Successful encapsulation of β-glucosidase during the synthesis of siliceous mesostructured materials

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ABSTRACT

BACKGROUND: The Biocatalysis field demands “universal supports” able to encapsulate enzymes with a straightforward methodology, and at the same time, being capable of keeping its catalytic activity. The employment of siliceous materials for such purpose is a big challenge because drastic synthesis conditions are required and besides, most of the times it is needed a functionalization to increase affinities towards the targeted enzyme. In this work, a compromise between the development of a well-formed mesostructured support and an acceptable enzymatic activity was attempted via the in-situ immobilization approach.

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RESULTS: The immobilization of β-glucosidase (EC 3.2.1.21) from Aspergillus niger was approached from different strategies. After trying immobilizing said enzyme with a post-synthesis approach, both with a covalent linkage (using epoxy activated supports) and with a non-covalent bonding (using amine-functionalized materials), non-high loadings were achieved (3.5 mgE/g and 7.6 mgE/g, respectively). Nevertheless, when the in-situ approach was attempted, success in reaching the highest enzyme loading (close to 200 mgE/g) was achieved.

CONCLUSION: In this work, the in-situ encapsulated enzyme within the support-cages fully prevented it to be released through the narrow windows connecting cages, achieving a less than 5% release of the initially desorbed protein, as well as a further total absence of leaching. This enabled the biocatalyst to be reused at least eight times more without any loss in activity.

Keywords: Enzyme immobilization; β-glucosidase; In-situ; Ordered mesoporous silica materials; Post-synthesis.
1. INTRODUCTION

It is very well-known that enzyme immobilization within solid supports improves the stability against different inactivation agents (microbial attack, presence of organic solvents…) and avoids the unwanted solubility of the enzyme in the reaction medium, so it can be efficiently recovered offering a reuse/reutilisation potentiality in subsequent reaction cycles 1-4. Nevertheless, the current scientific problem revolves around the fact of the lack of an existing straightforward “universal” approach to immobilise large enzymes such as the β-glucosidase employed in this work into mesoporous silica materials. This is the reason why herein it is pretended the usage of different immobilization methods based on siliceous materials as supports, in order to look for the most appropriate immobilization methodology. Additionally, provided the fact ordered mesoporous silica materials render high surface area with tuneable and uniform pores sizes, as well as excellent biocompatibility 5-8, employment in several applications such as supports for enzyme attachment once the material is rationally well-designed is more than justified. Moreover, added to the common advantages of using immobilized enzymes, high enzyme loadings, high catalytic efficiencies as well as high confinements can be achieved with these meso-silica structures while avoiding important substrate diffusional limitations 9.

Regarding the state of the art of enzyme immobilization, there exist different paths for the immobilization of enzymes in general and of β-glucosidase in particular. Concerning the post-synthesis approach, covalent and non-covalent attachment on previously synthesized mesoporous siliceous materials can be employed. Covalent bonding can be performed on epoxy-functionalized supports to make them react with the amine or sulfhydryl groups of the enzyme 10-12. For the case of non-covalent bonding, support functionalization with amine groups can be used to establish electrostatic interactions at a controlled immobilization pH with deprotonated carboxyl group of the polypeptide chain 13. Regarding the in-situ strategy, a “ship-in-a-bottle” encapsulation by synthesis of the mesostructured siliceous material in presence of the enzyme can be employed. The objective is to achieve a particular mesostructure where the
enzymes are efficiently physically-retained inside large homogeneous cavities. Ideally, leaching from these cages might be prevented by the narrow dimensions of the windows that are connecting the cages.

In this work, we have used the hyperthermostable commercial β-glucosidase (β-D-glucoside glucohydrolases, EC 3.2.1.21) known as Novozym 188 or Cellobiase produced by the fungus Aspergillus niger 14. This large enzyme has a stable homodimeric structure with a molecular weight of 240 kDa (each monomer: 120 kDa) (deduced by SDS-PAGE) and an isoelectric point of 4.0 15, 16. β-glucosidase enzymes are involved in the hydrolysis of terminal non-reduced β-D-glucosyl residues forming β-D-glucose products 17, 18. They have applications in the high temperature hydrolysis of cellulosic biomass to glucose in combination with two other types of enzymes (endo-β-1,4-glucanases and cellobiohydrolases) 14, 19-23. The main advantages of using immobilized β-glucosidase is the obtaining of high-value and highly-demanded antioxidant products like olive polyphenols (i.e. hydroxytyrosol, oleuropein) 24 or the saccharification of cellulosic and lignocellulosic materials into bioethanol to provide biofuel 21.

Since, synthesis of siliceous materials involves extreme reaction conditions (strong acid conditions, high temperatures) not compatible with preservation of enzyme activity, our group intended, and indeed, succeeded in developing a strategy for in-situ encapsulation of the robust Candida antarctica lipase B enzyme (CALB, EC 3.1.1.3) by controlling and adjusting experimental conditions for the synthesis of the silica carrier 25. However, one of the fundamental issues to deal with was how to efficiently load the enzyme into the pore space 25-27. For the encapsulation method, the enzyme either has to be compatible with the surfactant and additives used to template the pore architecture of the support or has to act itself as a template 26. In our previous work 25 we concluded that enzyme molecules, such as CALB lipase, should interact with the hydrophobic tail of the surfactant, acting thus as a cooperative hydrophobic guest. In this way, a certain amount of the enzyme should be accommodated into the cavities of material. However, when the same approach is tried with a labile enzyme, this one losses its
intrinsic activity obtaining non-active biocatalysts; or conversely, the enzyme does not participate in the self-assembling of the material \(^{28}\).

To the best of our knowledge, no detailed study has been done on investigating the in-situ encapsulation of \(\beta\)-glucosidase from \textit{Aspergillus niger} into mesoporous materials. Thus, the challenge in this work resides in the attempt to synthesize silica materials with large-pore structure in the presence of the bulky dimeric \(\beta\)-glucosidase enzyme being, at the same time, capable of effectively immobilize enzymes without dramatic loss of activity and using also mild conditions. Said conditions were soft enough to indeed avoid damage in the 3-dimensional enzyme structure, while keeping at the same time the formation of the meso-silica structure around enzyme molecules. The comparison of the different strategies performed here to immobilize this large enzyme (\(\beta\)-glucosidase) clearly implies a state-of-the-art advance, allowing thus the development of efficient active biocatalysts. Particularly, in-situ supports improves the benefits given by some other home-based rationally designed post-synthesis supports (like NH\(_2\)-FDU-12 or NH\(_2\)-SBA-15) having at the same time more active biocatalysts and lower enzyme leaching, what indeed brings these catalysts closer to their potential use in different applications.

2. EXPERIMENTAL

It is worth nothing that most of the experimental methodology performed in this work can be found in the Supporting Information. Additionally, said methodology consist in: 1) employed chemicals; 2) Pretreatment of \(\beta\)-glucosidase extract; 3) Protein determination; 4) Determination of \(\beta\)-glucosidase activity; 5) Effect of pH on the activity of \(\beta\)-glucosidase; 6) Thermal stability of \(\beta\)-glucosidase; 7) Evaluation of \(\beta\)-glucosidase stability in the presence of ethanol and acidic medium; 8) Synthesis of supports: Functionalization of amorphous silica by grafting with epoxy groups (MS-3030-g-epoxy); Functionalization of amorphous silica by grafting with amine groups (MS-3030-g-N); Synthesis of large-pore ordered mesoporous silica with cage-like pore structure (FDU-12); Functionalization of large-pore mesoporous silica with cage-like pore
structure (FDU-12) by grafting with amine groups; Synthesis of large-pore mesoporous silica with channel-like pore structure (SBA-15) functionalized with amino groups by co-condensation. 9) Post-synthesis immobilization of β-glucosidase on supports. 10) Characterization methods. 11) Surfactant and micelle expander removal: Mild extraction treatment. 12) Recycling experiments using IS-XYL-3.5 biocatalyst.

2.1.2.1. In-situ immobilization of β-glucosidase

IS-TMB-x samples: In a typical synthesis procedure, 1 g of triblock co-polymer Pluronic F127 was dissolved at room temperature in 20 mL of Milli-Q water inside a closed container made of Teflon, using a magnetic stirrer. In another flask, 5 g of KCl were dissolved in 20 mL of water. The later KCl solution was added to the first solution after 3 h. The container was then kept at a temperature of 15 °C and the solution was gently stirred for 1 h to allow temperature equilibration. Then, 10 mL of enzyme extract with a concentration of 24.76 mg/mL were added under gentle agitation (100 rpm). Afterwards, 1417 µL of the micelle expander TMB were added. After 30 min, the pH was adjusted to 3.5 or 4.0 by adding 1 M HCl solution dropwise and then water up to a total volume of 10 mL. Next, 4463.5 µL of TEOS were finally added. The resulting gel was gently stirred (120 rpm) for 24 h at 15 °C and subsequently heated at 50°C in the closed container under static conditions for 5 days. Enzymatic activity of the suspension, supernatant and blank was measured every 24 hours (by triplicate) to evaluate β-glucosidase stability/activity under the synthesis-encapsulation conditions. The mixture was then filtered and the resulting solid product was washed with water (Milli-Q grade), dried under vacuum and afterwards under dry nitrogen stream. Then it was stored at 4 °C for later analysis. The resulting samples were denoted as “IS-TMB-3.5” or “IS-TMB-4.0”, where the number denotes the pH of the synthesis medium.

IS-XYL-x samples: The synthesis of IS-XYL-x samples followed a similar procedure as the one described above, but this time with some modifications. 1 g of triblock co-polymer Pluronic F127 was dissolved at room temperature in 20 mL of Milli-Q water (Milli-Q) inside a closed container made of Teflon and using a magnetic stirrer. In another flask, 2.5 g of KCl were dissolved in
20°mL of water. This KCl solution was added to the first solution after 3 h. The container was then kept at a temperature of 14 °C and the solution was gently stirred for 1 hour to allow temperature equilibration. Then, 10 mL of enzyme extract with a concentration of 24.76°mg/mL were added under gentle agitation (100 rpm) prior to addition of 4262.7 µL of the micelle expander m-xylene. After 30 min, the pH was adjusted to 3.5 or 4.0 by adding 1 M HCl solution dropwise and then water up to a total volume of 10°mL. Finally, 4830 µL of TEOS were added. The gel obtained was gently stirred (120 rpm) for 24°h at 14°C and subsequently heated at 50°C in the closed container under static conditions for 5 days. The enzyme activities of the suspension, supernatant and blank were assayed by triplicate every 24 hours to evaluate β-glucosidase stability under the synthesis-encapsulation conditions. The mixture was then filtered and the resulting solid product was washed with Milli-Q water, dried under vacuum and afterwards under dry nitrogen stream. Then it was stored at 4 °C for later analysis. The resulting samples are denoted as “IS-XYL-3.5” or “IS-XYL-4.0”, where the number denotes the pH of the synthesis medium.

2.11 2.2. Drastic extraction and calcination treatments

After testing in-situ biocatalysts activities, organic compounds like surfactant, micelle expanders and enzyme molecules were removed by drastic extraction conditions and calcination, respectively, in order to characterize the material structure. An amount of 300 mg of each biocatalysts sample were suspended in 5 mL of HCl (35 %) and 45 mL of ethanol solution and afterwards, refluxed at 78 °C under stirring for 24 h. The solids were recovered by vacuum filtration and air dried. Besides, another 300 mg of each biocatalyst were calcined in a furnace at 550°C for 5 h (heating ramp: 2 °C/min).

2.12 2.3. Measurement of activity of immobilized enzyme

To determine the enzymatic activity of β-glucosidase immobilized on mesoporous silica materials, 10 mg of the respective dried biocatalysts were re-suspended in 1 mL of 50°mM buffer and analysed for remaining β-glucosidase activity in the p-NPG hydrolysis activity assay detailed in SI.
2.13 2.4. Enzyme leaching

To evaluate the ability of the supports to retain the non-covalently bonded enzyme, the biocatalysts were suspended for 48 h in conditions that favoured the enzyme leaching: very high dilution (1.25°mg/mL) in 50 mM acetic acid/sodium acetate at pH 7.0, i.e., above the isoelectric point of the enzyme (pI 4.0) to generate electrostatic repulsion with the silica surface. At different times, aliquots of the suspensions were withdrawn and centrifuged, and the protein content of the supernatants was measured through Bradford analyses (in triplicate).

After leaching tests (48 h) and checking the absence of further desorption, electrophoresis of the final catalysts was performed to confirm the presence of encapsulated enzyme. Samples of immobilized enzyme were submitted to denaturant conditions treatment, so the linear chain of amino acids may be released from the pores and the supernatant can be analysed by electrophoresis (EF). The enzyme was identified by SDS-polyacrylamide gel electrophoresis (10°% SDS-PAGE) by the method of Laemmly 29 using Bio-Rad mini-protean III electrophoresis unit.

3. RESULTS AND DISCUSSION

The results and discussion section has been divided in two approaches depending on the moment in which the enzyme is incorporated in the material; i) after the synthesis of the materials (post-synthesis immobilization) only for comparison purposes with the in-situ approach, and ii) the in-situ immobilization or “ship-in-a-bottle” itself where the enzyme is incorporated during the synthesis process of the support.

3.1. Post-synthesis immobilization of β-glucosidase (β-Glu)

3.1.1. Characterization of supports

The XRD patterns of silica LP-FDU-12-C and amino-functionalized silica LP-FDU-12-g-N is showed in Figure S2, in which two well-resolved peaks, which can be indexed as 111 and 311 reflections, respectively, are indicative of a face-centered cubic (fcc) mesostructure (space group...
These are similar to the XRD patterns of FDU-12 reported in the literature. The diffractogram of sample LP-FDU-12-g-N indicates that grafting of aminopropyl groups on sample LP-FDU-12-C does not affect the structural ordering.

The pore ordering of sample LP-FDU-12-g-N is also assessed by TEM images (Figure S3a, b) taken at different directions evidence the well-defined 3D cubic mesostructure of spherical mesopores (Fm3m symmetry) with a highly ordered lattice array over large domains. From these TEM images, the cage size can be estimated to be larger than 20 nm, in accordance with the nitrogen adsorption-desorption measurements Figure S4 (see inset). The arrangement of pore openings on the surface of the sample particles can also be observed in the SEM images (Figure S3c, d).

As shown in Figure S4, both LP-FDU-12-C and LP-FDU-12-g-N materials exhibit type IV nitrogen adsorption-desorption isotherms with a steep capillary condensation step at high relative pressures together with a H1 hysteresis loop, which is indicative of large mesopores and of a highly uniform size. Figure S4 inset shows the pore size distributions obtained from the adsorption and desorption branches of the isotherms based on the BJH model. Table S2 collects the pore sizes corresponding to the maximum of these distributions. The cage size, estimated from the pore size distribution of the adsorption branch, is close to 25 nm for sample LP-FDU-12-C. On the other hand, according to the pore size distribution obtained from the desorption branch, the windows that interconnect neighbouring cages have an estimated size of 17 nm. The results obtained for sample LP-FDU-12-g-N indicate that grafting of aminopropyl groups on the silica support provoke a slight decrease of the cage size to ca. 21 nm, as well as a small reduction of surface area and pore volume.

Chemical analysis was employed for the quantitative determination of the –NH₂ groups incorporated into LP-FDU-12-g-N by grafting. The nitrogen content determined is equivalent to 0.7 amine groups per gram of silica (Table S2).

For comparative purposes, two additional silica materials bearing surface amino groups were used in this work, namely an ordered mesoporous material with a channel-type structure (LP-
SBA-15-co-N) and an amorphous silica with disordered mesopore structure (MS-3030-g-N). The textural properties and content of amino groups of these samples have been previously reported and are summarized in Table S2. Sample LP-SBA-15-co-N possesses non-intersecting cylindrical channels with a diameter of 18 nm, close to the size of the apertures interconnecting cages in sample LP-FDU-12-g-N, and exhibits higher surface area and pore volume. In addition, compared to sample LP-FDU-12-g-N, the content of amino groups in sample LP-SBA-15-co-N (1.5 mmol N/g SiO$_2$) is two times higher. Sample MS-3030-g-N exhibits also a certain surface area and also the highest content of surface amino groups (1.8 mmol N/g SiO$_2$). In contrast with the ordered mesoporous silica materials, the MS-3030-g-N support possesses a broad pore size distribution and significantly larger mesopores (the maximum of the pore size distribution is close to 30 nm), which results also in ca. two times higher pore volume (Table S2).

Textural properties of mesoporous amorphous silica MS-3030 activated with epoxy groups (MS-3030-g-epoxy) are similar to those for MS-3030-g-N, with a surface area of 223 m$^2$/g and a total pore volume of 2.1 cm$^3$/g (Table S2, Figure S5). For this sample, the content of epoxide groups determined by titration corresponds to 1.26 mmol per gram of silica. Also for comparative purposes, a commercial epoxy activated poly(methacrylamide) copolymer in the form of macroporous beads (Eupergit C) was used in this work. This support has a surface area of 4.5 m$^2$/g, a pore volume of 0.06 cm$^3$/g, a main pore diameter of 10 nm and a content of epoxide groups of 0.6 mmol/g.

Different approaches to immobilize the large enzyme β-Glu onto the porous materials were studied: covalent attachment and non-covalent interaction or adsorption by the post-synthesis strategy and encapsulation by the in-situ strategy.

3.1.2. Post-synthesis immobilization of β-glucosidase (β-Glu)

Post-synthesis immobilization of β-Glu via covalent attachment on epoxy-activated supports (MS-3030-g-epoxy and Eupergit C) was disregarded, because it rendered poor enzyme loadings (3.5 mgE/g) and low specific activities (0.06 U/mgE) (see SI, Table S3).
Regarding the post-synthesis immobilization via non-covalent attachment, in order to promote interaction between the enzyme and the support, three amino-functionalized silica materials (MS-3030-g-N, LP-SBA-15-co-N and LP-FDU-12-g-N) were considered. The immobilization on these amino-functionalized silica supports was performed at pH 5.5 at which the surface of the support is positively charged due to protonation of amino groups. At this pH, higher than the isoelectric point of the enzyme (pI = 4.0), the enzyme surface would be negatively charged, so electrostatic interactions control the adsorption process (Scheme S5). Post-synthesis immobilization of β-Glu via electrostatic interactions also yielded low enzyme loadings (Table S3), reaching a maximum value of 7.6 mg/g for LP-SBA-15-co-N. However, while covalent bonding leads to deactivation of this enzyme (Table S3), the specific activity was maintained through adsorption in LP-SBA-15-co-N, LP-FDU-12-g-N, and MS-3030-g-N supports. Actually, the last two biocatalysts displayed higher specific activity than the free enzyme (higher than 2.9 U/mg enzyme). This phenomenon has also been observed by immobilization of this particular enzyme on MOFs.

Immobilized β-Glu onto the ordered mesostructures LP-SBA-15-co-N and LP-FDU-12-g-N were suspended in conditions that would favour enzyme leaching in order to study the effect of the structure (two dimensions channel for LP-SBA-15-co-N and three dimension cage-window cavity for LP-FDU-12-g-N, respectively) on the enzyme leaching (see Schemes S6 b and 6 c). Only at the beginning of the test, it was found that cylindrical pores of LP-SBA-15-co-N were not as efficient in retaining the enzyme as the large cages of LP-FDU-12-g-N, as shown in Figure 1. However, finally both materials succeeded to retain the enzyme within pores after the initial release (up to 8 h of testing). It is worth noting that, the released enzyme showed negligible activity. Consequently, the low leaching achieved (13% of encapsulated enzyme for LP-FDU-12-g-N and 17% for LP-SBA-15-co-N) may probably be due to enzyme molecules that could eventually be adsorbed onto the external surfaces of the particles during the immobilization process. Nevertheless, because the synthesized support materials were highly ordered, the enzymes located inside the pores or cavities were highly retained, being thus
evident that the support design plays an important role in the final properties of the resultant biocatalyst.

3.2. In-situ immobilization of \( \beta \)-Glucosidase (\( \beta \)-Glu)

Avoiding enzyme leaching is a handicap when the driving forces of immobilization are non-covalent interactions. But it is strictly necessary when the goal is the industrial application of the biocatalysts. For the immobilization by adsorption in FDU-12 materials, it is important to adjust the windows size and the interactions between the protein and support surface in order to enhance enzyme retention. However, the narrow windows preventing leaching are also the responsible for the low enzyme loading of these materials when enzyme immobilization is carried out by post-synthesis adsorption. So, the alternative solution might consist in synthesizing the support in presence of the enzyme in order to produce cages containing the enzyme, which would eventually allow obtaining higher enzyme loading. At the same time, narrow windows connecting the cages would prevent the exit of the enzyme molecules so, ideally, the physical encapsulation method reduced the leaching.

In order to rationalize the potential mechanism that would allow the efficient encapsulation of enzyme molecules during the synthesis of the silica matrix, the surface amino acids of the enzyme were studied by using a program for the visualization of proteins (PyMOL Win). Scheme S7 shows the presence of hydrophobic regions in \( \beta \)-Glu (white color). In solution, non-polar surface areas of the protein can interact with the hydrophobic micelle expanders (XYL or TMB) by hydrophobic interactions \(^{37, 38}\). Surfactant molecules in turn can cover these enzyme/organic species, having their apolar chains interacting with the hydrophobic regions and the polar heads oriented towards the external polar (aqueous) medium. Thus, silicate polymers can grow around these polar regions of the surfactant aggregates that actually cover the enzyme molecules, and the mesostructured silica material would be ideally formed in this way via cooperative self-assembly (see Scheme 1).

3.2.1.1. Stability of free \( \beta \)-glucosidase
As synthesis of the siliceous materials involves some harsh conditions, it is necessary to determine the range of experimental conditions of stability for soluble β-Glu in order to establish the adequate synthesis conditions to preserve enzyme activity. First, the effect of pH on the activity of β-Glu was evaluated. Figure S6 shows relative activity of the soluble β-Glu enzyme at different pH values between 3.0 and 9.0. The activity-pH profile shows a sharp optimum at pH 4.5. The enzyme exhibited highest activity at pH 4.5, although it maintained up to 90% and 70% of its activity at pH 4.0 and 3.5, respectively. Those are the pH values selected for in-situ encapsulation of β-Glu enzyme. It is worth stressing that the in-situ immobilizations requires long periods of incubation at mild acid pH in the range 3.5-4.0 in the synthesis medium, and that precipitation of silica and orderly formation of mesostructures did not occur at higher pH values 39, 40.

Thermal stability was also evaluated. β-Glu from A. niger is known to be a hyperthermostable enzyme, therefore the activity of the enzyme incubated at 50 °C or 60 °C was maintained higher than the one measured at room temperature (Figure S7 a). The increase of activity at the high temperature has also been observed by other authors 15. Only after 150 hours of incubation at 60 °C activity drop was detected. These results show that the enzyme in solution exhibits a high thermoresistence required to preserve enzyme activity for the long periods of incubation at the high temperature needed to carry out the in-situ immobilization.

During the hydrolysis and condensation reactions of the tetraethoxysilane (TEOS) in the presence of water and the catalyst (HCl), ethanol is formed in the reaction media 41. This unwanted product could affect enzyme stability; therefore soluble enzyme was incubated in 10% ethanol solution at acidic pH (3.5) and 35 °C. It was found that ethanol did not cause an important loss of the enzyme activity in p-NPG hydrolysis for periods of incubation up to 72 h (Figure S7 b) 17. The results could be useful to provide information about its tolerance for following studies of the enzyme encapsulated in an “in-situ” way, and also in additional tests for mild extraction of the surfactant/micelle expander (Figure S11, Table S4).

3.2.1.2. Synthesis of in-situ encapsulated β-glucosidase
The previous tests verified that the soluble enzyme keeps high activity at acidic pH, and is resistant to high temperatures and to the presence of ethanol, which are the experimental conditions required for in-situ immobilization.

The amount of $\beta$-Glu entrapped during the encapsulation process was followed by monitoring both, the enzyme activity and the protein concentration in the supernatant. Figure 2 shows the time course of encapsulation. The syntheses carried out at pH 3.5 allowed encapsulating nearly 100% of the enzyme in solution (measuring the protein concentration decrease by Bradford assay of the Blank (or control), suspension and supernatant), although it was found that the rate of encapsulation depended on the type of swelling agent used. Thus, sample IS-XYL-3.5 (obtained with $m$-xylene) reached the maximum percentage of encapsulation after 1 day and this amount was maintained practically constant for 5 days, while the biocatalyst IS-TMB-3.5 (prepared using 1,3,5-trimethylbenzene) showed a progressive enzyme loading for 5 days. In contrast, at pH 4.0 the enzyme loadings increased up to 2 days and remained nearly constant for longer synthesis times, with maximum enzyme loadings below 40% of the total enzyme.

The yields and activities of samples obtained by encapsulation after 5 days of ageing at 50°C are summarized in Table 1. It is worth noting that, while at pH 3.5 similar enzyme loadings were achieved with both micelle expanders, the biocatalyst activity and specific activity were much higher when using $m$-xylene. At pH 4.0, the values of enzyme loading and catalytic activity were rather favourable for 1,3,5-trimethylbenzene, although the specific activities were very close when using both micelle expanders.

In all cases, activity of encapsulated $\beta$-Glu is lower than that of the free enzyme (2.7°U/mgE). Synthesis conditions were modified to become relatively mild compared to the experimental conditions used in conventional synthesis of ordered mesoporous silica material (pH < 2, temperature over 80 °C, hydrothermal treatment) 42. However, this treatment at relatively high temperature (50 °C), acidic pH (between 3.5 and 4.0), presence of salts and organic compounds for a long period may cause partial distortion of the protein and thus lead to
a loss of activity. In spite of that, the decrease of the specific activity in IS-XYL-3.5 is only 56% with respect to the soluble enzyme, which becomes successfully encapsulated. Furthermore, for this particular biocatalyst additional steps to remove possible residues of surfactant / micelle expander were not necessary (Figure S11, Table S4). Additionally, the presence of the surfactant was not deleterious for enzyme catalytic activity, which also seemed to indicate that the diffusion of reactants and products was possible without the need of eliminating the surfactant and/or micelle expanders. This finding is in line with previous results reported by other groups and also from our own previous work on CALB lipase encapsulation in mesoporous silica materials, in which the specific activity was significantly diminished after the removal of surfactant. Consequently, IS-XYL-3.5 was the chosen candidate to conduct the subsequent experimentation. Finally, said biocatalyst was recycled 8 times in consecutives cycles and the release of p-nitrophenol measured after each cycle at 340 nm. In these recycling experiments, no significant relative absorbance decrease was observed (Figure 3). Conversely, after those 8 recycled cycles for LP-SBA-15-co-N a 60% of the initial activity was maintained, while for LP-FDU-12-g-N a 75% was preserved (data not shown).

3.2.1.3. Structural characterization of the silica supports

In order to determine the actual pore structure of the silica support built around the enzyme molecules during the encapsulation conditions, the samples were analysed by dark-field STEM electron microscopy. Representative high resolution STEM images (Figure 4, a-c) of sample IS-XYL-3.5 show a siliceous mesocellular foam structure (MCF) composed of a three dimensional pore system with large spherical cages of uniform size (~10-15 nm).

The four biocatalysts synthesized via the in-situ route exhibit similar mesocellular foam structure, with cages of uniform size. This is confirmed by the low-angle XRD patterns of the as-synthesized biocatalysts (Figure 5), which show an intense diffraction peak that can be attributed to the presence of a mesostructure in these solids due to the uniform size of cages and thickness of silica walls, without a clearly defined long distance cavity order that could eventually be associated to any specific symmetry, reason why it is difficult to index any
specific peak in said diffractograms. The interplanar spacing corresponding to this diffraction peak is ca. 14-15 nm for the samples synthesized at pH 3.5, a value that closely matches the regular distances between the centres of two neighbouring cages estimated from the STEM micrographs. The low-angle diffraction peak of samples obtained at pH 4.0 shows lower intensity, which indicates that the increase of pH leads to poor mesostructured materials. The peak also appears slightly shifted towards higher angle, denoting a decrease of the interplanar spacing close to 2 nm.

To carry out a direct measurement of the textural properties of the silica network, complete removal of the enzyme and other organic species trapped in the pores is required. This was attempted by calcination at 550 °C. However, XRD characterization (Figure 5) and nitrogen adsorption-desorption isotherms (Figure S12) showed that this treatment led to a strong modification of the silica mesostructure. In the case of samples synthesized at pH 3.5, the low-angle diffraction showed a marked shift to higher angle, corresponding to a decrease of the interplanar spacing close to 3.5 nm. This implies a significant shrinkage of the silica cages, which can be attributed to an increase of the condensation degree of the silica framework during the high temperature treatment, as the synthesis conditions employed are expected to favour the formation of poorly condensed silica. For samples synthesized at pH 4.0, the calcination treatment produced the loss of the mesostructure, as shown by the lack of diffraction peak in the low-angle XRD patterns. These results suggest that samples synthesized at pH 4.0 possess a very poorly condensed silica network that collapses (at least partially) upon calcination. Therefore, aiming to minimize the modification of the silica mesostructures, we used an alternative route for the removal of organics. Thus, we carried out the extraction of the surfactant, micelles expander, and enzyme in drastic conditions (solvent extraction with ethanol, under reflux for 24 h at 78 °C, followed by filtration, washing several times with ethanol and drying at room temperature for 24 h). As shown in Figure 5, the silica samples obtained after this extraction treatment exhibit a more intense low-angle diffraction peak compared to their as-synthesized counterparts, which is consistent with the successful removal of the organic species.
and the retention of the silica mesostructure. It can be seen that the extraction treatment produces a small shift of the diffraction peak for all samples, which indicates shortenings of the $d^2$ spacing of only 0.5-1 nm.

Therefore, nitrogen adsorption-desorption isotherms of the porous silica matrices obtained after extraction of the organics were recorded to carry out a direct measurement of the size of cages that host the enzyme molecules. The isotherms (Figure 6) show that the four samples possess a large adsorption capacity, with total pore volume around 0.5 cm$^3$/g (Table 2), which evidences that the enzyme, surfactant and swelling agent have been removed from the silica hosts, and that they possess an open porous network in which the cages (observed by STEM) should possess openings that interconnect them. This is also confirmed by the high BET surface area values obtained, close to 500 m$^2$/g for samples synthesized at pH 3.5. Lower surface area values shown by samples synthesized at pH 4.0 might be attributed to a lower contribution of micropores, as suggested by the lower adsorption volume obtained at low relative pressures. The shape of the isotherms also support that the silica matrices possess a mesostructured pore network. For all samples, the isotherms show a continuous increase of the adsorbed volume as the relative pressure increases, due to the multilayer adsorption on the inner silica surface, and a steep increase at relative pressure around 0.7-0.8 that can be attributed to capillary condensation in mesopores of uniform size. At higher relative pressures, the isotherms show the adsorption of nitrogen on the outer surface of particles and interparticle spaces. The steep adsorption step is notably more pronounced for samples synthesized at pH 3.5, which evidences the existence of cages with more uniform size than samples synthesized at pH 4.0. Accordingly, the BJH pore size distributions calculated from the adsorption branch of the isotherms are narrow, especially in the case of samples synthesized at pH 3.5. These pore size distributions indicate that the silica hosts possess cages with a diameter of 9.0 nm in the case of samples synthesized in the presence of $m$-xylene, while 1,3,5-trimethylbenzene led to slightly smaller cages (Table 2). All the isotherms show a hysteresis loop with an abrupt drop of the adsorbed volume in the desorption branch at a relative pressure value $ca.$ 0.4. This is known to occur due to the forced closure of
the hysteresis loop caused by the tensile strength effect.\textsuperscript{45} Therefore, the desorption isotherms obtained show that, in all samples, evacuation of nitrogen from the cages takes place through small openings of less than ca. 3.6 nm in diameter. Taking into account the enzyme dimensions, these results indicate that, on the one hand, the size of silica cages would allow to accommodate a single enzyme molecule per cavity and, on the other hand, that the small size of the cage windows would prevent the enzyme diffusing out of the cages. Consequently, the enzyme might be acting as a template for the mesoporous structure (Scheme 1).

3.2.1.4. Enzyme leaching tests

The aim to perform in-situ encapsulation is to build a cage-window silica matrix capable of confining the enzyme inside cages, in such a way that those enzymes are not able to diffuse through the narrow windows of the support, and to ensure permanent retention of the enzyme, despite the lack of covalent bonding with the support (Scheme S6a). To validate our hypothesis and demonstrate enzyme retention, the biocatalysts were incubated in conditions which are adverse for enzyme-support interaction. These conditions were high dilution (the ratio solid/total volume was 1.25 mg biocatalyst per°mL buffer) at pH 7.0 for 48 h at room temperature. Figure 1 summarizes the time course of enzyme release from the various in-situ β-Glu biocatalysts. It can be seen that in all cases a small fraction of the immobilized enzyme is leached during the first hour of treatment, probably corresponding to molecules adsorbed on the external surface of the particles. No further release of enzyme is detected at longer incubation time meaning that those encapsulated in the silica matrix would remain retained. The levels of enzyme leaching obtained are equivalent to as low as 1% and 4-5% of the total enzyme content of biocatalysts prepared at pH 3.5 and 4.0, respectively. This efficient retention of the enzyme is due to its occlusion in cages that possess narrow windows, smaller than the enzyme molecule.

The profiles plotted in Figure 1 show that both biocatalysts prepared by adsorption through the post-synthesis approach in supports that possess wider pores exhibit a continuous leaching that is prolonged for several hours. Nevertheless, the amount of enzyme leached eventually reached a constant level close to 14% for sample LP-FDU-12-g-N and 17% for LP-SBA-15-co-
These results show that the tight confinement of the enzyme in pores that are only slightly wider than the enzyme dimensions, combined with the chemical affinity provided by surface groups, allow these supports to retain the vast majority of enzyme molecules (83 - 86%), probably limiting the leaching only to protein molecules adsorbed on the external surface and the pore regions closest to the external surface. This effect seems to be slightly enhanced in the case of the support LP-FDU-12-g-N that possesses a porous network with higher tortuosity (see Scheme S6 b) than LP-SBA-15-co-N (see Scheme S6 c).

To ensure that the enzyme has been efficiently in-situ encapsulated within the materials cavities, an indirect test to check its presence was conducted. For that, after 48 h of incubation for leaching tests and once no more enzyme was released, biocatalysts obtained by the in-situ approach were submitted to SDS-PAGE Electrophoresis (10% polyacrylamide gel electrophoresis) in denaturing conditions (with sodium dodecyl sulphate and mercaptoethanol, at boiling temperature for 10 min). After this treatment, the resulting linear peptide chain was able to easily diffuse out of the porous support as it was verified by electrophoresis assay of the supernatants. The electrophoresis results (Figure S14) show a protein band of 120 KDa for all samples (lanes 2-6) corresponding to one subunit of the homodimeric structure of β-Glu. These results confirm the presence of the enzyme inside these silica matrices.

4. CONCLUSIONS

Immobilization of hyperthermophilic β-glucosidase from Aspergillus niger was approached from different strategies using siliceous materials as supports. By means of covalent bonding attachment of the enzyme to epoxy activated supports, poor enzyme loadings and low specific activities were rendered. Additionally, when compared with the post-synthesis immobilization by adsorption, although enzymatic loadings are low, the specific activities are within the same order of magnitude as the free enzyme. Both the channel-like structure biocatalyst (LP-SBA-15-co-N), and the windows-cage structure (LP-FDU-12-g-N) together with their associated
entrance pore size around 18 – 17 nm respectively, which by the way is tightly adjusted to the enzyme size, managed to retain the enzyme within their porous silica network.

The difficulty to achieve significant yields in terms of enzyme loading regarding β-glucosidase immobilization on pre-formed supports, either via covalent or non-covalent interactions, was overcome employing enzyme in-situ encapsulation by modifying the experimental conditions. In particular, the employed strategy involved the usage of non-ionic surfactants, micelle swelling agents, salts at mild synthesis conditions (pH°3.5 and pH°4.0 and 50°C). The synthesis method employed seems to be appropriate to encapsulate β-glucosidase avoiding enzyme agglomeration, thus preserving enzyme activity. Regarding the pH parameter, by reducing the amount of acid (HCl, pH 4.0) in the synthesis media a worse structural ordering was obtained. Nevertheless, the in-situ strategy at pH 3.5 yielded the highest enzyme loading in comparison with the covalent and non-covalent post-synthesis immobilization. Specifically, IS-XYL-3.5 biocatalyst finally reached loadings close to 200 mg per gram of catalyst while retaining 56 % of the initial activity of free enzyme. Regarding its recyclability, IS-XYL-3.5 can be reused without an activity loss of at least eight times. For every in-situ biocatalyst, enzyme encapsulation inside cage-window structures fully prevented the enzyme release, achieving only less than 5% protein initially desorbed and further total absence of leaching. Consequently, leaching is prevented despite the lack of covalent bonding with the matrix.

ACKNOWLEDGEMENTS

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### TABLES

#### Table 1. β-glucosidase encapsulation yield and catalytic performance of biocatalysts obtained after 5 days of ageing at 50 °C (in-situ immobilization approach).

<table>
<thead>
<tr>
<th>Biocatalyst</th>
<th>% Encapsulated(^{[a]}) (Bradford analysis)</th>
<th>Enzyme loading(^{[b]}) (mgE/g)</th>
<th>Biocatalyst activity(^{[c]}) (U/g)</th>
<th>Specific activity(^{[d]}) (U/mgE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS-TMB-pH3.5</td>
<td>96.9</td>
<td>200</td>
<td>24.3</td>
<td>0.12</td>
</tr>
<tr>
<td>IS-TMB-pH4.0</td>
<td>36.2</td>
<td>75</td>
<td>50.8</td>
<td>0.68</td>
</tr>
<tr>
<td>IS-XYL-pH3.5</td>
<td>91.7</td>
<td>175</td>
<td>262.6</td>
<td>1.50</td>
</tr>
<tr>
<td>IS-XYL-pH4.0</td>
<td>25.4</td>
<td>49</td>
<td>31.3</td>
<td>0.64</td>
</tr>
</tbody>
</table>

\(^{[a]}\) Percentage of encapsulated enzyme relative to the total amount of enzyme initially added in the immobilization medium (determined by Bradford analysis).  
\(^{[b]}\) Milligrams of enzyme immobilized per gram of support.  
\(^{[c]}\) Activity Units per gram of support.  
\(^{[d]}\) Activity Units per milligram of enzyme calculated as Biocatalytic activity (U/g) against Enzyme loading (mgE/g). Reference activity of the free enzymatic extract: 2.7 U/mgE (C = 24.76 mgE/mL). Tested reaction: Hydrolysis of p-NPG (4-nitrophenyl-β-D-glucopyranoside).

#### Table 2. Textural properties of porous silica matrices obtained after removal of organics from in-situ encapsulated β-glucosidase biocatalysts using drastic extraction conditions (solvent extraction with ethanol, under reflux at 78 °C for 24 h, followed by vacuum filtration, washing several times with ethanol and drying at room temperature for 24 h).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Pore size(^{[a]}) (nm)</th>
<th>Pore volume(^{[b]}) (cm(^3)/g)</th>
<th>BET surface area(^{[c]}) (m(^2)/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS-TMB-3.5-Ext</td>
<td>8.5</td>
<td>0.4</td>
<td>467</td>
</tr>
<tr>
<td>IS-TMB-4.0-Ext</td>
<td>7.6</td>
<td>0.5</td>
<td>266</td>
</tr>
<tr>
<td>IS-XYL-3.5-Ext</td>
<td>9.0</td>
<td>0.6</td>
<td>491</td>
</tr>
<tr>
<td>IS-XYL-4.0-Ext</td>
<td>9.0</td>
<td>0.6</td>
<td>390</td>
</tr>
</tbody>
</table>

\(^{[a]}\) BJH Pore size estimated from the adsorption branches of the isotherms.  
\(^{[b]}\) Pore volume (cm\(^3\)/g) estimated from the amount of nitrogen adsorbed at the relative pressure of 0.97.  
\(^{[c]}\) BET specific surface area (m\(^2\)/g).
FIGURE CAPTIONS

Figure 1. Leaching of β-glucosidase from biocatalysts prepared by non-covalent post-synthesis immobilization (β-Glu immobilized in LP-SBA-15-co-N and LP-FDU-12-g-N) and in-situ encapsulation (IS-XYL-4.0, IS-XYL-3.5, IS-TMB-3.5, IS-TMB-4.0), expressed as percent of total amount of enzyme immobilized on each support. Y-axes error bars are plotted.

Figure 2. Percentage of encapsulated β-glucosidase enzyme versus synthesis time (days). Y-axes error bars are plotted.

Figure 3. Relative absorbance at 340 nm as a function of the number of reaction cycles using IS-XYL-pH3.5 biocatalyst. Vertical error bar at each data point is graphically represented.

Figure 4. Dark-field STEM micrographs (a, b and c) and SEM micrograph (d) of the sample IS-XYL-3.5.

Figure 5. Low-angle X-ray diffraction patterns of as-synthesized silica-encapsulated β-glucosidase samples and porous silica solids obtained after removal of organics by calcination or drastic extraction.

Figure 6. N₂ adsorption-desorption isotherms of porous silica matrices obtained after removal of organics from encapsulated β-glucosidase samples by drastic extraction conditions. The isotherms have been shifted vertically for clarity (IS-XYL-3.5 +200 cm³/g and IS-TMB-3.5 +120 cm³/g, respectively).

SCHEME CAPTIONS

Scheme 1. Schematic representation of hypothesis regarding in-situ encapsulation procedure: Firstly, organic micelle expanders (TMB or XYL) are put in contact with the enzymatic solution and, afterwards the hydrophobic aminoacids of the enzyme would interact with said expanders creating hydrophobic superficial regions around enzymes. Later on, surfactant hydrophobic tails would surround said enzyme molecules together with the expanders and, once the silica source is added, condensation would take place and thus, enzymes molecules encapsulation could be possible within a siliceous mesocellular foam material (MCF) with a cage-windows structure. Resultant in-situ biocatalysts: IS-XYL-x or IS-TMB-x, where x refers to pH 3.5 or pH 4.0 synthesis conditions.
Figure 1.

![Figure 1](image1.png)

Figure 2.

![Figure 2](image2.png)
Figure 3.

Figure 4.
Figure 5.

Figure 6.
REFERENCES


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