ACS Sustainable Chemistry & Engineering

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Research Article

Reutilization of the Most Stable Coimmobilized Enzyme Using Glutaraldehyde Chemistry to Produce a New Combi-biocatalyst When the Coimmobilized Enzyme with a Lower Stability Is Inactivated

Diego Carballares,[‖] Pedro Abellanas-Perez,[‖] Diandra de Andrades, Maria de Lourdes Teixeira de Moraes Polizeli, Javier Rocha-Martin, and Roberto Fernandez-Lafuente*

Cite This: *ACS Sustainable Chem. Eng.* 2024, 12, 6564−6572

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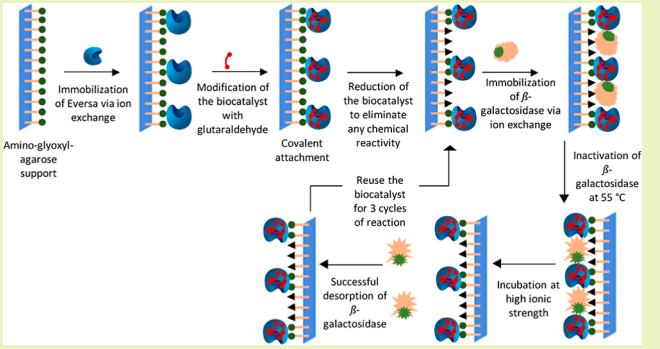
ABSTRACT: In the present article, glutaraldehyde was used to covalently coimmobilize the lipase Eversa Transform 2.0 and the β-galactosidase from *Aspergillus oryzae*. Both enzymes were adsorbed on amino supports and modified with glutaraldehyde. However, the first enzyme remained almost fully active under stress conditions, while the β-galactosidase lost a large percentage of its activity. To prevent the necessity of discarding both enzymes, the lipase was covalently immobilized following this immobilization strategy. The biocatalyst was reduced to eliminate its chemical reactivity, and the β-galactosidase was then coimmobilized via ion exchange. The incubation at high concentrations of salt desorbed the β-galactosidase from the support. This combi-biocatalyst was used in three inactivation/rebuilding cycles where the inactivated β-galactosidase was liberated from the combi-biocatalyst by washing at high ionic strength and replaced with a fresh enzyme, while the immobilized lipase maintained its activity throughout the 3 cycles. That way, it was possible to use this strategy to reuse the immobilized Eversa Transform 2.0 to build new combi-biocatalysts after β-galactosidase inactivation.



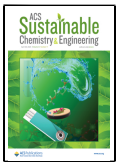
KEYWORDS: *enzyme coimmobilization, dissimilar enzyme stabilities, reuse of stable coimmobilized enzymes, enzyme release from combi-biocatalysts*

INTRODUCTION

The development of biocatalysis[1−5] and the focus on cascade reactions[6−10] have promoted a growing interest in the design of coimmobilized enzymes.[11−14] As the enzymes involved in the cascade are in a confined space after coimmobilization, this permits all coimmobilized enzymes to have available high concentrations of their substrates from the beginning of the reaction, avoiding the lag time usually found when utilizing independently immobilized enzymes.[15,16] When some intermediate product is unstable, this lag time can define the obtained yields and the level of contamination by the unwanted byproduct.[17] Otherwise, enzyme coimmobilization mainly allows a reduction in the reaction cycle time, and this time saving may depend on the enzyme kinetic features and biocatalyst features.[17]

In the design of coenzyme-immobilized biocatalysts, some facts need to be considered. The first one is that enzyme immobilization is much more than a technique to separate the enzyme from the reaction medium, reutilize it, and facilitate downstream processing of the product.[18] Immobilization is the last opportunity to tune the properties of the enzyme biocatalyst:[19−25] adequate enzyme immobilization may enhance its stability (e.g., by promoting many enzyme−support bonds)[26] or be associated with enzyme purification,[27] avoiding spending time and expenses in the building of the biocatalysts. Immobilization can distort the enzyme structure, producing new enzyme conformations that in some instances can present enhanced activity, specificity, or selectivity.[28] However, in enzyme coimmobilization, it is not very likely that the same protocols may permit achievement of full benefits for all involved enzymes. There are some other problems related to building or use of coimmobilized enzymes that can be found in

Received: December 14, 2023
Revised: March 15, 2024
Accepted: April 9, 2024
Published: April 18, 2024



different reviews.[17,19] That way, enzyme coimmobilization should be performed only in cases where the advantages outweigh the problems.[17,19]

Now, we want to focus on a specific problem: the coimmobilization of two enzymes that, after immobilization, exhibit very different stabilities; e.g., one is almost fully inactivated after some reaction cycles, while the other immobilized enzyme remains almost fully active.[17,19] Using conventional coimmobilization protocols, all the enzymes that are coimmobilized must be cast off, although some of them remain in full activity.[17,19] Some strategies have been recently launched to overcome this matter.[29−36] All are founded on the coimmobilization of the diverse enzymes utilizing diverse immobilization strategies in such a way that the least stable coimmobilized enzyme may be eliminated from the biocatalysts without affecting the stable and almost fully active coimmobilized enzyme(s). Some strategies modify the previously immobilized most stable enzyme to convert it into an ion exchanger.[30,31,33] Other strategies modify the support used for previous covalent immobilization of the most stable immobilized enzyme to transform it into an anion exchanger.[32,34] Other strategies use heterofunctional supports.[35,36] The latter is similar to the proposal we present in this research effort. Here, we launch the use of aminated supports to coimmobilize enzymes using glutaraldehyde chemistry.[37−39] Glutaraldehyde is a quite adaptable immobilization reagent. It can be utilized to obtain a glutaraldehyde-preactivated support. For this purpose, each amino moiety in the support will be modified by two glutaraldehyde molecules.[40] This is perhaps the most popular strategy. However, in a previous paper, we found that the mixed ionic-hydrophobic adsorption of enzymes on a reduced amino-glutaraldehyde−glutaraldehyde (without chemical reactivity) support causes the following: after covalent attachment of the most stable enzyme(s) and adsorption of the least stable one(s), the release of the poorly stable and inactivated enzyme is not possible under conditions compatible with the enzyme stabilities.[41] However, another use of glutaraldehyde to covalently attach enzymes to a support consists of the glutaraldehyde modification of the enzymes that have been previously ionically exchanged on aminated supports.[37] In this case, glutaraldehyde must be utilized involving just a molecule of glutaraldehyde per amino group in both the enzyme and the support as they are very reactive with other amino-glutaraldehyde moieties but not with amino groups.[42] In our previous research, we found it possible to release enzymes from reduced amino-glutaraldehyde supports.[41]

That way, it may be possible to immobilize the most stable enzyme(s) on an aminated matrix, treat the biocatalyst to attach one molecule of glutaraldehyde to each primary amino moiety of both the support and the enzyme to obtain covalent enzyme−support bonds, reduce the biocatalyst to eliminate the possibility to establish covalent bonds, adsorb the least stable enzyme(s) on the support, and, after its inactivation, release it (them) just by incubating it at high ion strength.[41] This way, this strategy could allow that when the least stable enzyme is inactivated, the most stable and covalently insolubilized enzyme can be utilized in the preparation of a new combi-biocatalyst identical to the first one. As model enzymes, we have used Eversa Transform 2.0 (EVR), a very stable lipase that is commercially available, which has been prepared by genetic modification of the lipase from *Thermomyces lanuginosus*[43−45] and the β-galactosidase from *Aspergillus oryzae* as an enzyme showing a lower stability.[46−51] Although the enzymes have been chosen just as a model system, we can envisage some joint applications of both enzymes. One likely application may be the simultaneous modification of the fats and lactose of milk to produce free-lactose milk (or lactose enriched in prebiotic galactosides)[52−55] that could also bear flavors produced by the lipase action, decrease the triglyceride concentration,[56−60] or be protected by the presence of some free fatty acids with antimicrobial agent activity.[61−64] Other application may be the production of galactosyl-diglycerides using lipase to produce specific diglycerides from the corresponding triglyceride (e.g., 1,2 diacetin) and the β-galactosidase from *A. oryzae* as the catalyst to produce the O-link[52,65−67] between galactose and diglyceride via kinetically controlled synthesis.[68−73] In this instance, the high concentration inside the particle of the diglyceride may be critical to have good reaction yields,[71] and the immediate modification can prevent the fact that the acyl migration results in 1,3 diacetin.[74]

MATERIALS AND METHODS

Materials. Eversa Transform 2.0 (40.9 mg of protein per mL) was gifted by Novozymes Spain (Alcobendas, Madrid), and β-galactosidase from *A. oryzae* (20 units of *o*NPG/mg of protein), glutaraldehyde (GLU), ethylenediamine, *p*-nitrophenyl butyrate (*p*-NPB), 2-nitrophenyl β-D-galactopyranoside (*o*NPG), and sodium borohydride were supplied by Sigma-Aldrich (Madrid, Spain). ABT Technology (Madrid, Spain) was the supplier of agarose CL-4B. The determination of the protein concentration was carried out by employing the Bradford method.[75] Bovine serum albumin was used to prepare the calibration curves. An "analytical grade" quality was employed for other chemicals employed in this paper. The enzymes were used without any further treatment.

Methods. All experiments were carried out at least three times. The results are shown as their average value and standard deviation.

Protocols to Quantify Enzyme Activities. To quantify the activity of the lipase at pH 7.0 and 25 °C, the augmentation in absorbance at 348 nm (the isosbestic point of *p*-NP, 5150 M^{-1} cm^{-1} being the extinction coefficient[76,77]) caused by the production of *p*-nitrophenol by the hydrolysis of 0.4 mM *p*-NPB in 25 mM sodium phosphate was recorded. To begin the experiment, 25−50 μL of the enzyme solution/suspension was added to a substrate solution prepared by mixing 2.5 mL of 25 mM sodium phosphate and 50 μL of 50 mM *p*-NPB dissolved in acetonitrile.

The augmentation in absorbance at 380 nm at 25 °C and pH 5.0 caused by the liberation of *o*-nitrophenol in the hydrolysis of 10 mM *o*NPG in 50 mM sodium acetate (ε under these conditions was 10,493 M^{-1} cm^{-1}) was utilized to determine the β-galactosidase activity. To initialize the reaction, a volume of 2.5 mL of the substrate solution was added to a cuvette, and then, 50−100 μL of the enzyme suspension/solution was added.

Immobilization of Enzymes on MANAE-Agarose. The protocol described by Fernández-Lafuente et al.[78] was employed to prepare amino-agarose (MANAE-agarose). A mass of 1 g of the support was added to 10 mL of 3 mg/g Eversa Transform 2.0 or β-galactosidase extract solutions in 5 mM Tris−HCl at pH 7.0. The mixture was shaken at 25 °C using a roller, different samples of the supernatant and suspension were taken periodically, and their activities were determined by employing *p*-NPB or *o*NPG as described above. Finally, the biocatalysts were recovered using a sintered filter funnel and stored at 4−6 °C. The used loadings of the enzymes are well under the maximum loadings of the support (over 20 mg/g) to avoid substrate diffusional matters that can alter the apparent stability/activity of the enzymes.

Treatment of the Lipase Biocatalysts with Glutaraldehyde. A mass of 1 g of MANAE-Eversa Transform 2.0 (EVR) biocatalyst was incorporated into 10 mL of 50 mM sodium phosphate at pH 7.0

https://doi.org/10.1021/acssuschemeng.3c08231
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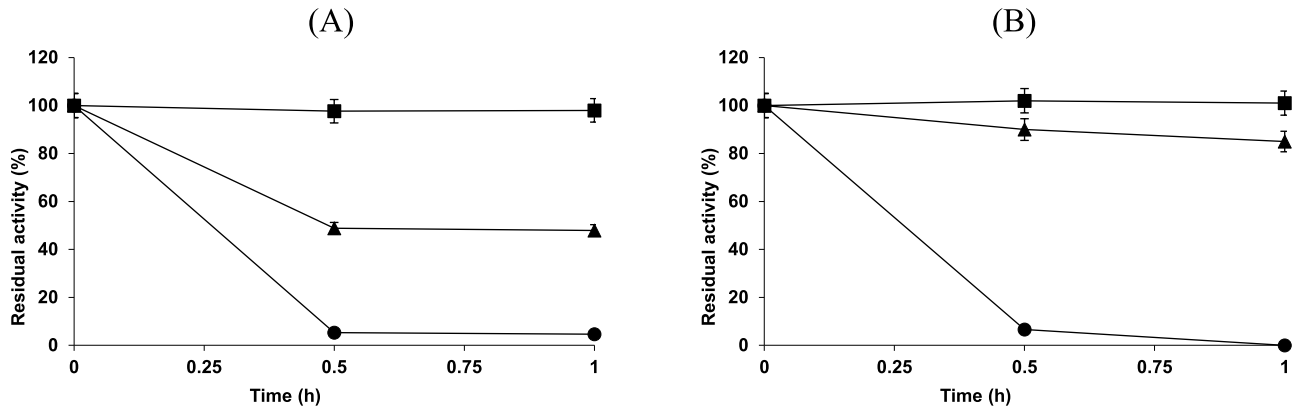

Figure 1. Immobilization course of enzymes over the MANAE support. (A) Eversa Transform 2.0 and (B) β-galactosidase. The experiment was performed using 5 mM Tris−HCl buffer at pH 7.0 and 25 °C. Other specifications can be found in the Materials and Methods section. Solid triangles: suspension; solid squares: reference; and solid circles: supernatant.

containing 1% (v/v) GLU. The suspension was stirred for 1 h at 25 °C. This protocol ensures the full modification of the primary amino groups of the enzyme(s) and the support mainly with only one molecule of glutaraldehyde.[79] The reactivity of amino-glutaraldehyde versus other amino-glutaraldehyde groups is quite high.[80,81] To permit the covalent reaction, the immobilized enzyme was left to react with the support for 5 h after washing and resuspension of the modified enzyme. Then, in order to eliminate any chemical reactivity, the biocatalyst was recovered and incubated in 0.10 M sodium carbonate at pH 9 containing 1 mg/mL sodium borohydride under mild stirring. After these modifications, the biocatalysts were washed by employing a sintered filter with distilled water and stowed at 6 °C.

Immobilization of β-Galactosidase on Reduced MANAE-Agarose-GLU. β-Galactosidase was immobilized using 3 mg of enzyme per gram of MANAE-agarose-GLU support suspended in 5 mM Tris−HCl at pH 7.0 and 25 °C for 1 h. Samples of the supernatant and suspension were taken to quantify their activities in a Jasco V-730 spectrophotometer using *o*NPG as the substrate as described above. Finally, the biocatalyst was washed in a sintered filter funnel utilizing distilled water and deposited in a fridge until its use. The biocatalyst was called MANAE-EVR-GLU-β-galactosidase.

Release of β-Galactosidase from MANAE-Agarose-GLU. To release β-galactosidase from MANAE-agarose-GLU, 1 g of the biocatalyst was incubated for 2 h at 25 °C in 10 mL of 5 mM Tris−HCl/300 mM sodium chloride at pH 7.0. Then, the biocatalyst was washed 10 times with 10 volumes of desorption solution utilizing a sintered glass funnel under vacuum. Next, exhaustive washing with distilled water was carried out before performing a sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) experiment.

SDS-PAGE Analysis of the Reversibly Immobilized Enzymes. The method proposed by Laemmli[82] was utilized in the SDS-PAGE experiments. 5% polyacrylamide was utilized for the concentration gel, and 12% polyacrylamide was used for the resolution gel. To get the samples used in these experiments, a maximum final protein concentration of 0.25−0.3 mg of protein per mL of solution was utilized. The samples were prepared by incubating the enzyme preparations in a solution containing 8% (w/v) SDS and 10% (v/v) mercaptoethanol (the rupture buffer) in a bath with boiling water. To ensure the liberation of all enzyme molecules adsorbed on the support, these suspensions were boiled for 8 min, followed by centrifugation to discard the support.[83] As samples to inject into the gel, 15 μL samples of the supernatants were utilized. A sample of 3 μL of the low-molecular-weight marker was employed. The system was subjected to 100 V, and the gels were incubated in a Coomassie brilliant blue solution to stain the protein bands.

Thermal Inactivation, Desorption, and Reuse of Coimmobilized Enzymes. One gram of the MANAE-EVR-GLU-β-galactosidase biocatalyst was added to 10 mL of 10 mM sodium carbonate, at pH 8.0, and the temperature was set to 55 °C. At specified times, samples of the inactivation suspensions were withdrawn to quantify their maintained activities (utilizing *o*NPG for β-galactosidase and *p*-NPB for lipase as substrates). Residual activities are presented as ratios with the initial activities of the biocatalysts (in percentage).

When utilizing the combi-biocatalysts, and to study if the covalently immobilized enzyme could be reused, after the indicated inactivation time, the combi-biocatalyst was incubated in 5 mM Tris−HCl containing 300 mM sodium chloride at pH 7.0 for 30 min (1 g of derivate/10 mL of desorption solution) to release the partially inactivated β-galactosidase from the combi-biocatalyst and reutilize the immobilized and almost fully active Eversa Transform 2.0. Next, the desorption solution was employed to wash the combi-biocatalyst. Afterward, exhaustive washing with distilled water was performed. Then, a fresh batch of β-galactosidase was immobilized to produce a new combi-biocatalyst. These cycles were repeated three times.

RESULTS

Preparation of a Combi-biocatalyst of Eversa Transform 2.0 and β-Galactosidase Using the Glutaraldehyde Chemistry. Figure 1 shows the immobilization course of Eversa Transform 2.0 and β-galactosidase on MANAE-agarose beads. Immobilization proceeds very rapidly in both cases, and the β-galactosidase almost maintains its activity intact, while Eversa Transform 2.0 decreased the activity by around 50%. The modification with 1% (v/v) glutaraldehyde has a scarce effect on both enzyme activities. The inactivation courses of both covalent biocatalysts at pH 8 and 55 °C can be observed in Figure 2. While Eversa Transform 2.0 almost maintained its activity intact after 2 h, the β-galactosidase lost 75% of its activity after only 30 min. That way, both enzymes could be coimmobilized using this strategy, but immobilized Eversa Transform 2.0 should be discarded after only 30 min even when it maintains its initial activity almost intact. Figure 3 shows that the immobilization of Eversa Transform 2.0 on the MANAE support produces some enzyme stabilization. The modification with glutaraldehyde of the adsorbed enzyme to achieve the promotion of covalent cross-linking between amino-GLU groups located in the support and the enzyme produces a significant stabilization of the enzyme, higher than when using MANAE−glutaraldehyde−glutaraldehyde-preactivated supports, which yielded the stability of Eversa Transform 2.0 even lower than the just ionically adsorbed enzyme. This enzyme stabilization by modification with glutaraldehyde of enzymes that are adsorbed on aminated supports occurs in many instances and suggests the production of a relatively

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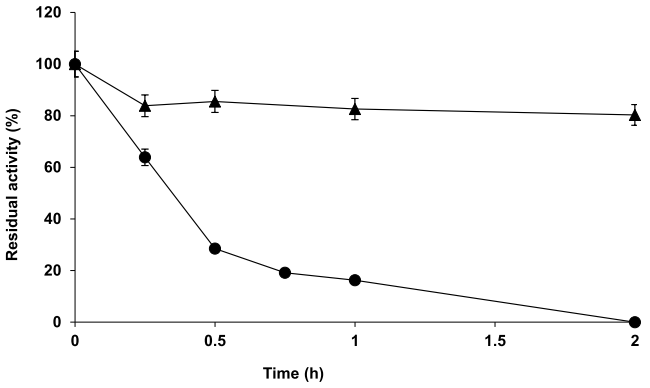

Figure 2. Inactivation course of different enzyme biocatalysts immobilized over the MANAE support. The experiment was performed using 10 mM sodium carbonate buffer at pH 8.0 and 55 °C. Other specifications can be found in the Materials and Methods section. Solid triangles: MANAE-EVR-GLU and solid circles: MANAE-β-galactosidase-GLU.

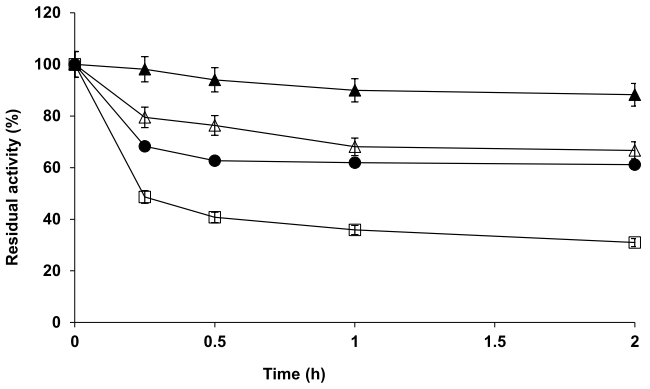

Figure 3. Thermal inactivation of different EVR biocatalysts at pH 8 and 67 °C. Other specifications can be found in the Materials and Methods section. Squares: free EVR; triangles: MANAE-EVR; solid triangles: MANAE-EVR-GLU, and solid circles: MANAE-GLU-GLU-EVR.

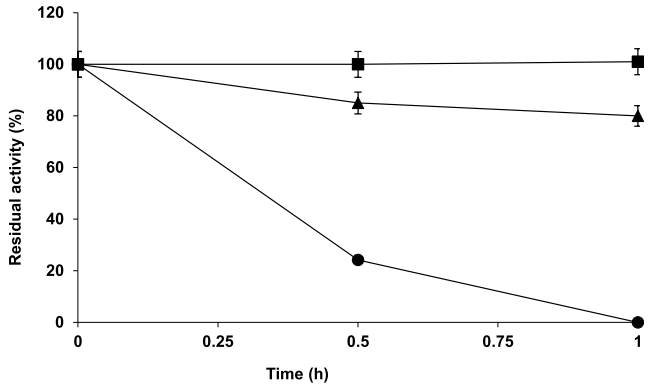

Figure 4. Immobilization course of β-galactosidase over the MANAE-GLU support. The experiment was performed using 5 mM Tris−HCl buffer at pH 7.0 and 25 °C. Other specifications can be found in the Materials and Methods section. Solid triangles: suspension; solid squares: reference; and solid circles: supernatant.

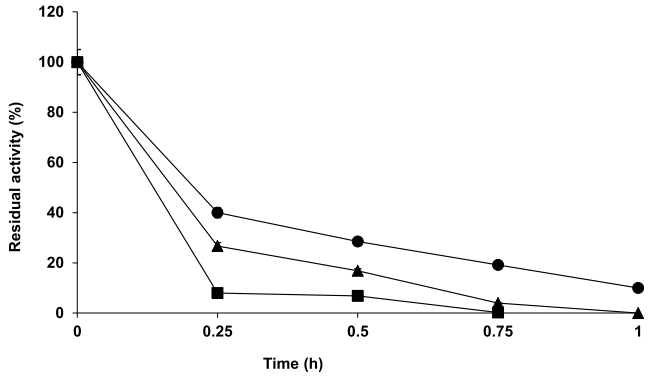

Figure 5. Inactivation course of different biocatalysts of β-galactosidase. The experiment was performed using 10 mM sodium carbonate buffer at pH 8.0 and 55 °C. Other specifications can be found in the Materials and Methods section. Solid circles: MANAE-β-galactosidase-GLU. Solid triangles: MANAE-GLU-β-galactosidase and solid squares: MANAE-β-galactosidase.

intense multipoint covalent immobilization plus the possibility of getting some intramolecular cross-linking.[84]

That way, the coimmobilization of these enzymes using this glutaraldehyde technique could be a good example to analyze the possibilities of covalently immobilizing Eversa Transform 2.0 on the support via glutaraldehyde and, after reduction, coimmobilizing the β-galactosidase to produce a combi-biocatalyst.

Step by Step Preparation of a Combi-biocatalyst of Eversa Transform 2.0 and β-Galactosidase Using the Glutaraldehyde Chemistry and Ion Exchange. *Reversible Immobilization of β-Galactosidase on Reduced MANAE-GLU Agarose Beads.* The strategy requires the possibility of immobilizing the β-galactosidase on the MANAE-GLU support (after reduction), with a performance similar to the one obtained using MANAE. Figure 4 shows that the β-galactosidase immobilization course on this support is fairly similar to the immobilization in MANAE, with slightly higher losses of enzyme activity. The stability of the enzyme adsorbed on reduced MANAE-GLU was even slightly higher than that when immobilized on MANAE (Figure 5), although it was less stable than the enzyme adsorbed on MANAE and then modified with glutaraldehyde. That way, in this specific case, it is necessary to choose between the reutilization of the most stable enzyme or to improve the stability of the least stable enzyme. As a model case, we have continued with this bienzymatic system as an example of the reuse of the most stable enzyme.

Immobilization of Eversa Transform 2.0 Following the Glutaraldehyde Chemistry and the Coimmobilization of the β-Galactosidase via Ion Exchange. Next, we immobilized Eversa Transform 2.0 on MANAE-agarose beads, modified it with glutaraldehyde, and reduced the biocatalyst. Figure 6 shows that all Eversa Transform 2.0 enzyme molecules were covalently immobilized (no protein band could be observed in the SDS-PAGE analysis). Then, β-galactosidase was immobilized on the MANAE-Eversa-GLU biocatalyst via ion exchange. First, it was checked that the β-galactosidase did not immobilize on the Eversa-glyoxyl (reduced) biocatalyst to ensure that adsorption was via interactions with the support.

Figure 7 shows the inactivation course of both enzymes in the combi-biocatalysts; results were fairly similar to the inactivation courses of the individually immobilized enzymes. Eversa Transform 2.0 maintained over 90% of the initial activity after 75 min of inactivation; at that time, almost all the activity of the β-galactosidase had been lost.

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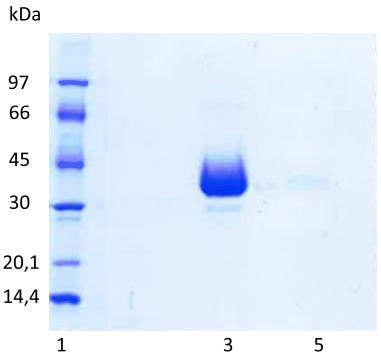

Figure 6. SDS-PAGE analysis of different Eversa Transform 2.0 preparations. Lane 1: low molecular marker; lane 3: MANAE-EVR; and lane 5: MANAE-EVR-GLU (1%). Other specifications can be found in the Materials and Methods section.

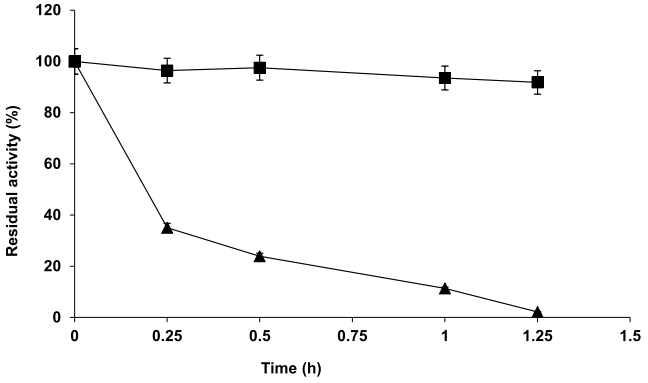

Figure 7. Inactivation course of MANAE-EVR-GLU-β-galactosidase. The experiment was performed using 10 mM carbonate buffer at pH 8.0 and 55 °C. Other specifications can be found in the Materials and Methods section. Solid triangles: β-galactosidase and solid squares: EVR.

Next, we checked the possibility of releasing β-galactosidase from the combi-biocatalyst. For this purpose, the combi-biocatalyst was incubated in 300 mM NaCl. After SDS-PAGE analysis of the samples (Figure 8), it was visualized that all of the proteins from the β-galactosidase extract could be released

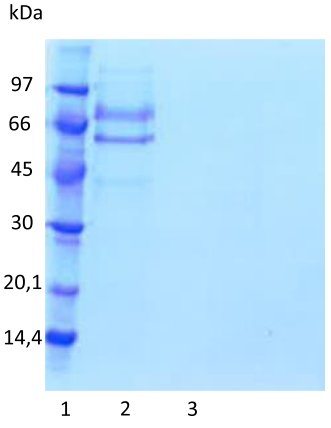

Figure 8. SDS-PAGE analysis of MANAE-EVR-GLU-β-galactosidase after incubation and washing with 0.3 M sodium chloride at pH 7.0. Lane 1: low molecular marker; lane 2: initial combi-biocatalyst; lane 3: combi-biocatalyst after incubation in 0.3 M sodium chloride. Other specifications can be found in the Materials and Methods section.

from the combi-biocatalyst. Two main bands and some minor ones could be visualized in the gel (lane 2) as the galactosidase was not submitted to any purification process. The activity of Eversa Transform 2.0 remained intact after this incubation.

That way, it was possible to use this combi-biocatalyst to reuse Eversa Transform 2.0 to build new combi-biocatalysts when β-galactosidase was inactivated.

Reuse of Eversa Transform 2.0 Coimmobilized with β-Galactosidase to Build a New Combi-Biocatalyst after β-Galactosidase Inactivation. Figure 9 shows several cycles of

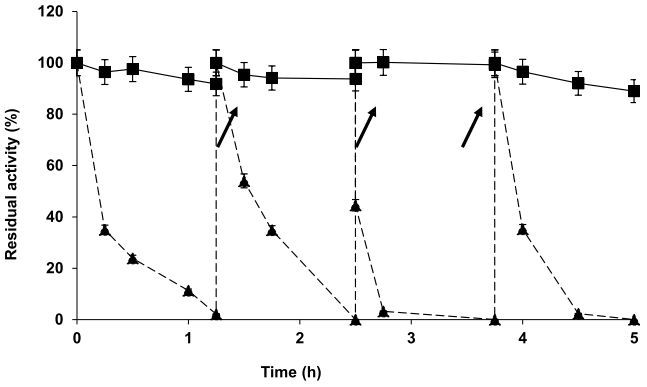

Figure 9. Cycles of thermal inactivation at 55 °C and pH 8.0 of MANAE-EVR-GLU-β-galactosidase and β-galactosidase desorption by incubation and washing with 0.3 M sodium chloride and immobilization of a new batch of β-galactosidase (indicated with an arrow). Other specifications can be found in the Materials and Methods section. Solid symbols and dashed lines: β-galactosidase and solid symbols and solid lines: EVR.

combi-biocatalyst Eversa Transform 2.0/β-galactosidase incubation at 55 °C and pH 8. After inactivation of the β-galactosidase, the combi-biocatalyst was washed with 0.3 M NaCl, and then a new batch of β-galactosidase was immobilized on the Eversa Transform 2.0 biocatalyst. The results showed that, as using the individually immobilized enzymes, the β-galactosidase was almost fully inactivated after 75 min of incubation under these conditions, while Eversa Transform 2.0 maintained around 90% of the initial activity. After washing with a 0.3 M NaCl solution and immobilization of a new batch of β-galactosidase, the activity of Eversa Transform 2.0 recovered the initial values (the washing under mild conditions may permit the activity recovery by enabling the enzyme refolding),[85−89] and several cycles could be repeated with similar results (Figure 8). Figure 9 shows that after the three cycles of biocatalyst recovery of the biocatalyst, washing with 0.3 M NaCl was still able to eliminate almost all the inactivated β-galactosidase from the biocatalyst. The small bands that can be observed may be a consequence of some β-galactosidase molecules that could be unfolded by thermal inactivation and the interaction with the support that can establish a higher number of enzyme−support interactions.[90,91] Washing with 0.6 M NaCl permitted us to have an almost fully clean lane after three cycles of reuse of the immobilized Eversa Transform 2.0 to produce new combi-biocatalysts (Figure 10).

CONCLUSIONS

The use of amino-glutaraldehyde chemistry makes coimmobilizing enzymes following different mechanisms feasible. The first immobilized enzyme (the enzyme that will be the most stable one) can be covalently attached to the support if, after

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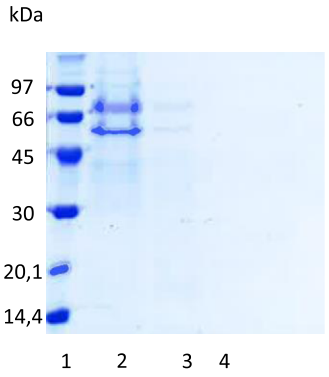

Figure 10. SDS-PAGE analysis of MANAE-EVR-GLU-β-galactosidase after three cycles of thermal inactivation at 55 °C and pH 8.0 and incubation and washing with 0.3 M sodium chloride at pH 7.0. Lane 1: low molecular marker; lane 2: initial combi-biocatalyst; lane 3: combi-biocatalyst after incubation in 0.3 M sodium chloride. Lane 4: combi-biocatalyst after incubation with 0.6 M sodium chloride. Other specifications can be found in the Materials and Methods section.

enzyme immobilization via ion exchange, the biocatalyst is treated with glutaraldehyde. After borohydride reduction to eradicate any chemical reactivity of the support, a second enzyme (exhibiting lower stability) may be coimmobilized in the biocatalyst via ion exchange. The incubation in solutions bearing high ionic strength enables the release of this enzyme from the biocatalyst after its inactivation.

This way, if the first enzyme remains almost fully active when the second enzyme is fully inactivated, this strategy may be utilized to reuse the covalently immobilized enzyme to prepare a new combi-biocatalyst. The inactivation favors the establishment of additional enzyme−support interactions of the inactivated enzyme, making the use of a higher ionic strength necessary to release most of the ionically immobilized and inactivated enzyme. The reaction conditions should be chosen in a way that the least stable enzyme can be reutilized in a minimum number of reaction cycles to make the process economically viable. This strategy can have a strong effect on the sustainability of the combi-biocatalyst. Its advantages will be higher if the most expensive or most difficult to produce enzyme is the most stable one, and that way, it is the one that prolongs its operational lifetime. The reuse of the stable enzyme that is immobilized saves the costs (economical and environmental ones) derived from the production of the enzyme, the support, and the immobilization (the glutaraldehyde cross-linking will be performed just once on the global operational life of the combi-biocatalyst). The rebuilding of the combi-biocatalysts will be very simple, as is any physical immobilization technique. The number of "stable" and "unstable" enzymes will depend on the specific process where the combi-biocatalyst is going to be utilized; the requirement is that the difference in stabilities of the utilized enzymes is large enough to have some enzymes inactivated when the other enzymes remain almost fully active. These advantages are achieved just by using water−saline solutions with high ionic strength that at first glance could be reused if a strategy for the precipitation of the least stable inactivated enzyme in the solution is developed. That way, the reuse of the immobilized and almost fully active enzymes can have a great impact on the sustainability of the process. Due to the general use of this glutaraldehyde treatment of the adsorbed enzymes as a technique to stabilize enzymes via an intense multipoint covalent attachment, this strategy may be of general use.

AUTHOR INFORMATION

Corresponding Author

Roberto Fernandez-Lafuente − *Departamento de Biocatálisis, ICP-CSIC, 28049 Madrid, Spain;* orcid.org/0000-0003-4976-7096; Email: rfl@icp.csic.es

Authors

Diego Carballares − *Departamento de Biocatálisis, ICP-CSIC, 28049 Madrid, Spain;* Present Address: Chemical and Materials Engineering Department, Faculty of Chemical Sciences, Complutense University of Madrid, Complutense Ave., Madrid, 28,040, Spain

Pedro Abellanas-Perez − *Departamento de Biocatálisis, ICP-CSIC, 28049 Madrid, Spain*

Diandra de Andrades − *Departamento de Biocatálisis, ICP-CSIC, 28049 Madrid, Spain; Department of Biology, Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo, São Paulo 14040-901, Brazil*

Maria de Lourdes Teixeira de Moraes Polizeli − *Department of Biology, Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo, São Paulo 14040-901, Brazil;* orcid.org/0000-0002-5026-6363

Javier Rocha-Martin − *Department of Biochemistry and Molecular Biology, Faculty of Biological Sciences, Complutense University of Madrid, Madrid 28040, Spain*

Complete contact information is available at: https://pubs.acs.org/10.1021/acssuschemeng.3c08231

Author Contributions

‖D.C. and P.A.-P. have evenly contributed to this paper.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We gratefully recognize the financial support from Ministerio de Ciencia e Innovación and Agencia Estatal de Investigación (Spanish Government) (PID2022-136535OB-I00 and CNS2022-135135). D.C. holds a FPI fellowship from this project. The authors gratefully acknowledge FAPESP (São Paulo Research Foundation) by research scholarship to D.A. (grant no.: 2023/01338-7). The help and suggestions from Dr. Angel Berenguer (Departamento de Química Inorgánica, Universidad de Alicante) are gratefully recognized.

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https://doi.org/10.1021/acssuschemeng.3c08231
ACS Sustainable Chem. Eng. 2024, 12, 6564−6572