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High Stabilization of Enzymes Immobilized on Rigid Hydrophobic Glyoxyl-Supports: Generation of Hydrophilic Environments on Support Surfaces

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Abstract: Very rigid supports are useful for enzyme immobilization to design continuous flow reactors and/or to work in non-conventional media. Among them, epoxy-methacrylic supports are easily functionalized with glyoxyl groups, which makes them ideal candidates for enzyme stabilization via multipoint covalent immobilization. However, these supports present highly hydrophobic surfaces, which might promote very undesirable effects on enzyme activity and/or stability. The hydrophilization of the support surface after multipoint enzyme immobilization is proposed here as an alternative to reduce these undesirable effects. The remaining aldehyde groups on the support are modified with aminated hydrophilic small molecules (glycine, lysine or aspartic acid) in the presence of 2-picoline borane. The penicillin G acylase from *Escherichia coli* (PGA) and alcohol dehydrogenase from *Thermus thermophilus* HB27 (ADH2) were immobilized on glyoxyl-functionalized agarose, Relizyme and Relisorb. Despite the similar density of aldehyde groups displayed by functionalized supports, their stabilization effects on immobilized enzymes were quite different: up to 300-fold lower by hydrophobic supports than by highly hydrophilic glyoxyl-agarose. A dramatic increase in the protein stabilities was shown when a hydrophilization treatment of the hydrophobic support surface was done. The PGA immobilized on the glyoxyl-Relisorb hydrophilized with aspartic acid becomes 280-fold more stable than without any treatment, and it is even more stable than the PGA immobilized on the glyoxyl agarose.

Keywords: protein stabilization; protein immobilization; 2-picoline borane; methacrylic support; microenvironment; biocatalysis; support hydrophilization

1. Introduction

In recent years, the use of very rigid supports for enzyme immobilization has gained interest in the design of heterogeneous biocatalysts for chemical processes [1–4]. On the one hand, it allows the design of continuous flow enzymatic reactors with the corresponding intensification of bioprocesses [5,6]. On the other hand, the rigid supports and immobilized enzyme biocatalysts can be completely dried (e.g., with cold acetone) while keeping their porous structure intact [7,8]. Dry immobilized enzyme biocatalysts can be used in solvent-free reaction systems or completely anhydrous reaction media (organic solvents, supercritical fluids, etc.) [7,9,10]. The combination of the enzymatic catalysis,

enzyme stabilization, continuous flow reactors and solvent-free reactions is highly desirable for a more sustainable chemical industry [6,11].

An ideal rigid support must contain easily derivatizable reactive groups on its surface to enable the design of different immobilization protocols [4,12]. It is also interesting that these supports are resistant to mechanical agitation and different reaction conditions [2]. In this way, simple derivatization processes of the support surface, enzyme immobilization protocols or additional modifications of immobilized enzymes could be designed. These immobilized biocatalysts could also be used in stirred tank reactors, where they might face several challenges: the biphasic reaction systems, the need for the continuous control of pH, the need for oxygen bubbling, etc. [13,14].

The highly activated methacrylate carriers with epoxy groups meet almost all essential requirements and are commercially available (Purolite ECR from Purolite; Sepabeads and Relizymes from Resindion) with different porosity and particle size. The epoxy groups can be hydrolyzed with acid to yield diol groups, and these can generate the aldehyde groups by oxidation with the periodate (Figure 1) [4]. Under alkaline conditions, these aldehyde-supports promote the very intense stabilization of proteins by multipoint covalent attachment, orientating the enzyme through its richest region in lysine residues [15].

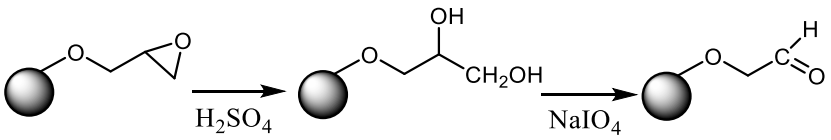

Figure 1. The functionalization of epoxy-activated supports based on methacrylate polymers with glyoxyl groups.

The hydrophilic/hydrophobic nature of methacrylate carriers is strongly determined by their polymeric composition. As a result, interactions with polar or hydrophobic regions of specific enzyme surfaces will be more or less favoured [16]. For example, highly hydrophobic methacrylic supports, such as octadecyl-activated ones, can confer an optimal hydrophobic microenvironment to lipase activity and stability [3,9]. Unlike other enzymes, the lipase surface is characterized by significantly extended lipophilic regions that can interact effectively with the hydrophobic supports [17]. In contrast, most enzymes immobilized in close contact with the hydrophobic surfaces can undergo conformational changes that seriously affect their activity and stability [18–20]. For example, some hydrophobic pockets located near the surface of the immobilized enzymes can become completely exposed to the medium due to the hydrophobic nature of the support. In general, enzymes are adversely affected by the presence of hydrophobic surfaces [20–22].

We envisioned a solution to this problem that consisted of the endpoint hydrophilization of the surface of these hydrophobic supports activated with aldehyde groups. This hydrophilization must also include a large part of the surface of the support that is close to the surface of the enzyme. For this purpose, we proposed the modification of the aldehyde groups that have not reacted with the enzyme, with small hydrophilic amino ligands (e.g., glycine, lysine and aspartic) as blocking agents to generate microenvironments (cationic, anionic or neutral net charge) on the aldehyde-activated support surfaces. The uni-punctual reaction of these hydrophilic amino ligands with the aldehyde groups of the support form very unstable Schiff bases that can be reduced in the presence of mild reducing agents such as 2-picoline borane [23]. In a previous study, a reductive amination with 2-picoline borane (2-PB) and the glycine of glyoxyl-activated supports was developed as an alternative methodology for the borohydride-sensitive enzymes, allowing the immobilization of those enzymes on the aldehyde-activated supports [23]. The immobilization of the enzymes by using this alternative reduction step presented an equal orientation and an equal number of covalent bonds between the enzymes and supports regarding the conventional reduction step with the sodium borohydride.

At this point, we immobilized two enzymes, penicillin G acylase from *E. coli* (PGA) and alcohol dehydrogenases from *Thermus thermophilus* HB27 (ADH2), by multipoint covalent attachment on three highly activated supports with the aldehyde groups. One support presented a very hydrophilic surface (agarose), while the other two commercial supports were methacrylate-derivatives and presented hydrophobic surfaces (Relizyme and Relisorb). In addition, the effect of each microenvironment on the activity and stability of the enzymes immobilized for every support was evaluated. Therefore, this work aimed to develop different microenvironments (cationic, anionic or neutral) on the surface of the methacrylate-derivative supports to avoid or reduce the adverse effects promoted by their hydrophobic surfaces.

2. Results and Discussion

2.1. Comparison of the Physical Properties of the Different Glyoxyl-Activated Supports

Previous studies from our group reported the physical characterization of the Relizyme and agarose-glyoxyl activated supports. More specifically, the characterization was done by mercury intrusion porosimetry (MIP) and the adsorption isotherms to the agarose 6BCL (AG) [24] and Relizyme EP403/S (ReG) [4]. In this work, the physical characterization of the Relisorb HG-400/SS (RbG) was developed, and the values of the physical parameters were compared for the three supports (Table 1).

Table 1. Physical-chemical parameters of different immobilization supports.

Support	BET Area (m^2/g) [1]	Aldehyde Density (Molecules of CHO/ 1000Å^2) [2]	Number of Immobilized Lysine Residues [3]	Reference
Agarose 6BCL (AG)	36	16.7	8	[24]
Relizyme EP403/S (ReG)	69	8.7	5	[4]
Relisorb HG-400/SS (RbG)	62	9.7	5	This work

[1] The BET area was determined by adsorption isotherms (AI). [2] The number of molecules of aldehydes per area of the support surface with a functionalization of 100 μmoles of $NaIO_4$/g of support.. [3] The estimation of the lysine residues involved in the immobilization of the PGA was determined as described in the Materials and Methods section. All the data are the mean values of three separate experiments where the error value was never as high as 5%.

According to the manufacturer, the average pore diameter for the three supports was similar, around 40–60 nm. Additionally, the three supports share the absence of the micropores; this was confirmed by the AI for the AG [24], ReG [4] and RbG (this work). The results from the two analytical procedures showed similar BET area values for the methacrylic supports (around 65 m^2/g). These values were higher than the BET area value for the AG (36 m^2/g). Notwithstanding, if the supports are functionalized at 100% of their capacity (around 100 μmoles CHO/g), the aldehyde group's density for the agarose is approximately 2-fold higher than both methacrylic supports resulting in almost 2-fold more of the aldehyde molecules under every molecule of the PGA on the agarose support. This higher density of aldehydes on the exposed surface could explain the results obtained from the quantification of the number of lysine residues of the PGA that were covalently attached to the support, which was slightly higher in the AG than the ReG and RbG (Table 1). These differences in the number of covalent bonds between the enzyme and the supports could show differences in the stabilization of the enzymes as previously described [25,26].

2.2. Effect of the Support Surface on the Activity of the Enzymes

The immobilization of the PGA and ADH2 was carried out on the three different glyoxyl-activated supports (AG, ReG and RbG) and their expressed activity and immobilization yield was determined. All the three supports have a similar surface density of aldehyde groups. We prepared conventional biocatalysts in which, after prolonged multipoint covalent enzyme-support bonding, the biocatalyst was reduced with sodium borohydride so that the Schiff bases formed between each enzyme molecule,

the supports were redirected to the very stable secondary amino bonds, and the free aldehyde groups were reduced to alcohols [27,28]. We also prepared non-conventional derivatives in which, after multipoint binding, the Schiff bases were reduced as usual. However, the aldehyde-remaining groups of the support were blocked with the glycine, or with the lysine, or with the aspartic acid in the presence of the 2-picoline borane (Figure 2) [23].

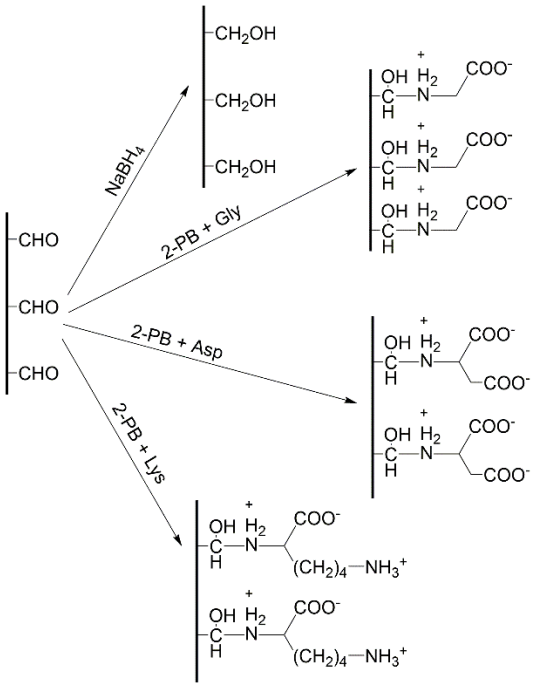

Figure 2. The schematic representation of the different microenvironments generated on the support surfaces.

Since the immobilization chemistry is the same, the orientation of the enzymes on the three different supports is also expected to be the same through the richest region in lysine residues of the enzyme surface [15,29]. In all cases, the immobilization was successful, and the immobilization yields reached values over 97% (Tables 2 and 3).

Table 2. Immobilization summary for PGA on different supports and blocking agents.

Enzyme	Preparation [1]	Immobilization Yield [2] (%)	Expressed Activity before Reducing Step [3] (%)	Expressed Activity after Reducing Step [3] (%)
PGA	AG-B	100	40	40
	AG-G			45
	AG-L			43
	AG-A			21
	ReG-B	98	23	23
	ReG-G			24
	ReG-L			24
	ReG-A			10
	RbG-B	100	37	37
	RbG-G			42
	RbG-L			47
	RbG-A			11

All the preparations were done as described in the Materials and Methods section. [1] B—preparation was reduced with sodium borohydride; G—preparation was reduced and blocked with 2-picoline borane (2-PB) in the presence of glycine; L—preparation was reduced and blocked with 2-PB in the presence of lysine; A—preparation was reduced and blocked with 2-PB in the presence of aspartic acid; AG—glyoxyl-activated agarose; ReG—glyoxyl-activated Relizyme; RbG—glyoxyl-activated Relisorb. [2] The immobilization yield = (offered activity – supernatant activity)/offered activity. [3] The expressed activity = (immobilized activity before or after reducing step/offered activity – final supernatant activity). All the data are the mean values of three separate experiments where the error value was never higher than 5%.

Table 3. Immobilization summary of the ADH2 on the different supports and blocking agents.

Enzyme	Preparation [1]	Immobilization Yield [2] (%)	Expressed Activity before Reducing Step [3] (%)	Expressed Activity after Reducing Step [3] (%)
ADH2	AG-B	100	75	75
	AG-G			79
	AG-L			83
	AG-A			75
	ReG-B	97	21	20
	ReG-G			25
	ReG-L			27
	ReG-A			21
	RbG-B	98	52	52
	RbG-G			52
	RbG-L			54
	RbG-A			50

All the preparations were done as described in the Materials and Methods section. [1] B—preparation was reduced with sodium borohydride; G—preparation was reduced and blocked with 2-PB in the presence of glycine; L—preparation was reduced and blocked with 2-PB in the presence of lysine; A—preparation was reduced and blocked with 2-PB in the presence of aspartic acid; AG—glyoxyl-activated agarose; ReG—glyoxyl-activated Relizyme; RbG—glyoxyl-activated Relisorb. [2] The immobilization yield = (offered activity – supernatant activity)/offered activity. [3] The expressed activity = (immobilized activity before or after reducing step/offered activity – final supernatant activity). All the data are the mean values of three separate experiments where the error value was never higher than 5%.

On the one hand, the expressed activities of the PGA preparations were the same for the AG and the RbG, which were 2-fold higher than the ReG. Regarding the reducing step, when the support was reduced with 2-PB in the presence of lysine or glycine, the expressed activity was slightly higher than that with the conventional reduction step with borohydride. However, the use of aspartic acid resulted in a 2-fold reduction of the enzymatic activity (Table 2).

On the other hand, similar results were obtained for the ADH2 preparations with few differences (Table 3). Firstly, the ReG and RbG presented reduced expressed activities, by 50% and 25%, respectively, compared to the AG preparation. Secondly, the expressed activities obtained with the reduction step with the 2-PB in the presence of an amino acid were similar or higher to those with the conventional reduction step.

Given that the enzyme orientation for the three supports is the same, the differences in the expressed activities could only be explained by the differences in the conformation of the immobilized enzymes, since the hydrophilicity of the supports is different [12]. Although it has been previously described that the increase in the number of bonds between the enzyme and AG support can lead to an excessive rigidification of the enzyme structure with the subsequent loss of activity, the expressed activities for the methacrylic supports were lower than for the agarose [26,30]. Therefore, the generation of a microenvironment after the immobilization step by using the 2-PB and an amino acid could be positive, neutral or negative for the enzymatic activity depending on the enzyme and amino acid studied.

2.3. Effect of the Support on the Thermal Stability of the Enzymes

As shown in Table 4, there was a high variability between the thermal stabilities of the immobilized biocatalysts given the differences in the nature of the support and the microenvironments generated.

The immobilization of the PGA by the conventional protocol (the reduction step with sodium borohydride) on the ReG or RbG supports negatively affected the thermal stability, since it decreased 100-fold regarding the immobilization on agarose. These results were expected because the higher hydrophilicity of the agarose polymer makes it more biocompatible than the methacrylate polymer [12]. Thus, the immobilization of the PGA on the AG led to higher recovered activity and a higher stabilization factor than the immobilization on the ReG and RbG. This could be explained by the higher hydrophobicity of the methacrylate polymer that would lead to the immobilization of a different and less "natural" conformation of the PGA on its surface [12]. Nevertheless, the immobilized biocatalysts

on the same supports, using the reduction step in the presence of 2-PB and aspartic acid, showed higher thermal stabilities than the other microenvironments. It seems that a negative net charged microenvironment was favorable for improving the thermal stability of the PGA on the hydrophobic supports (Table 4 and Figure 2). By generating this microenvironment, the thermal stability of these preparations improved by 14- and 281-fold for the ReG and RbG preparations, respectively. Nonetheless, the RbG preparation was the most stable preparation of the PGA, with a half-life of 169 h at 60 ºC and pH 7.0 and a stabilization factor of 1.4-fold regarding the AG-B preparation, which was the most stable preparation of wild-type PGA to date [31].

Table 4. Stabilization parameters of the PGA and ADH2 preparations.

Enzyme	PGA		ADH2	
Preparation [1]	Half-Life [2] (h) at 60 °C pH 7.0	Stabilization Factor [3]	Half-Life [2] (h) at 80 °C pH 7.0	Stabilization Factor [3]
AG-B	124	1.0	16	1.0
AG-G	7.4	0.06	58	3.6
AG-L	0.9	0.01	106	6.6
AG-A	29	0.2	29	1.8
ReG-B	1.1	1.0	4.4	1.0
ReG-G	1.5	1.3	22	5.0
ReG-L	1.5	1.4	175	40
ReG-A	16	14	12	2.8
RbG-B	0.6	1.0	6.7	1.0
RbG-G	0.4	0.7	7.3	1.1
RbG-L	0.7	1.2	36	5.4
RbG-A	169	281	1.8	0.3

All the preparations were done as described in the Materials and Methods section. [1] B—preparation was reduced with sodium borohydride; G—preparation was reduced and blocked with the 2-PB in the presence of glycine; L—preparation was reduced and blocked with the 2-PB in the presence of lysine; A—preparation was reduced and blocked with the 2-PB in the presence of aspartic acid; AG—glyoxyl-activated agarose; ReG—glyoxyl-activated Relizyme; RbG—glyoxyl-activated Relisorb. [2] The half-life times were calculated according to the Methods section. [3] The stabilization factor is the ratio between the half-lives taking as reference the immobilized biocatalysts reduced with sodium borohydride. All the data are the mean value of three separate experiments where the error value was never higher than 5%.

The ADH2 preparations obtained by the conventional protocol (final reduction step using borohydride) showed that the agarose preparation was 3–4-fold more stable than the other preparations on the ReG and RbG. Once again, immobilization supports with a hydrophobic surface promoted less stabilization of the enzyme. In this case, a net positively charged microenvironment, obtained by reducing in the presence of 2-PB and lysine, led to a further improvement in thermal stability (Table 4 and Figure 2). Thus, this microenvironment increased the thermal stability by 40- and 5.4-fold on the ReG and RbG, respectively, regarding the absence of microenvironment (conventional reduction methodology). For the ADH2, the most thermal stable preparation was the ReG-L, with a half-life of 175 h at 80 °C and pH 7, which was 40-fold more stable than the ADH2-ReG-B, 1.5-fold more stable than the ADH2-AG-L and 11-fold more stable than the ADH2-AG-B (Table 4).

Despite the higher number of covalent bonds on the agarose preparations (Table 1), the most stable preparations were obtained by immobilizing the PGA and ADH2 on methacrylic supports (RbG-A for the PGA and ReG-L for ADH2; Table 4). The generation of highly hydrophilic microenvironments on the support surfaces overcame the negative effects of the hydrophobic surfaces of the methacrylic supports, especially during the thermal stability assays, in which the hydrophobic pockets of the enzymes could interact with the hydrophobic surfaces of the methacrylic supports.

The generation of different microenvironments enables the creation of new highly stabilized and active biocatalysts on rigid supports, which could work in continuous flow reactors. The use of these reactors may accelerate biotransformation, improve productivities, improve yields, and minimize waste generation regarding the stirred-tank reactors [6,32].

3. Materials and Methods

3.1. Materials

Penicillin G acylase (PGA) from *E. coli* was kindly provided by Antibioticos S.A. (León, Spain). Prof. José Berenguer (Centro de Biología Molecular Severo Ochoa, CSIC-UAM, Madrid, Spain) kindly donated the expression plasmid containing the ADH2 encoding sequence of *T. thermophilus* HB27. The agarose beads, 6%, were obtained from Agarose Beads Technology (Madrid, Spain). The Relizyme EP403/S and Relisorb HG-400/SS were purchased from Resindion (Milan, Italy). The 2-Picoline borane (2-PB) complex was purchased from TCI (Tokyo, Japan). The dimethyl sulfoxide (DMSO) was purchased from Applichem (Darmstadt, Germany). The 6-nitro-3-phenylacetamidobenzoic acid (NIPAB) was purchased from Fluorochem (Hadfield, United Kingdom). The sodium borohydride, glycine, aspartic acid, lysine, glycidol, 2,2,2-trifluoroacetophenone, and β-nicotinamide adenine dinucleotide reduced form (NADH) were purchased from Sigma-Aldrich Co. (St. Louis, IL, USA). The buffers and other reagents were obtained from Sigma-Aldrich Co. (St. Louis, IL, USA).

3.2. Methods

3.2.1. Protein Production

The ADH2 was expressed and purified according to the literature [33].

3.2.2. Enzymatic Assays

The enzymatic activity of the PGA was measured spectrophotometrically using 0.15 mM NIPAB in 50 mM sodium phosphate at pH 7 and 25 °C at 405 nm, as previously described [31].

The enzymatic activity of the ADH2 was measured spectrophotometrically using 10 mM 2,2,2-trifluoroacetophenone and 0.25 mM NADH in 50 mM sodium phosphate at pH 7 and 65 °C at 340 nm, as previously described [33].

3.2.3. Support Preparations

The glyoxyl-agarose (GA) support was prepared by the esterification of agarose 6BCL with glycidol and the further oxidation of the resulting glyceryl agarose with the appropriate amount of periodate, as previously described [27].

The glyoxyl-Relizyme (ReG) was prepared as previously described [4]. Firstly, the Relizyme EP403/S support was incubated with 0.5 M H_2SO_4 for 24 h at 25 °C to hydrolyze the epoxy groups of the support. Then, the resulting glyceryl Relizyme was further oxidized with the appropriate amount of sodium periodate.

The glyoxyl-Relisorb (RbG) was prepared by the direct oxidation of the glyceryl groups with the appropriate amount of sodium periodate [15].

The aldehyde groups were quantified by the $NaIO_4$ consumption method [27]. All the supports contain around 100 μmoles of aldehyde per gram.

The physical characterization of the Relisorb HG-400/SS support was carried out by mercury intrusion porosimetry (MIP) and adsorption isotherms (AI) in the same conditions that were previously described for the Relizyme EP403/S [4].

3.2.4. Protein Immobilization

The PGA [31] and ADH2 [33] were immobilized as previously described. Both the enzymes were immobilized at pH 10 and 25 °C, gently stirred for 4.5 h (PGA) and 3 h (ADH2). The immobilization times remained constant for the three different supports.

To finish the protein immobilization process, all the immobilized preparations were reduced with the 2-picoline borane (2-PB) or the sodium borohydride, as previously described [23]. The reduction step was performed in the presence of a 0.8 M concentration of a blocking agent (glycine, lysine or

aspartic acid), 30 mM 2-PB and 20% DMSO for 24 h under gentle stirring at 25 °C. The presence or absence of the aldehyde groups was qualitatively measured by using Schiff's reagent.

3.2.5. Thermal Inactivation Assays

The thermal stability assays of the different preparations were performed at pH 7 and 60 °C or 80 °C for PGA or ADH2, respectively. Samples were periodically withdrawn, and their activities were assayed. The half-lives and stabilization factors were calculated as previously described [25].

3.2.6. Estimation of the Lysine Residues Involved in the Immobilization of the PGA

The number of lysine residues of the PGA involved in the covalent attachment was calculated according to the literature [23,26].

4. Conclusions

Most enzymes immobilized in close contact with supports that present hydrophobic surfaces can undergo conformational changes that severely affect their activity and stability. The generation of highly hydrophilic microenvironments on the surfaces of methacrylic supports diminished the negative effects exerted by hydrophobic surfaces of this type of support. The PGA immobilized on the RbG and reduced in the presence of the 2-PB and aspartic acid was respectively 1.4- and 281-fold more stable than the AG and RbG preparations reduced with borohydride (conventional protocol). Considering that the PGA immobilized on agarose was 10,000-fold more stable than the soluble enzyme [27,34], it could be assumed that the new immobilized PGA biocatalyst showed a half-life that was 13,500 times higher than that of the soluble wild-type enzyme. In addition, the ADH2 immobilized on the ReG and reduced in the presence of the 2-PB and lysine was respectively 11- and 40-fold more stable than the AG and ReG biocatalysts reduced by the conventional protocol. Therefore, new highly stabilized and active immobilized biocatalysts can be fabricated on rigid supports. The approach developed here is based on the smart-hydrophilization of immobilized enzyme biocatalyst surface to generate specific microenvironments; it has huge potential for enzyme stabilization and activity preservation upon immobilization. This is presented as an alternative way to fabricate robust heterogeneous biocatalysts, which could respond to the growing demand of novel biocatalytic applications such as reactions in non-conventional media, solvent-free reaction systems, continuous flow reactors, etc. We believe that this work will contribute to the bridging of the gap between biocatalysis and the industrial implementation of more sustainable solutions.

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