoenvironment, Process Biochemistry 51 (2016) 865–874, https://doi.org/10.1016/J.PROCBIO.2016.04.002.

[69] H. Zaak, M. Sassi, R. Fernandez-Lafuente, A new heterofunctional amino-vinyl sulfone support to immobilize enzymes: application to the stabilization of *β*-galactosidase from *aspergillus oryzae*, Process Biochem. 64 (2018) 200–205, https://doi.org/10.1016/j.procbio.2017.09.020.

[70] T.N. da Rocha, R. Morellon-Sterling, L.R.B. Gonçalves, J.M. Bolivar, A. R. Alcántara, J. Rocha-Martin, R. Fernández-Lafuente, Synergy of ion exchange and covalent reaction: immobilization of penicillin G Acylase on Heterofunctional amino-vinyl sulfone agarose, Catalysts 13 (2023), https://doi.org/10.3390/catal13010151.

[71] T.N. Da Rocha, R. Morellon-Sterling, J. Rocha-Martin, J.M. Bolivar, R. Gonçalves, Luciana R.B. Fernandez-Lafuente, Immobilization of penicillin G acylase on vinyl sulfone-agarose. An unexpected effect of the ionic strength on the performance of the immobilization process, Molecules 27 (2022) 7587.

[72] D. Carballares, J. Rocha-Martín, R. Fernandez-Lafuente, Preparation of a six-enzymes multilayer combi-biocatalyst. Reuse of the most stable enzymes after inactivation of the least stable one. ACS Sustain Chem Eng (n.d.).

[73] S. Arana-Peña, C. Mendez-Sanchez, N.S. Rios, C. Ortiz, L.R.B. Gonçalves, R. Fernandez-Lafuente, New applications of glyoxyl-octyl agarose in lipases co-immobilization: strategies to reuse the most stable lipase, Int. J. Biol. Macromol. 131 (2019) 989–997, https://doi.org/10.1016/j.ijbiomac.2019.03.163.

[74] N.S. Rios, S. Arana-Peña, C. Mendez-Sanchez, C. Ortiz, L.R.B. Gonçalves, R. Fernandez-Lafuente, Reuse of lipase from *Pseudomonas fluorescens* via its step-by-step coimmobilization on glyoxyl-octyl agarose beads with least stable lipases, Catalysts 9 (2019) 487, https://doi.org/10.3390/catal9050487.