

***In situ* and post-synthesis immobilization of enzymes on nanocrystalline MOF platforms to yield active biocatalysts**

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Abstract

BACKGROUND: Very recently, metal-organic framework (MOF) materials have been postulated as emerging supports to achieve solid-state enzyme-contained biocatalysts. In this work, post-synthesis and *in situ* strategies to immobilize β -glucosidase and laccase on different MOF materials were studied. The MOF-based supports, i.e. MIL-53(Al), NH₂-MIL-53(Al) and Mg-MOF-74, were prepared under soft and sustainable conditions (room temperature and pH values compatible with enzymatic activity), allowing development of the *in situ* strategy.

RESULTS: In both post-synthesis and *in situ* approaches, the intercrystalline mesoporosity of the MOF-based support favored the immobilization efficiency or the specific activity. The latter expressed as units per milligram of immobilized enzyme was higher in the post-synthesis immobilization, whereas the biocatalysts prepared *in situ* gave much higher enzyme loading (over 85%) and lower enzyme leaching (around 5%). The *in situ* approach even worked in a non-aqueous (N,N-dimethylformamide) media in which the free enzyme was completely inactive. The immobilized enzymes are much larger than the structural pores of the MOFs.

CONCLUSIONS: Enzymes can be efficiently immobilized on nanocrystalline MOFs prepared under soft and sustainable conditions despite the supports lacking large enough pores to host the enzymes. The *in situ* approach is very efficient capturing enzymes and preserving some of their activity even under adverse conditions

INTRODUCTION

Obviating notable exceptions,^{1–3} catalysts designed by humans are far from being as selective as those given by nature, i.e. enzymes. Lability and solubility of enzymes limit their application and justify the efforts to immobilize enzymes with potential industrial interest in several supports.^{4–12} Numerous attempts to solve leaching and inactivation problems once immobilized have been reported.^{13–15} In the last decade, the use of siliceous ordered mesoporous materials as enzyme supports have provided new and improved properties to the resultant biocatalysts. Controlled pore size and surface chemistry together with regular structure give rise to high enzyme loadings, low diffusional limitations, high specific activities and scarce enzyme leaching even when the enzyme is non-covalently anchored to the supports.^{16–30} Metal-organic frameworks (MOFs) are porous materials that have strongly emerged in the last few years. They are composed of metal ions or clusters and organic linkers. The increasing number of applications in the last few years confirms the great potential of these new materials.^{31–35} More recently MOFs have been also used as a promising host matrix of enzyme immobilization.^{36–43} The most immediate approach has been to synthesize MOFs with pores large enough to host enzymes.^{44–48} However, this approach is far from being universal, as it entails two essential problems: (i) just a few (expensive and/or hard-to-prepare) MOF materials possess sufficiently large mesopores;^{45,47,48} and (ii) it is restricted to enzymes with small molecular dimensions that are able to be accommodated inside these pores.^{37,38,44} Nevertheless, very recently, some studies have opened the possibility of synthesizing enzyme-MOF biocatalysts without the requirement of hosting the enzymes within the structural pores of the MOF material, through either post-synthesis^{49–53} or *in situ*^{54–57} approaches. In other words, the formation of the enzyme-MOF composites can now take advantage of the extraordinary structural and compositional versatility as well as hierarchical porosity of MOF materials due to the use of a huge variety of organic functional moieties, metal nature, particle size, synthesis conditions, etc.^{41,58} In this work, we have compared post-synthesis and *in situ* methodologies to immobilize a given large-sized enzyme (β -glucosidase) in two MOF materials (X-MIL-53(Al) with X = H or NH₂) at room temperature and in water as the unique solvent.^{59–62} In these mild

conditions the immobilized enzymes preserve at least part of their activity. The influence of different parameters such as intercrystalline mesoporosity of the MOF support, its organic functionalization, the systematic change of the synthesis conditions in the case of the *in situ* approach, etc. have been studied and discussed. Experiments involving other enzyme (laccase) or different MOF support (nanocrystalline Mg-MOF-74)^{61,62} are also presented.

Some strategies involving MOFs as enzyme supports reported so far are based on different approaches, such as: (i) the mixture of enzymes and MOF materials promotes undetermined kinds of interaction between enzymes and already formed MOFs;^{50,52} (ii) the quick and controlled growing of MOF crystals is able to randomly capture certain amounts of enzyme molecules;^{54,56,57,63} (iii) the enzymes (or other biomolecules) induce the formation of MOF-based coatings;^{55,64} (iv) the enzyme is part of the MOF framework acting as a node;^{65,66} or (v) the protection of the resultant enzyme-MOF composite using polymers (i.e. polydopamine) to enhance hydrophilicity and biocompatibility of the support and to increase the biocatalyst's stability and reusability.^{67–69} Our approach⁷⁰ is based on trapping the enzymes into the relatively ordered mesopores created during the agglomeration/aggregation of MOF nanocrystals (intercrystalline mesoporosity).^{59–62} Unlike the *in situ* methods, our *in situ* approach does not generate embedded enzymes within the MOF crystals but they are possibly located in the hollow space between nanocrystals. Unlike the reported post-synthesis approaches, the possibility to encapsulate the enzymes within the intercrystalline mesoporosity is added to the simple interaction between the enzyme and the external surface of the MOF-based support. In the case of *in situ* immobilization approach, the possibility of preparing MOFs at room temperature and in water was taken as starting point,^{54,63} in order to preserve enzymatic activity. We have also explored the synthesis in a non-aqueous system,⁵⁵ namely DMF (N,N-dimethylformamide), and its effect on the activity of the free β -glucosidase and the MOF-enzyme composite. The colloidal-like nature of the synthesis media may avoid the escape of the enzymes from the support, giving very high immobilization efficiency for the *in situ* methodology. Since our system allows the enzyme immobilization by both post-synthesis and *in situ* approaches, this work compares both strategies in terms of immobilization efficiency, the specific catalytic activity or enzyme leaching.

EXPERIMENTAL

Detailed description of the as-received reactants and enzyme extracts, schemes of the different synthesis approaches, details of the characterization techniques as well as characterization and pretreatments of enzyme extracts, are given in Supporting information.

Synthesis of MOF materials

Enzyme-free MOF materials were prepared according to the methods described elsewhere.^{59–62} In particular, MIL-53(Al) and NH₂-MIL-53(Al) were prepared in water and at room temperature,^{59,60} whereas nanocrystalline Mg-MOF-74 was also prepared at room temperature but using N,N-dimethylformamide (DMF) as solvent.^{61,62} These MOF materials were used as supports for the post-synthesis immobilization of enzymes and for comparison with the corresponding biocatalysts.

Synthesis of enzyme-MOF biocatalysts

β -glucosidase (β -Glu) was selected to compare post-synthesis (immobilization after the synthesis of the material) and *in situ* (one-pot synthesis) immobilization methods. For the post-synthesis approach a non-functionalized MIL-53(Al) and a NH₂-MIL-53(Al) materials were prepared in water at room temperature.⁶⁰ Laccase (Lac) was also studied for comparative purposes because of its affinity for amino-functionalized materials.²⁹ *Post-synthesis immobilization: biocatalysts enzyme#MOF* Post-synthesis immobilization of either β -glucosidase or laccase on any of the three MOF materials (MIL-53(Al), NH₂-MIL-53(Al) or Mg-MOF-74) was carried out by suspending 50mg of the MOF material in 10 mL of an enzyme aqueous solution in 50 mmol L⁻¹ acetic acid/sodium acetate buffer at pH 5.5 (Scheme S3). The suspension was kept under mild stirring for 0–27 hours (no further enzyme molecules were immobilized after 4 h). The amount of enzyme immobilized onto the support was calculated by the difference between the respective activities of blank or suspension and the supernatant. Aliquots were withdrawn at given times, and the enzymatic activity of the blank, suspension and supernatant (after separating it by centrifugation) were assayed spectrophotometrically either by the hydrolysis of p-Nitrophenyl glucoside (p-NPG) by β -glucosidase or by the oxidation of the 2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) di-ammonium salt by laccase (see Supporting information for details). The decrease in activity of the supernatant to a minimum and constant value indicated the end of the immobilization process. Protein content of the supernatant before and after the immobilization process was also measured in order to calculate the immobilization yield. Then, the suspension was filtered and the solid biocatalysts were washed with the same buffer used for immobilization. No protein was detected in the washing residues. The solid samples were then dried under vacuum and were stored at 4 °C. In order to determine the activity of the immobilized enzyme, 10 mg of the corresponding biocatalysts were re-suspended in 0.5 mL of 50 mmol L⁻¹ acetate buffer pH 5.5 or deionized water and catalytically analyzed following the processes described in Supporting information. All the measurements were performed at least three times and their averaged value was taken for shown results and discussion.

In situ immobilization: biocatalysts β -Glu@MOF

The method to prepare the biocatalysts β -Glu@NH₂-MIL-53(Al) is similar to that described for this MOF material at room temperature in water,^{59,60} but slightly modified by the incorporation of the enzyme extract and by the order of addition of the solutions to prevent the exposure of the enzymes to extreme pH values (Scheme S4). In a typical synthesis procedure, two clear aqueous solutions were prepared separately. The metal solution (pH ~ 2.0) was formed by 2.0 g of Al(NO₃)₃·9H₂O in 6.030 g of deionized

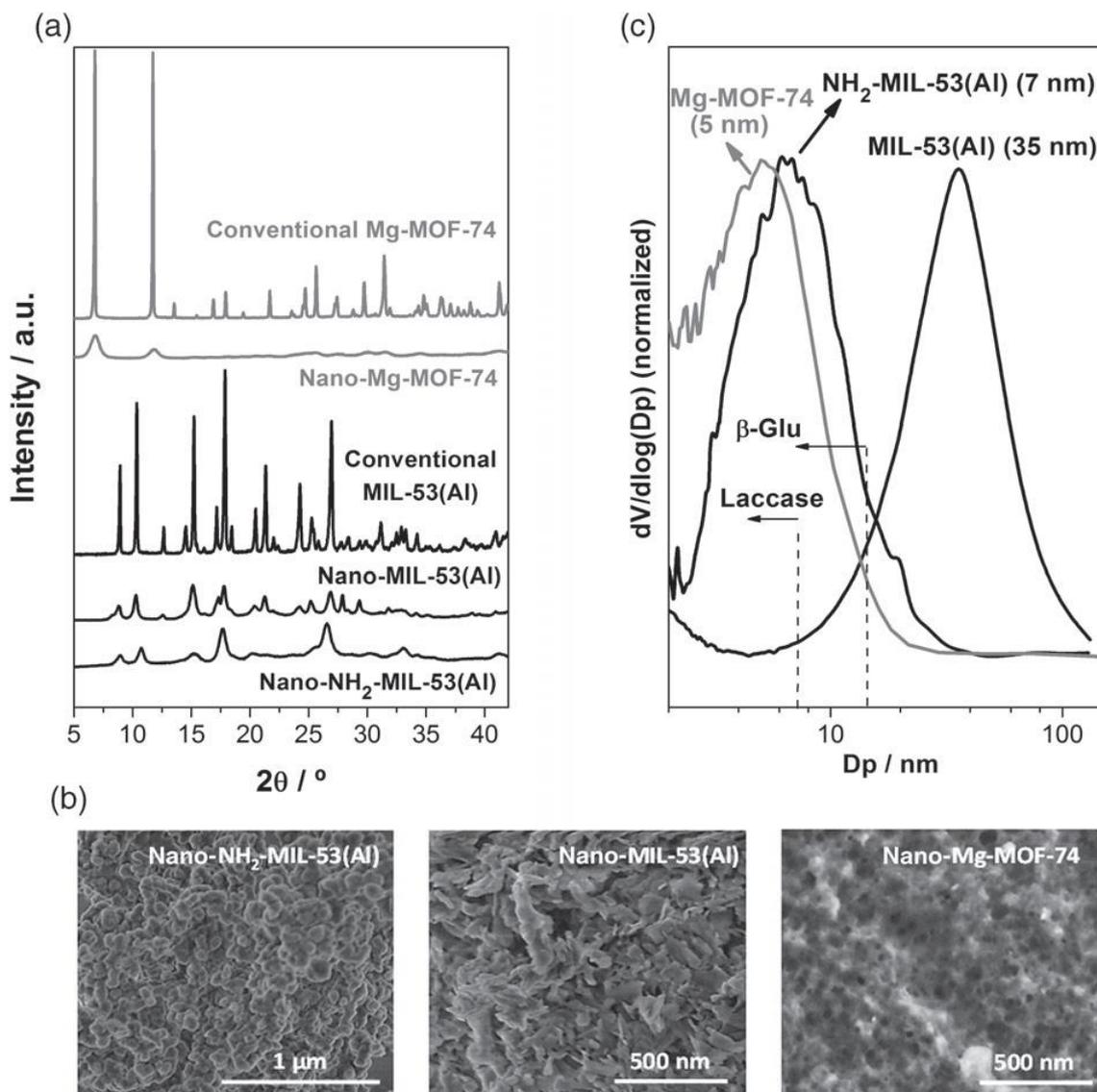


Figure 1. (a) PXRD patterns of the nanocrystalline MOFs used as support for enzyme immobilization: NH₂-MIL-53(Al) (black line, at the bottom), MIL-53(Al) (also black), and Mg-MOF-74 (grey), compared with the conventional micrometer-sized MIL-53(Al) (black) and Mg-MOF-74 (grey). (b) Representative SEM images of the three MOFs samples showing the intercrystalline mesoporosity. (c) Normalized pore size distribution curves arisen from the adsorption branches of the isotherms of the three nanocrystalline MOF materials. The size of the tested enzymes is indicated by dashed red lines.

water. The linker solution was prepared by dissolving 0.483 g 2-aminoterephthalic acid (NH₂-H₂BDC) in 13.246 g deionized water, assisted by one of the following three deprotonating agents: triethylamine (TEA, 0.538 g), ammonium hydroxide (NH₄OH 25%, 0.362 g) or sodium hydroxide (NaOH 1 mol L⁻¹, 5.206 g). The amount of water was corrected taking into account the water content, if any, contained in the deprotonating agent source. In the absence of these agents, the linker is not soluble, whereas in the presence of any of them the mixture becomes a clear solution after some time, from ca. 10 min in the case of NaOH up to several hours in the case of NH₄OH. The pH of this solution is near neutral (around 6) and higher for stronger bases used as deprotonating agents. β -glucosidase extract (2.75 mL of an extract concentration of 14.54 mg protein mL⁻¹ of aqueous solution) is added over this linker solution, which entails

a slight pH decrease (ca. 0.5 units) of the mixture. Later, the metal solution was dropwise added over the linker-enzyme solution under stirring at room temperature, causing the immediate appearance of a yellowish solid. The pH of the resultant suspension was rather acid, slightly above 3. Different aliquots were extracted from this suspension between 5 min and 48 h. The solid biocatalysts were separated from this suspension by centrifugation (12 500 rpm for 1 min), and they were denoted as β -Glu@NH₂-MIL-53(Al)-Base-time, where Base can be TEA, NH₃ or NaOH, and time is substituted by the particular synthesis time (1 h, 2 h, 48 h, etc.) in the name of the sample.

In the preparation of the biocatalysts β -Glu@Mg-MOF-74, the metal solution is formed by dissolving 0.561 g of magnesium acetate tetrahydrate in 10 g of N,N-dimethylformamide (DMF). 0.5 mL of the β -glucosidase extract (14.54 mg protein mL⁻¹ in aqueous solution) were added over this solution. Next, the linker solution, which is composed of 0.2 g of 2,5-dihydroxyterephthalic acid (dhtp) in 10 g of DMF, was added dropwise over the metal-enzyme solution, giving rise to a yellow suspension. The biocatalysts, denoted as β -Glu@Mg-MOF-74-2h or -24h, were isolated by centrifugation (12 500 rpm for 45 s) of the corresponding aliquots after 2 or 24 h reaction.

Electrophoresis test of enzyme retention in biocatalysts

Biocatalyst	Enzyme immobiliz. ^a / %	Enzyme loading ^b / mg g ⁻¹	Catalytic activity ^c / U g ⁻¹	Specific activity ^d / U mg ⁻¹
β -Glu@NH ₂ -MIL-53	1.2	0.6	11.4	19.30
β -Glu#MIL-53	14.3	7.1	30.0	4.22
β -Glu#MS-3030	8.8	4.4	14	3.2
Lac#NH ₂ -MIL-53	20.4	10.2	3.1	0.30
Lac#MIL-53	10.7	5.3	0.5	0.10
Lac#Mg-MOF-74	32.8	16.4	2.6	0.16

^a Percentage of immobilized enzyme in the solid biocatalyst against the amount of added enzyme.
^b mg of enzyme per g of biocatalyst.
^c Catalytic activity (in activity units U per g of biocatalyst) in the hydrolysis of p-NPG (para-nitrophenyl-beta-D-glucopyranoside) for β -glucosidase-containing biocatalysts and in the ABTS (2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) oxidation for laccase-containing ones.
^d Specific activity (in activity units U per mg of contained enzyme) in the hydrolysis of para-nitrophenyl-beta-D-glucopyranoside for β -glucosidase-containing biocatalysts and in the ABTS oxidation for laccase-containing ones. Reference activity of enzymatic extracts: 2.93 U mg⁻¹ for β -glucosidase (C = 24.76 mg mL⁻¹) and 1.074 U mg⁻¹ for laccase (C = 1.27 mg mL⁻¹).

The efficiency of the enzyme encapsulation was studied by means of SDS-PAGE electrophoresis. Since the solid samples are not suitable for electrophoresis, the enzyme was forced to leave the pores by the following procedure. First, the biocatalysts were suspended in the electrophoresis buffer solution (containing sodium dodecyl sulfate, mercaptoethanol, bromophenol blue, tris buffer pH 6.8 and glycerol) and boiled for 10 min. In such denaturing conditions including the split of disulfide bonds, the tertiary structure of the protein should be lost and the random coil chain should then be easily released from the pores. The supernatants of these suspensions were withdrawn and run in 10 % SDS-PAGE electrophoresis.

Leaching tests

The resistance of the enzyme to leaching from the support was studied under conditions that presumably favor the release of the protein, namely high dilution and low ionic strength. The biocatalysts were incubated in 50 mmol L⁻¹ phosphoric acid/trisodium citrate buffer at pH 5.0 with a content of 1.25 mg of solid per mL of buffer. Enzyme leaching was calculated by monitoring the appearance of protein in the supernatant utilizing the Bradford assay.⁷¹

RESULTS AND DISCUSSION

The results and discussion section has been divided in two different main sub-sections corresponding to the post-synthesis (immobilization of the enzyme in the pre-existent support) and *in situ* (the enzyme is present during the formation of the MOF-based support) strategies. In each approach, different parameters have been systematically varied. The advantages and disadvantages of the post-synthesis vs the *in situ* methods are discussed in a third sub-section.

Post-synthesis immobilization: biocatalysts Enzyme#MOF

Figure 1 shows some characterization results of the three MOF-based nanomaterials used as supports: MIL-53(Al), NH₂-MIL-53(Al) and Mg-MOF-74. The total surface area of all these materials is very high (near 1000 m² g⁻¹),^{60,62} but most of it corresponds to MOF structural micropores, which cannot host proteins. Only the external surface ranging between 90 and 100 m² g⁻¹ in the three MOF samples,^{60,62} is available for that aim. MIL-53(Al) and NH₂-MIL-53(Al) having very different interparticle mesopore size distribution,⁶⁰ were used to study the effect of confinement of the enzyme. These materials were also used to study the influence of organic functionalization, particularly the affinity of laccase for amino groups of the supports.^{29,72} On the other hand, Mg-MOF-74 could provide some information about the possible role of the open metal sites in the enzyme immobilization. From a sustainable point of view, it is remarkable that all three materials were prepared at room temperature according to two recent publications of our group.^{59–62}

The nanocrystallinity of the samples can be inferred from the powder XRD (X-ray diffraction) patterns compared with those of the corresponding conventional micrometer-sized homologues (Fig. 1(a)). Such nanocrystalline nature of the supports acts as driving force to either agglomerate^{59,60} or aggregate⁶² the crystalline nanocrystals in particles of several microns (Fig. 1(b)). The fused particles possess relatively ordered intercrystalline mesoporosity as indicated by pore size distribution (Fig. 1(c)) and visualized by SEM images (Fig. 1(b)). The maximum of the PSD (pore size distribution) curves for NH₂-MIL-53(Al) (7 nm) and Mg-MOF-74 (5 nm) materials (Fig. 1(c)) is below the dimensions of the studied enzymes (6.3 nm × 7.2 nm × 8.9 nm for laccase,²⁹ and 12.3 nm × 10.7 nm × 8.1 nm for β -glucosidase according to the same estimation method applied to laccase). Furthermore, it is well known that the BJH method underestimates the pore dimension by at least 20%.^{73,74} In addition, Mg-MOF-74 and NH₂-MIL-53(Al) materials contain certain intercrystalline mesopores as large as 20 and 30 nm, respectively, (Fig. 1(c)). Therefore both MOF-based supports possess some mesopores large enough to accommodate these enzymes.

Table 1 shows some relevant data of the biocatalysts generated by the post-synthesis immobilization procedures (enzyme#MOF). Entries 1, 2, 4 and 5 of Table 1 compare the immobilization of the enzymes β -glucosidase and laccase on MIL-53(Al) and on its amino-functionalized counterpart materials. MIL-53(Al) was able to immobilize 14.3% of β -Glu (Fig. 1(c)), probably due to their intercrystalline mesoporosity. However, this dimeric protein is too large to enter into most of the pores of NH₂-MIL-53(Al) (Fig. 1(c)), so no improvement in the immobilization yield was observed upon functionalization with amine groups. In this case the immobilization yield was very low but an unexpected effect of increased activity of the enzyme was observed, which could possibly be related to the removal of some inhibitors present in the enzyme extract. Some other recent studies in which enzymes have been immobilized in MOF supports, also found an increase of activity per enzyme molecule (unpublished results). A commercial amorphous (MS-3030) amine-functionalized mesoporous silica (MS-3030) support with surface area of 236 m² g⁻¹ and an average pore size of 29 nm was also used to immobilize β -glucosidase for comparative purposes. As shown in Table 1, values of enzyme loading and catalytic activity obtained with this support were lower than those with β -Glu#MOF. double on NH₂-MIL-53(Al) than on MIL-53(Al), probably due to the smaller size of laccase (with a monomeric protein structure) and also because laccase does not show the extensive glycosylation of beta-glucosidase, which might cause steric hindrance.⁷⁵ In addition, the interaction between laccase and the amino groups favors the immobilization of this enzyme as shown by entries 4 and 5 of Table 1, which indicate that immobilization yield is double in amino-functionalized MOF. The importance of the confinement is ratified in the laccase immobilization procedure over the NH₂-free Mg-MOF-74 material (entry 6 of Table 1). The presence of open metal (Mg) sites in this MOF⁶⁰ could play a role as important as that of amino groups in the Lac#NH₂-MIL-53(Al) biocatalyst (entry 4 of Table 1).

In summary, the post-synthesis enzyme immobilization over nanocrystalline MOF-based supports opens up numerous possibilities of tailoring the properties of the support (porosity, organic functionalization, metal coordination, etc.) and seems to be a promising strategy to immobilize enzymes. The preservation of catalytic activity indicates that the protein structure is maintained, corroborating the good prospects of the enzyme immobilization on these sustainable MOFs. These results encourage working on the improvement of both enzyme loadings and specific activity.

***In situ* immobilization: biocatalysts β -Glu@MOF**

Our methodology entails an indirect and extra industrial advantage, i.e. the sustainability of the process, since these materials were prepared at room temperature.^{59,60} That aspect also inspires a strategy to immobilize enzymes on MOF materials by an *in situ* approach, which has been reported to have some advantages/singularities in comparison with the post-synthesis approaches for some other supports.^{20,41,76} Moreover, in other reported *in situ* immobilization systems, the enzymes, far from being mere spectators, can have active roles such as template in the generated pores.²⁰ Based on these two premises, the following *in situ* studies focus on the immobilization of the large enzyme β -glucosidase on both NH₂-MIL-53(Al) and Mg-MOF-74 materials.

***In situ* immobilization of β -glucosidase on NH₂-MIL-53(Al)**

Our reported method to prepare carboxylate-based MOFs at room temperature and in water as the unique solvent implies starting from two solutions of very different pH values.^{59,60} The choice of the solution in which the enzyme is added as well as the order of addition of the solutions to form the final mixture, are crucial to optimize the preservation of catalytic activity of the enzyme, which could become irreversibly affected by extreme acidity/basicity of the media. On the other hand the linker and metal solutions form a precipitate as soon as they are mixed, so the enzymatic extract must be ideally present in this medium in order to favor the enzyme immobilization. For these two reasons, the enzyme was added in the linker solution previously and carefully prepared in order to reach pH values (not higher than 6) just above the pK_{a2} value of the amino-terephthalic/amino-terephthalate system. The metal solution was added over the linker solution containing the enzyme and not the opposite as usually reported.^{59,60} Thus, the enzyme was present from the first moment of the formation of the MOF material and it does not suffer the extreme acidic conditions of the metal solution (pH ca. 2).

The role of the chemical base is to deprotonate the carboxylic groups of the linker sources,^{59,60} and it is the only chemical that is not present in the final MOF material.

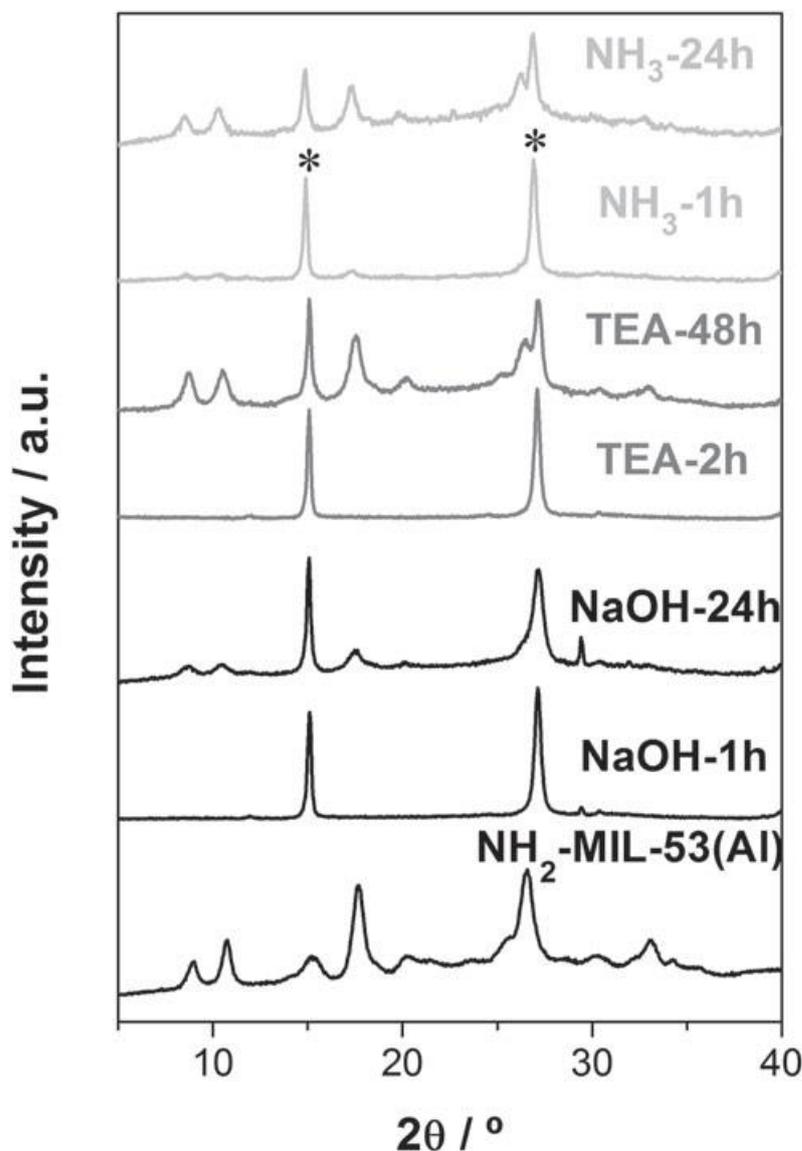


Figure 2. Normalized XRD patterns of the biocatalysts β -Glu@NH₂-MIL-53(Al) prepared with different bases (NaOH, TEA or NH₃) and after different synthesis times (from 1 h to 48 h). The pattern of the enzyme-free NH₂-MIL-53(Al) is also shown for comparison purposes. The two most intense reflections of the protonated linker H₂BDC are indicated with asterisks over the top pattern.

However, the nature of that base could have a key influence on the enzyme activity, so various deprotonating agents were tested, i.e. NaOH, trimethylamine (TEA) and NH₄OH. Figure 2 shows the XRD patterns of the six biocatalysts β -Glu@NH₂-MIL-53(Al) prepared with three deprotonating agents and after two synthesis times. They are compared with the XRD pattern of an enzyme-free nanocrystalline NH₂-MIL-53(Al) material, also prepared at room temperature according to the method described elsewhere.^{59,60} The diffractograms are dominated by two phases: the protonated organic linker NH₂-H₂BDC and the desired nanocrystalline NH₂-MIL-53(Al) material. The preparation of X-MIL-53(Al) materials at room temperature and in water assisted by non-strong bases as deprotonating agents (TEA or NH₃) is a novelty of this work.

In good agreement with the evolution of crystalline phases along the synthesis time in similar systems,^{59,60} NH₂-H₂BDC is the predominant phase at short times, whereas the proportion of the NH₂-MIL-53(Al) phase increases at the expense of the former with synthesis time. The formation of the NH₂-H₂BDC is reasonable considering that the final pH (around 3) of the mixture is lower than the pK_{a2} of the organic linker (4.8). After the tested times (not longer than 48 h), the MOF phase becomes dominant. Surprisingly, the amount of NH₂-H₂BDC phase is higher than that of the MOF material when the strongest base NaOH is used as the deprotonating agent.

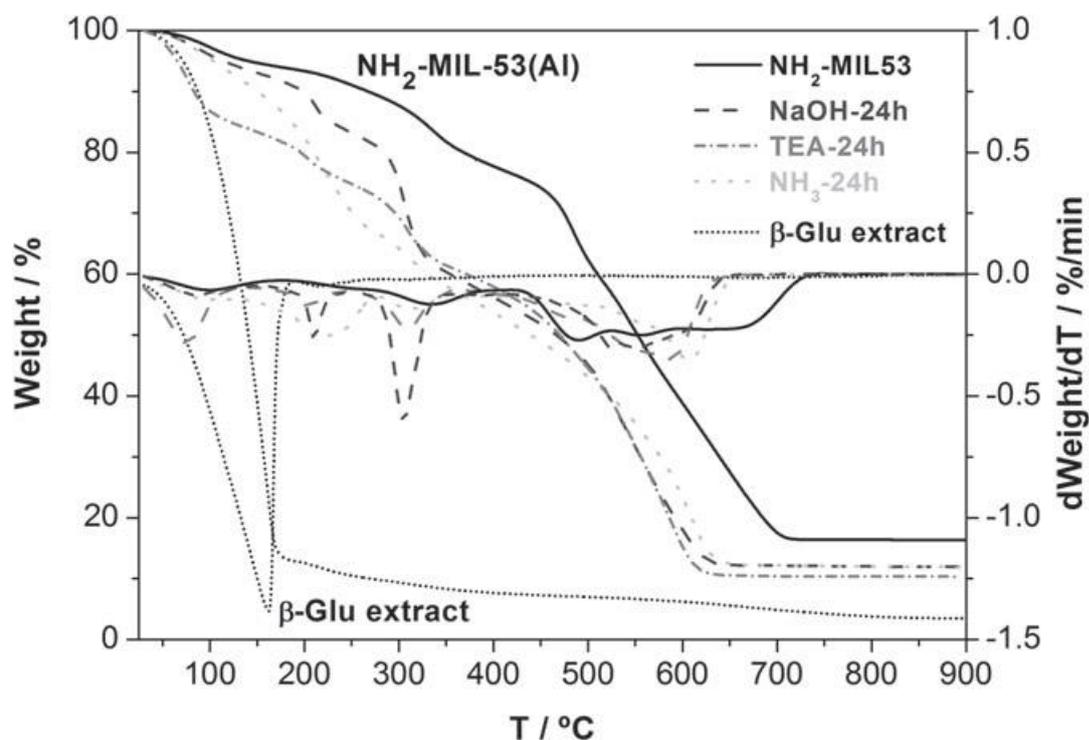


Figure 3. TGA (left Y-axis) and DTG (right Y-axis) curves of the $\text{NH}_2\text{-MIL-53(AI)}$ material (solid black lines), the $\beta\text{-Glu}$ extract (dot black lines) and the biocatalysts $\beta\text{-Glu@NH}_2\text{-MIL-53(AI)}$ prepared with different base (NaOH, TEA or NH_3) at long times.

The presence of the enzyme in these biocatalysts was inferred by different characterization techniques. Figure 3 shows the thermogravimetric curves and the corresponding derivatives of some biocatalysts compared with those of the enzymatic extract and the enzyme-free $\text{NH}_2\text{-MIL-53(AI)}$ material. The TGA plot of the $\beta\text{-Glu}$ extract is evidently dominated by a large weight loss of water, and it contains some other minor weight losses. One of them is abrupt enough to be detected in the derivative curve. That loss was centered at 217°C and tentatively attributed to the enzyme as described by others authors.^{54,57} Validating that assignation, the TGA curve of the enzyme-free MOF material does not show any loss close to that temperature, whereas the TGA plots of all $\beta\text{-Glu}$ -containing biocatalysts show sharp weight losses centered in the range $205\text{--}215^\circ\text{C}$, which must be related to the presence of the enzyme. The relatively low weight losses and the presumable overlap of this loss with that of NH_3 in the $\beta\text{-Glu@NH}_2\text{-MIL-53(AI)-NH}_3$ biocatalysts discouraged us to quantify the enzymatic charge with this technique. That quantification was accurately achieved by means of the Bradford method (Table 1). Nevertheless, TGA provides qualitative and quick valuable information that could even become quantitative in other systems. TGA could also provide some extra information about: (i) the thermal stability of the MOF-based support through the linker decomposition temperature; (ii) the MOF composition, through the linker loss/residual weight ratio; (iii) the possible presence of impurities, such as the undesired protonated linker; or (iv) the possible interaction enzyme-MOF. Thus, from the TGA/DTG curves of the biocatalysts $\beta\text{-Glu@NH}_2\text{-MIL-53(AI)}$, it is evident that the MOF supports have similar Al/linker ratio and similar stability to those of the enzyme-free $\text{NH}_2\text{-MIL-53(AI)}$ material irrespective of the deprotonating agent nature, although with different linker decomposition pattern. Moreover, the amount of deprotonated linker (weight loss slightly above 300°C)⁶⁰ is in good agreement with the phase identification/quantification by means of powder XRD (Fig. 2). CHNS chemical analysis was also used to prove the presence of $\beta\text{-glucosidase}$ in the biocatalysts. Although the presence of N atoms in the amino-containing supports prevents the use of N content as an enzymatic probe, sulfur content (S) could however play that role. Indeed, the $\text{NH}_2\text{-MIL-53(AI)}$ material was completely free of S whereas the enzymatic extract (concentration of 14.54 mg of enzyme per mL of solution) gave a S content of $0.24\text{ wt.}\%$. The measured S content of the biocatalysts $\beta\text{-Glu@NH}_2\text{-MIL-53(AI)-NaOH-24h}$, -TEA-48h and $\text{NH}_3\text{-24h}$ were 0.17 , 0.19 , and $0.15\text{ wt.}\%$, respectively, in good qualitative agreement with the enzyme content estimated by the Bradford method (Table 2). The series of the $\beta\text{-Glu@NH}_2\text{-MIL-53(AI)}$ biocatalysts were tested in the hydrolysis of para-nitrophenyl-beta-D-glucopyranoside (p-NPG) (Table 2, Scheme S1). Regardless of the used deprotonating agent, the capacity of the solid support to immobilize the enzyme $\beta\text{-glucosidase}$ increases with synthesis time, reaching an

immobilization percentages of almost 100% of the enzyme after 24 h (Table 2). We think that a combination of two factors contributes to the high enzyme retention: (i) the colloidal-like nature of the MOF suspension preventing enzyme release;⁴¹ and (ii) a favorable enzyme–MOF interaction.⁷⁷ At shorter times, the immobilization yield is much lower, which must be related with the low proportion of NH₂-MIL-53(AI) phase with respect to the linker NH₂-H₂BDC (Fig. 2). In other words, the MOF material, and not the protonated linker, is responsible for retaining the enzyme molecules. Despite none of our samples being purely MOF phase, all of them succeed in retaining relatively high enzyme loadings. The activity of the biocatalysts prepared for longer synthesis times was higher because of the higher enzyme loadings (Table 2). But the reaction conditions and/or diffusional problems of reactants not reaching more hindered enzyme caused a progressive inactivation of the β -glucosidase. So the specific activity (activity per milligram of immobilized enzyme) was lower (Table 2). It should be noted that, unlike the post-synthesis immobilization procedure, the *in situ* synthesis conditions had to be designed to favor formation of the nanocrystalline MOF and not only to maintain enzyme activity. That is why longer experiments were not planned since 24 or 48 h were enough to immobilize almost the whole amount of the enzyme present. NaOH is the only deprotonating agent reported so far for the synthesis of X-MIL-53(AI) materials under these conditions.^{59,60} However, the biocatalysts prepared in the presence of NaOH gave the lowest catalytic activity. This could be in part due to the high basic character of the solution in which the enzyme is added. *In situ* immobilization of β -glucosidase on Mg-MOF-74 Mg-MOF-74 was chosen as an *in situ* support to study a possible template role of the intercrystalline mesopores played by the enzyme because: (i) the relative narrow mesopore size distribution in Mg-MOF-74 (Figure 1), with a maximum smaller than the β -glucosidase dimensions; and (ii) the high sensitivity of the textural properties of the M-MOF-74 nanomaterials to any minor change in the synthesis conditions such as metal source solubility or nature of the metal anion source.⁶² However, the organic medium in which the nanocrystalline Mg-MOF-74 is prepared, i.e. N,N-dimethylformamide (DMF), leads to fast, complete and irreversible inactivation of the free β -glucosidase.

Figure 4 compares the powder XRD patterns, the N₂ adsorption/ desorption isotherms at –196 °C and the pore size distribution (PSD) curves of the enzyme-free nanocrystalline Mg-MOF-74⁶¹ and the biocatalyst β -Glu@MOF-74, both obtained after 24 h synthesis time. XRD patterns indicate that MOF-74 is the unique detected crystalline phase irrespective of the presence/absence of β -glucosidase. However, the presence of this enzyme noticeably affects the nature of the phase, as the XRD peaks are broader, more intense and noisier, which is probably related to its smaller crystal size and lower crystal size homogeneity. Nevertheless, the biocatalyst is almost as porous as the β -glucosidase-free MOF-74 sample (993 vs 1007 m² g⁻¹, respectively) but having different features in the mesopore region (Fig. 4(b) and (c)). The pore size distribution of the biocatalyst is broader and shifted to larger pores compared with Mg-MOF-74, suggesting that certain intercrystalline mesopores are able to accommodate β -glucosidase. This experimental fact suggests that enzyme might have a template-like role that would favor the consistence of the biocatalyst presumably through non-covalent interactions and minimizing the enzyme leaching. In this sense, it has been demonstrated recently that bio-macromolecules can induce the formation of MOF coatings around them.^{36,40,55,56}

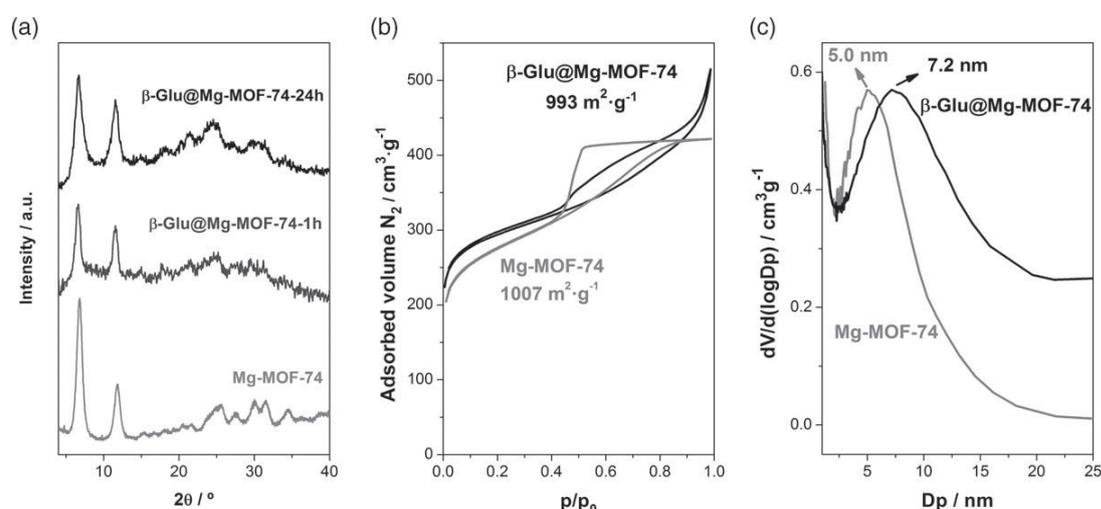


Figure 4. (a) Normalized XRD patterns of the Mg-MOF-74 and the biocatalysts β -Glu@Mg-MOF-74 after 1 h and 24 h of preparation. (b) and (c) N₂ adsorption/desorption isotherms at –196°C and the PSD curves arisen from the adsorption branches, respectively, of the enzyme-free (grey) and β -glucosidase-containing (black) Mg-MOF-74 after 24 h of synthesis time. Estimated BET areas and PSD maxima are indicated in (b) and (c), respectively

Table 2. Enzyme immobilization percentage (calculated by Bradford method), enzyme loading, catalytic activity and specific activity of the biocatalysts β -Glu@NH₂-MIL-53(Al) prepared by *in situ* strategy

Biocatalyst	Enzyme immobiliz. ^a / %	Enzyme loading ^b /mg g ⁻¹	Catalytic activity ^c /U g ⁻¹	Specific activity ^d /U mg ⁻¹
β -Glu@NH ₂ -MIL-53				
NaOH-1h	33	28	6	0.21
NaOH-24h	96	79	31	0.39
TEA-2h	17	18	40	2.17
TEA-48h	99	108	94	0.87
NH ₃ -1h	5	7	18	2.57
NH ₃ -24h	98	56	51	0.91

^a Percentage of immobilized enzyme in the solid biocatalyst against the amount of added enzyme.
^b mg of enzyme per g of biocatalyst.
^c Catalytic activity (in activity units U per g of biocatalyst) in the hydrolysis of para-nitrophenyl-beta-D-glucopyranoside.
^d Specific activity (in activity units U per mg of contained enzyme) in the hydrolysis of para-nitrophenyl-beta-D-glucopyranoside. Reference activity of enzymatic extracts: 6.93 U mg⁻¹ (C = 14.54 mg mL⁻¹).

These biocatalysts were tested in the hydrolysis of the substrate p-NPG to p-nitrophenol (Table 3). Catalytic activity of the enzymatic extract was instantly and fully lost in DMF. Remarkably, both MOF-supported biocatalysts prepared *in situ* in DMF for 1 and 24 h catalyzed the reaction to some extent, which means that the MOF support protects β -glucosidase activity from total inactivation in this adverse medium.⁵⁵ High value applications can be derived from this result since the range of industrial reactions catalyzed by enzymes might be expanded to some organic media.³⁵ The catalytic activity of these biocatalysts noticeably decreases with time, as expected because of the adverse immobilization media for the enzyme.⁷⁸ Nevertheless, after 24 h of synthesis, the solid biocatalyst maintains almost half the conversion reached after 1 h. Although the catalytic activity of these biocatalysts is rather limited, the one obtained after 1 h gave the same activity as β -Glu@NH₂-MIL-53(Al)-NH₃-1h, and three times higher activity than that of β -Glu@NH₂-MIL-53(Al)-NaOH-1h (Table 2), both prepared in aqueous medium over the same time. Of course, that comparison is in favor of the MOF-74-supported biocatalyst as, unlike its counterparts, this sample contains MOF as the unique crystalline phase detected by XRD. This fact promisingly leaves some room for improvement of the catalytic activity at even shorter times. On the other hand, once again, it certifies that the MOF phase is the only (or, at least, the foremost) responsible solid phase in the immobilization of enzymes. Likewise, it suggests that both the MOF formation and enzyme immobilization occur simultaneously, and that the enzyme–MOF interconnection is favored in the *in situ* strategy.

The easiest characterization test to prove the presence of enzyme within the samples β -Glu@Mg-MOF-74 was the electrophoresis under enzyme denaturant conditions by SDS-PAGE.⁷⁹ Figure 5 shows the electrophoresis gel images of the biocatalysts β -Glu@Mg-MOF-74-1 h and -24 h compared with the β -glucosidase extract. The widest band in the electrophoresis gel of the enzymatic extract corresponds to the monomer of β -glucosidase (120 kDa), whereas the rest of the signals must be attributed to other proteins present in the extract. Before immobilization the enzymatic extract was ultrafiltrated as shown in Figure S1, so only a major band of 120 kDa corresponding to the monomer of β -glucosidase remains, whereas some minor bands can be neglected. The gels of both biocatalysts β -Glu@Mg-MOF-74-1h and -24h also show the main signal of 120 kDa, undoubtedly evidencing the presence of the enzyme β -glucosidase in these biocatalysts. Sulfur content of the biocatalysts β -Glu@Mg-MOF-74-24h (0.13 wt%) compared with those of the β -glucosidase extract (0.24 wt% for a concentration of 14.54 mg mL⁻¹) and of the enzyme-free Mg-MOF-74 (0.00 wt%) gave extra qualitative but unequivocal evidence of the presence of the enzyme in these Mg-MOF-74-supported biocatalysts.

Table 3. Enzyme immobilization percentage (calculated by Bradford method), enzyme loading, catalytic activity and specific activity of the biocatalysts β -Glu@Mg-MOF-74 prepared by *in situ* strategy in DMF as solvent

Biocatalyst	Enzyme immobiliz. ^a / %	Enzyme loading ^b /mg g ⁻¹	Catalytic activity ^c /U g ⁻¹	Specific activity ^d / U mg ⁻¹
β -Glu@Mg-MOF-74				
1h	86	112	18	0.16
24h	86	113	7	0.07
Extract (14.54 mg mL ⁻¹)	0	0	0	0

^a Percentage of immobilized enzyme in the solid biocatalyst against the amount of added enzyme.
^b mg of enzyme per g of biocatalyst.
^c Catalytic activity (in activity units U per g of biocatalyst) in the hydrolysis of para-nitrophenyl-beta-D-glucopyranoside.

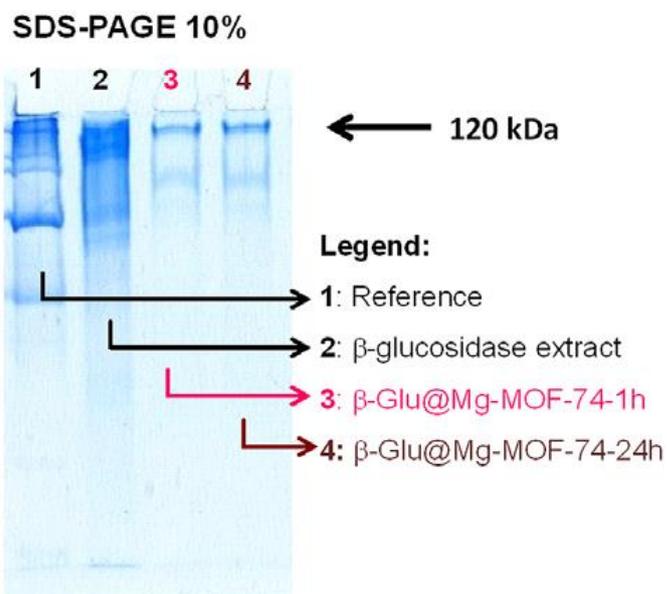


Figure 5. Electrophoresis gel of (from the left to the right): (a) a protein marker, (b) β -glucosidase extract (concentration 14.54mgmL⁻¹), (c) biocatalyst β -Glu@Mg-MOF-74-1h, and (d) biocatalyst β -Glu@Mg-MOF-74-24h

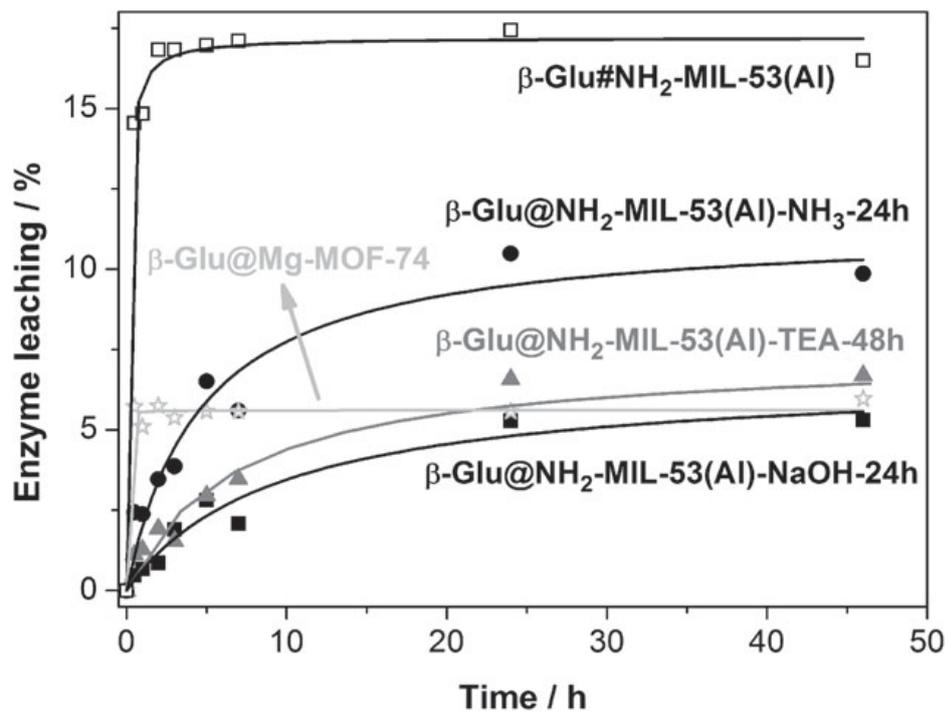


Figure 6. Percentage enzyme leaching of some β -glucosidase-containing biocatalysts of this work along the tested time (from top to bottom): post-synthesis β -Glu#NH₂-MIL-53(Al) and *in situ* β -Glu@NH₂-MIL-53(Al)-NH₃-24h, β -Glu@NH₂-MIL-53(Al)-TEA-48h, β -Glu@Mg-MOF-74, β -Glu@NH₂-MIL-53(Al)-NaOH-24h.

Post-synthesis vs *in situ* enzyme immobilization on nanocrystalline MOF supports One of the main strengths of this study is the diversity of methodologies proposed to immobilize enzymes on nanocrystalline MOFs after the study of a number of variables. Namely, post-synthesis and *in situ* strategies, synthesis medium either aqueous or adverse organic solvent (DMF), two enzymes with different size/affinities and MOF supports with different physico-chemical properties (functionalization, pore size, etc.). Having discussed these strategies during this article, it seems noteworthy to make a systematic comparison between post-synthesis and *in situ* strategies. To this aim, three key aspects of the enzyme immobilization will be discussed: enzyme leaching, enzyme loading and catalytic activity

The representative biocatalysts selected were post-synthesis β -Glu#NH₂-MIL-53(AI) (entry 1 of Table 1) and *in situ* β -Glu@NH₂-MIL-53(AI)-TEA-48h (entry 4 of Table 2). Figure 6 shows the leaching of β -glucosidase from the different MOF. All the biocatalysts share the same shape with an initial desorption during the first hours of treatment, and then a plateau is reached. Longer incubation times showed no further increase of leached protein. Probably, the initial desorption observed is mainly due to the removal of enzyme molecules immobilized on the external surface of the MOF particles. However, the amount of released β -glucosidase from post-synthesis β -Glu#NH₂-MIL-53(AI) is significantly higher than from *in situ* ones, as expected from the encapsulation model assumed from the results presented along this work.^{40,41} This is especially evident for β -Glu@NH₂-MIL-53(AI)-TEA-48h, where the immobilization yield is as high as 99% (see Table 2).

The immobilization method also has different effects on the catalytic activity. As seen in entry 1 of Table 1 and entry 4 of Table 2, the activity of β -glucosidase is better preserved via post-synthesis immobilization (19.30 units per milligram of immobilized enzyme). This is an expected result since the immobilization medium is mild whereas *in situ* immobilization is performed in less favorable conditions for enzymatic activity.

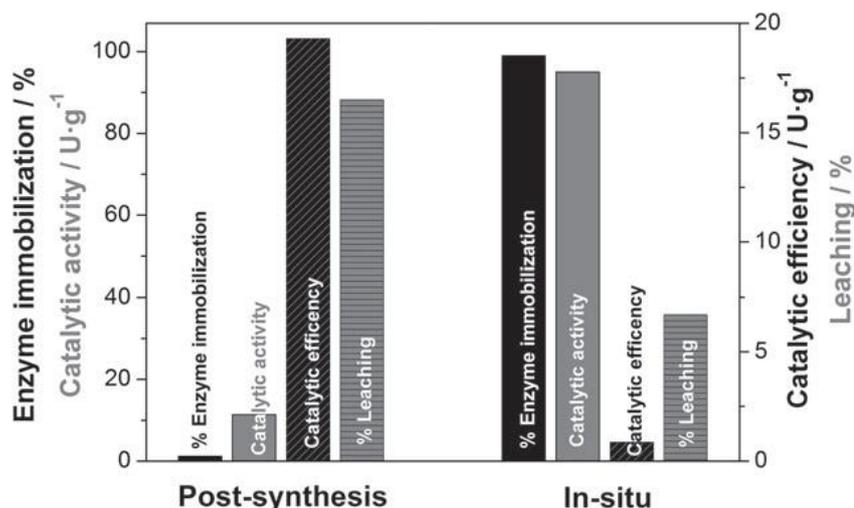


Figure 7. Percentage enzyme immobilization (black columns, left Y-axis), catalytic activity (grey, left Y-axis), catalytic efficiency (striped black, right Y-axis) and percentage enzyme leaching (striped grey, right Y-axis) of the biocatalyst β -Glu#NH₂-MIL-53(AI) prepared by post-synthesis (left) and *in situ* (right) approaches.

Figure 7 graphically compares the most remarkable catalytic data of the two selected biocatalysts in the hydrolysis of the p-NPG to p-nitrophenol at 35 °C. At first glance, the marked differences between the catalytic performance of *in situ* and post-synthesis biocatalysts are very clear. Thus, enzyme immobilization yield (black columns in Fig. 7) is much higher when the biocatalyst is prepared *in situ*, and is able to retain practically all available enzymes in the media for the studied enzyme concentrations. In contrast, in the post-synthesis approach, enzymes found restrictions to entering the cavities, which is logical paying attention to their dimensions (12.3 nm × 10.7 nm × 8.1 nm) and the pore size distribution of the NH₂-MIL-53(AI) (centered at 7 nm). As a result of the immobilization yield, catalytic activity considered in units per gram of biocatalyst of the *in situ* biocatalyst is much higher (grey columns in Fig. 7) than its post-synthesis counterpart, although the specific activity (units per mg of immobilized β -glucosidase, striped black columns in Fig. 7) of the *in situ* one was lower. Finally, as commented above,

the leaching of β -glucosidase is three times lower in the *in situ* approach, retaining almost 93% of the enzyme immobilized (striped grey columns in Fig. 7).

CONCLUSIONS

Post-synthesis and *in situ* approaches to immobilize the enzymes β -glucosidase and (post-synthesis one for) laccase on sustainable MOF materials has been described and compared in this work. The studied MOF supports, NH₂-functionalized and non-functionalized MIL-53(Al) and Mg-MOF-74, are characterized by two key features: (i) high external surface area (mainly as intercrystalline mesoporosity); and (ii) preparation at room temperature. Biocatalysts prepared *in situ* became richer in enzyme loadings (> 85% of the enzyme added to the synthesis media), more catalytically active and leaked less of the immobilized enzymes, whereas the intrinsic catalytic activity per enzyme molecule was higher in the post-synthesis biocatalysts. The *in situ* approach even led to active biocatalysts in a non-aqueous adverse media for enzymes, N,N-dimethylformamide. These methods are of general application because the enzymes do not have to be smaller than the MOF pores. The wide structural, compositional and synthetic versatility of MOFs opens up many possibilities for their use as enzyme supports.

ACKNOWLEDGEMENT

This work has been partially financed by the Spanish State Research Agency (Agencia Española de Investigación, AEI) and the European Regional Development Fund (Fondo Europeo de Desarrollo Regional, FEDER) through the Project MAT2016-77496-R (AEI/FEDER, UE). The authors thank X. Xiao, T. Siepenkoetter and C. Carucci for critical comments on the manuscript.

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