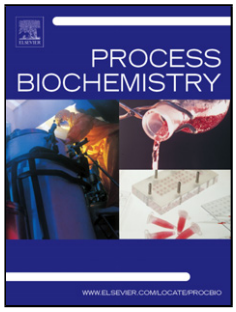

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Covalent immobilization-stabilization of β-1,4-endoxylanases from *Trichoderma reesei*: Production of xylooligosaccharides

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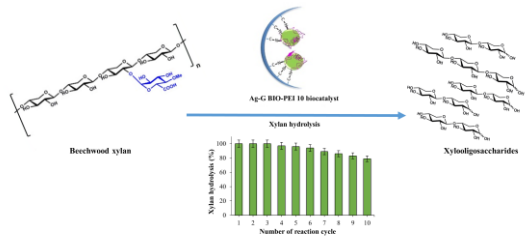
Graphical abstract



HIGHLIGHTS

- A xylanases-rich commercial cocktail, BIO, was immobilized on Ag-G.
- Ag-G BIO was 10-fold more stable than its soluble counterpart.
- Physical amination of Ag-G BIO was >100-fold more stable than the soluble form.
- Physically aminated Ag-G BIO was reused for 10 cycles of xylan hydrolysis.

ABSTRACT

The production of xylooligosaccharides (XOS) was evaluated using immobilized and stabilized biocatalysts of a commercial enzymatic cocktail, Bioxilanase L PLUS (BIO), which is based on the xylanolytic enzymes produced by *Trichoderma reesei*. BIO was immobilized by multipoint covalent attachment on agarose beads highly activated with aldehyde groups under alkaline conditions (Ag-G BIO) resulting in a highly active and stable biocatalyst. Ag-G BIO was 10-fold more stable than soluble preparation at pH 7.0 and 60 ºC. Ag-G BIO was also physically modified by surface coating with polyethyleneimine (PEI) which promotes an ionic interaction with the anionic groups of the enzyme surface. Ag-G BIO covered with a layer of PEI 10 kDa (Ag-G BIO-PEI 10) was >100-fold more stable than soluble BIO preparation. The optimal biocatalyst (Ag-G BIO-PEI 10) allowed to perform ten cycles of beechwood xylan hydrolysis reaction at high concentration (4% (w/v)) with a high conversion degree (> 80%). Moreover, Ag-G BIO-PEI 10 reached 90% of conversion in only 8 hours and so, it could be used in short reaction times, which would extend its useful life, thus allowing its application for industrial processes.

KEYWORDS: enzyme immobilization-stabilization; xylan hydrolysis; β-1,4-endoxylanases; xylooligosaccharides

1. Introduction

Xylanases (endo-1,4-β-*D*-xylanase) are enzymes that catalyze the hydrolysis of glycosidic bonds between xylose units in the hemicellulose fraction of plant cell walls. Endoxylanases hydrolyze the main chain of the polymer, while β-*D*-xylosidases act on non-reducing ends, releasing xylose [1, 2]. These enzymes are produced by different microorganisms (filamentous fungi, yeasts, and bacteria), notably those from genera *Aspergillus*, *Trichoderma*, *Fibrobacter*, *Clostridium* and *Bacillus* [3, 4]. Although, most commercial xylanase cocktails are from fungal origin (Allzyme PT, produced by *Aspergillus niger*; Multifect Xylanse, Depol D333MDP and Bioxylanase, produced by *Trichoderma reesei*).

Xylooligosaccharides (XOS) chain length can vary from 2 to 10 xylose residues linked by β-(1-4) bonds and with different substituents such as acetyl groups, uronic acids and arabinose residues among others. They are naturally present in fruits, bamboo, vegetables, bamboo, honey and milk, and can also be produced at industrial scale via chemical or enzymatic routes from xylan-rich materials, which are available widely and at low cost [3]. The enzymatic production is preferred in food industry due to the lack of undesirable side reactions and products [3]. For XOS production, xylan degradation should be partial, *i.e.*, the production of xylose should be avoided. Thus, enzyme preparations with low β-xylosidase and exo-xylanase activities are required [5, 6]. XOS are currently considered important prebiotic ingredients which can promote an increase in the number of bifidobacteria, which are among the first microbes to colonize the human gastrointestinal tract and are believed to exert positive health benefits on their host [3, 7, 8]. They are advantageous over other non-digestible

oligosaccharides regarding health as well as technologically related properties. However, the currently high cost of production indicates the necessity of further development in processing and purification technologies [3, 5].

An ideal process of enzyme immobilization may confer not only biocatalyst insolubilizing and reuse, but also an improvement of its stability at different reaction conditions. Thus, supports and immobilization techniques must be carefully evaluated considering both enzyme properties and the intended application. For this purpose, the mechanism of enzyme-support interaction, the structural characteristics, the chemical purity and the biochemical properties should be taken into account to achieve a successful immobilization of the enzyme. Glyoxyl agarose support is activated with short aliphatic aldehyde groups and presents excellent and well-known properties for enzyme immobilization [9]. The glyoxyl groups are very stable at alkaline pH, which allows for a long-term incubation between the enzyme and the activated support to promote a very intense enzyme-support multipoint covalent attachment [9]. Glyoxyl groups are capable of reversibly reacting with primary amino groups to form imines, which can be converted into irreversible bonds by reduction. Thus, secondary amines are formed, while reducing aldehydes, in order to produce inert hydroxyl groups. This immobilization protocol, consisting of using highly activated surface support at alkaline pH, promotes the formation of several simultaneous bonds per enzyme molecule [10]. After the first multipoint attachment process, it has been reported that controlling parameters such as incubation time of enzyme-support, temperature and density of reactive groups on the support surface can increase or decrease the number of interactions between enzyme and support [10]. Therefore, increasing these parameters can drive to a very intense multipoint covalent attachment enzyme-support after reduction step. The attachment of the enzymes on the support occurs through the lysine-rich region of the enzyme surface. While lysine residues are abundant on the surface of many

enzymes, others have very few of them. In these cases, amino groups can be introduced by chemical modification [12].

The strategies for enzyme stabilization also include modification of enzyme surface and the creation of a protecting external environment that allows the concomitant substrate input and product output. Chemical modification of proteins has been widely used to alter their physicochemical and biological properties [13, 14]. An alternative for further improvement on enzyme stability is adding a layer of a hydrophilic polycationic polymer such as polyethyleneimine (PEI). PEI presents a high density of primary, secondary and tertiary amino groups which create a physicochemical modification on enzyme surface by adding numerous positive charges. This fact may permit the strong ionic exchange of the polymer with any area of the protein surface containing anionic groups [15], *i.e.*, aspartic and glutamic acid residues [16-18].

The immobilization and stabilization of a xylanase-rich commercial cocktail, Bioxylanase (BIO), on glyoxyl-activated agarose is described in this paper. The surface of the immobilized enzymes was modified by chemical and physical amination. The activity and stability of the immobilized derivatives were evaluated and compared to Depol D333MDP (D333MDP). Then, the most promising immobilized biocatalyst of BIO and D333MDP was then applied to beechwood xylan hydrolysis during various cycles, and the released products were evaluated by liquid high-performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD).

2. Materials

10% cross-linked agarose beads (10 BCL) was from Agarose Bead Technologies (Madrid, Spain). Low molecular weight electrophoresis markers were purchased from GE

Healthcare (Uppsala, Sweden). Ethylenediaminetetraacetic acid (EDA), 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide (EDAC), sodium borohydride, sodium periodate, sodium hydroxide, dinitrosalicylic acid (DNS), sodium acetate, sodium chloride, sodium phosphate, glycidol, polyethyleneimine (PEI) (1,300, 10,000 and 25,000 Da) and xylan from beechwood were supplied by Sigma-Aldrich Chem. Co (St. Louis, MO, USA). Depol™ 333MDP (D333MDP) was provided as a powder by Biocatalysts (Wale, UK). Bioxilanase L Plus (BIO) was provided as a liquid preparation by Biocon (Spain). Pierce™ Coomassie (Bradford) Protein Assay Kit was purchased from Thermo Fisher Scientific (Rockford, Illinois, USA). Purified standards xylobiose, xylotriose, xylotetraose, xylopentaose, and xylohexaose were purchased from Megazyme (Wicklow, Ireland). All other reagents were analytical or HPLC grade.

3. Methods

3. 1. Preparation of enzyme solutions

One gram of D333MDP freeze-dried powder was diluted in 100 mL of a 0.005 M sodium phosphate buffer at pH 7.0. The enzyme solution was centrifuged for 1 hour at 3000 rpm (Beckman Avanti-JA.14), and the supernatant was recovered, retaining 100% of its initial activity. BIO was diluted 1:100 (v/v) in a 0.1 M sodium bicarbonate buffer pH 10.0.

3. 2. Enzyme assay and protein determination

Xylanolytic activity was determined using a suspension of 4% (w/v) beechwood xylan in 0.05 M sodium acetate buffer pH 5.0. This supension was stirred for 1 hour at 25 ºC and then centrifuged at 3750 rpm for 1 hour (Beckman Avanti-JA.14). The precipitate was removed, and

the soluble xylan fraction was used as the substrate. Xylanase activity was colorimetrically measured by the dinitrosalicylic (DNS) acid method [19], using xylose as standard.

β-Xylosidase activity from BIO and D333MDP was assayed using 5 mM p-nitrophenyl-β-D-xylopyranoside (pNPX) in 0.05 M sodium acetate buffer pH 5.0 and appropriately diluted enzyme solution or immobilized biocatalyst to 1 mL final volume [20]. At appropriate time intervals, aliquots were taken and the reaction was stopped with 1 mL of a saturated potassium tetraborate solution and the absorbance was read at 405 nm. The released p-nitrophenolate was determined by reading absorbance at 405 nm. One unit of activity was defined as the amount of enzyme required to release 1 μmol of product equivalent per min in the assay conditions at 25 °C ($\varepsilon = 17198\ M^{-1}\ cm^{-1}$). Assays were performed in triplicate.

Protein was determined using the Bradford method [21], using bovine serum albumin as standard.

3. 3. SDS-PAGE analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out following the previously described method [22]. Different preparations contained the same amount of protein. Samples were analyzed using 12% polyacrylamide gels, and proteins were detected by Coomassie blue staining.

3. 4. Preparation of glyoxyl-activated agarose

Firstly, 10 BCL agarose gel was activated by introducing diol groups. Distilled water was added to 105 g agarose to 180 ml final volume. After homogenization, 50 mL of cold 1.7 M NaOH solution was added to this suspension. Subsequently, 1.425 g BH_4Na was added to the mixture. Then, 36 mL glycidol was dropwise added in an ice-cold bath. The suspension was gently stirred for 15-18 hours at room temperature. After incubation, the mixture was

abundantly washed with distilled water. Next, 3.21 g $NaIO_4$ was dissolved in 1.5 L of distilled water. Then, the glyceryl-activated agarose was added to the $NaIO_4$ solution. After 2 hours, the support was abundantly washed with distilled water, filtered under vacuum and stored at 4 °C [11].

3. 5. Enzyme immobilization

An aliquot of the enzymatic cocktail containing 100 UI was diluted in 10 mL of 0.1 M sodium bicarbonate buffer pH 10.0. This mixture was added to 1 g of support, and the mixture was incubated at 25 ºC under mild agitation. At different time points, the xylanase activity was measured in the supernatant and suspension. Blanks (distilled water instead of enzymatic samples) were included as controls for the immobilization processes. After immobilization, the derivatives were reduced for 30 min at room temperature with 1 mg/mL sodium borohydride in the presence of a 1% (w/v) beechwood xylan aqueous solution. D333MDP was immobilized as previously described by Martins de Oliveira et al. [23]. Finally, the derivatives were vacuum-filtered, washed abundantly with water and stored at 4 °C until use. All experiments were performed in triplicate with an error lower than 5%. The immobilization yield (Y) and the expressed activity (EA) were determined as:

$$Y\ (\%) = \frac{\text{Total xylanase activity offered to the support} - \text{unbound xylanse activity}}{\text{Total xylanase activity offered to the support}} \times 100$$

$$EA\ (\%) = \frac{\text{Total xylanase activity observed on the conjugates}}{\text{Total xylanase activity offered to the support}} \times 100$$

3. 6. Physical amination of immobilized enzymes with polyethyleneimine

Immobilized enzymes were modified with PEI of different molecular weights: 1,300, 10,000 and 25,000 Da. One gram of the immobilized glyoxyl derivatives was suspended in 20

ml of a 5% (w/v) PEI solution prepared in 0.025 M phosphate buffer at pH 8.0. After 90 min of gentle stirring at room temperature, the derivatives were abundantly washed with distilled water, filtered under vacuum and stored at 4 °C.

3. 7. Chemical amination of immobilized D333MDP

The surface of the immobilized D333MDP was chemically aminated in solid-phase according to [23].

3. 8. Thermal stability of the enzymatic preparations

The thermal stability assays were evaluated under two different conditions of pH (10.0 and 7.0) and temperature (25 ºC and 60 ºC). 1 g of the immobilized derivative, of D333MDP and BIO, was suspended in 10 mL of 0.1 M of sodium bicarbonate buffer pH 10.0 or 10 mL of 0.025 M of sodium phosphate buffer pH 7.0. In all cases, suspension samples were withdrawn at different set times, and activity was measured as described above. The residual activity was calculated as the ratio between the activities over the time in relation to the initial activity. Half-life times was calculated as described previously [24].

3. 9. Xylan hydrolysis

0.1 g of each derivative from the enzymes D333MDP and BIO were added to 10 mL of 4% (w/v) beechwood xylan (prepared as described above). The reaction was allowed to stand at 50 ºC pH 5.0 under agitation of 150 rpm until reaching the maximum release of reducing sugars. After different set times, samples were withdrawn, and the release or reducing sugars was measured as described above.

3. 10. Analysis of the xylan hydrolysis products

The XOS content of samples taken at different times periods during xylan hydrolysis was analyzed by high-performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) as previously described [23]. The analysis was carried out in an ICS3000 Dionex system consisting of a SP gradient pump, an AS-HV autosampler and an electrochemical detector with a gold working electrode and Ag/AgCl as reference electrode. An anion-exchange 3 × 250 mm Carbo-Pack PA-200 column (Dionex) was used at 30 °C. The initial mobile phase was 15 mM NaOH for 12 min. Then, gradients (8 min) from 15-75 mM NaOH and from 0-80 mM sodium acetate were applied. After that, the mobile phase composition varied from 75 to 100 mM NaOH and from 80 to 320 mM sodium acetate for 10 min. The flow rate was constant at 0.5 mL/min. The peaks were analyzed using the Chromeleon Software. Identification and quantification of XOS were based on external calibration using commercial standards (degree of polymerization from 1 to 6) and the calibration curve regression coefficients that were higher than 0.99. All samples were previously filtrated through 0.45 µm nylon filters and conveniently diluted with distilled H_2O. All analyses were carried out in duplicate, and data were expressed as the mean value. Standard deviation was never higher than 5%

3. 11. Operational stability of the biocatalysts

The reuse assay of the immobilized biocatalysts was performed under the same conditions as described above. When the production of reducing sugars became constant, the immobilized biocatalysts were easily recovered by filtering and washing with distilled water. Then, fresh substrate solution was added to begin a new reaction cycle [23]. The retained

xylanase activity was calculated as the ratio between activity at the end of each cycle and activity before the first cycle (expressed as percentages).

4. Results

4. 1. Characterization of the enzymatic cocktails with xylanolytic activity

The filamentous fungus *Trichoderma reesei* (anamorph *Hypocrea jecorina*) is one of the best producers of cellulolytic and hemicellulolytic enzymes, producing all necessary enzymes for cellulose and hemicellulose degradation [25, 26]. Several commercial enzyme preparations are currently manufactured using *T. reesei* strains [27]. D333MDP (Biocatalysts) and BIO (Biocon) are commercial enzymatic cocktails with high pentosanase/hemicellulase activity based on *T. reesei* as the producer platform, having xylanase as the main enzymatic activity. A previous characterization of the enzymatic cocktails is essential in order to evaluate the differences in their protein concentration, purity, the presence of different xylanolytic enzymes, as well as their proportions in each sample. According to the suppliers, both cocktails present enzymes with an optimal pH range between 4.0-6.0 and an optimal temperature range between 50-60 ºC. In this sense, BIO presented 32.6 U/mg of specific xylanolytic activity. While the D333MDP preparation presented 5.5 U/mg of xylanolytic activity. SDS-PAGE analysis of both cocktails (Figure 1) revealed that D333MDP is mainly composed of a protein with 21 kDa. In contrast, BIO is a more complex preparation consisting of the same 21 kDa protein and also by others proteins with a molecular weight approximately of 21, 38, 43 and 65 kDa.

The stained 21 kDa protein band in both cocktails showed the same molecular weight described for Xyn II (GH11) from *T. reesei* [28]. Besides, this fungus also produces other xylanases such as Xyn III (GH10) and Xyn IV (GH30), which present a molecular weight of

38 and 43 kDa, respectively [29]. Very low β-xylosidase activity was detected in BIO and D333MDP preparations, 0.01 and 0.03 U/mg respectively. According to the manufacturer of the BIO cocktail, this also contents an accessory enzyme with α-L-arabinofuranosidase activity. The α-L-arabinofuranosyl groups of xylans are formed by α-1,3 linkages with the xylan backbone [30]. Although the amount of L-arabinose in the xylan chains is low [31], its presence in side chains restricts the activity of xylanases, particularly of GH 11 family. In contrast, it has been observed that GH10 xylanases are able to act near the substitutions, releasing XOS that can content substituents at the non-reducing terminal [31]. In this manner, the presence of α-L-arabinofuranosidase and GH 10 xylanase probably contributes to a higher specific activity of the BIO cocktail.

4. 2. Enzyme immobilization

Covalent immobilization of enzymes is a powerful tool for enzyme stabilization. Both xylanase preparations were immobilized under alkaline conditions through their lysine-rich region of the enzyme surface onto glyoxyl agarose support. Enzyme immobilization was carried out on agarose highly activated with glyoxyl groups (short aliphatic aldehyde groups) to achieve an intense multipoint covalent attachment of enzyme-support. This intense attachment may increase the rigidity of the immobilized tertiary structure of the enzymes, hence inducing a higher stability against small conformational changes caused by denaturing agents, for example, heat and organic solvents [9, 32]. Thus, a multipoint covalently immobilized enzyme might be much more stable than their soluble counterparts [33, 34].

BIO preparation was efficiently immobilized using this immobilization protocol (Ag-G BIO). We investigated the time-course of the immobilization by monitoring the enzyme activity of suspension and supernatant under these immobilization conditions. The immobilization yield

of the BIO preparation increased from 35% after 1 hour of incubation up to 87% after 5 hours of incubation. After 24 hours of incubation, the biocatalyst expressed 89% of its initial catalytic activity and the immobilization yield was 87% after the final reduction step. An increase of the incubation time under alkaline conditions led to obtain less active biocatalysts. Therefore, longer incubation times under these immobilization conditions negatively affected the expressed activity due to the increase in the number of interactions between the enzyme and the support resulting in a greater rigidification of the tertiary structure of the enzyme. This effect has been observed previously in D333MDP [23]. D333MDP preparation was immobilized on glyoxyl agarose support as previously described [23], in order to compare to BIO preparation. Thus, the D333MDP cocktail was incubated 24 hours under alkaline conditions (Ag-G D333MDP) to obtain the optimal immobilized biocatalyst (immobilization yield was 66%, and the expressed activity was 86%).

It is noteworthy that β-xylosidase activity was not detected in the immobilized biocatalysts. A possible explanation is that the enzyme responsible for β-xylosidase activity is not stable at the alkaline pH values required for immobilization on agarose activated with glyoxyl groups.

4. 3. Study of the stability of immobilized biocatalysts

Several factors can affect the enzyme activity and stability. They can undergo structural changes caused by extreme variations in the pH values or the presence of denaturing agents. According to previously reported [1, 35], most xylanases have optimum activity at pH between 5.0 and 7.0, and generally, they lose their catalytic activity at pH values far from this range. In addition, xylanase stability at high temperatures is advantageous for industrial applications,

since the enzyme reaction could be carried out at elevated temperatures, reducing the risk of microbial contamination [37].

Thus, the stability of the immobilized BIO was studied under two different conditions. First, the Ag-G BIO biocatalyst was incubated in sodium bicarbonate buffer at pH 10.0 and 25 ºC (Figure 2A). Ag-G BIO showed similar stability to Ag-G D333MDP [23] when they were incubated under these conditions. Their half-lives were approximately 50 hours, whereas the half-life of their soluble counterparts were 12 and 9 hours for D333MDP and BIO, respectively. Thus, BIO immobilized preparation was 5.5-fold more stable than the soluble preparation.

Second, the same immobilized biocatalyst was incubated at pH 7.0 and 60 ºC (Figure 2B). The soluble BIO preparation retained 14% of their initial activities after 24 hours of incubation. In contrast, Ag-G BIO retained 56% of its initial activity after 24 hours under the same conditions. Thus, the half-life of the Ag-G BIO biocatalyst was 48 hours. Under the same incubation conditions, Ag-G D333MDP was 2-fold less stable than Ag-G BIO biocatalyst.

Both immobilized preparations of BIO and D333MDP had a rapid loss of catalytic activity in the first 4 hours of the thermal inactivation. During the following hours, the inactivation of the biocatalysts proceeded much more slowly (Figure 2 and 3). These results show a biphasic mechanism of inactivation for the different immobilized biocatalysts. This result could be due to the different stabilities of the enzymes that compose both enzymatic cocktails.

4. 4. Post-immobilization modification of the immobilized biocatalysts: Physical amination of immobilized biocatalysts

PEI has been used to stabilize monomeric and multimeric enzymes [36, 37] and even monomeric proteins in solution, by preventing different effects such as aggregation, oxidations,

interaction with interfaces, and so on [38]. BIO and D333 MDP enzymatic cocktails were incubated in the presence of three PEI with different sizes (1.3, 10 and 25 kDa), at low ionic strength. Enzyme activity of BIO remained fully unaltered during this incubation. In contrast, the incubation of PEI with D333MDP had an adverse effect on the enzymatic activity. The catalytic activity decreased between 30% (PEI 1.3 kDa and 10 kDa) and 45% (PEI 25 kDa) after modification with the cationic polymer.

Next, surfaces of Ag-G BIO and Ag-G D333MDP were coated with PEI by ionic exchange and then, they were inactivated at 60 ºC and pH 7.0 (Figure 3A and 3B). On the one hand, after evaluating different PEI sizes, a coating of the Ag-G BIO surface using the 10 kDa polymer (Ag-G BIO-PEI 10) showed the greatest stability. Unmodified Ag-G BIO retained 31% of its initial activity after 90 hours of incubation, while Ag-G BIO-PEI 10 biocatalyst retained 70% under the same incubation conditions (Figure 3A). PEI is a cationic polymer formed by tertiary, secondary and primary amino groups, which promote a very strong ionic interaction with the anionic groups of the enzyme surface. The formation of a hydrophilic microenvironment surrounding the enzyme molecules and the strong attenuation of conformational changes in the areas of the protein covered by the PEI promoted by high temperatures greatly improved the stability. The positive effect of the PEI-coating on the enzyme stability has been previously described for different enzymes avoiding the inactivation by strong stirring, interactions with gas bubbles, solvents and high temperatures [36, 37]. It is noteworthy to mention that desorption of PEI from the enzyme surface can only occur under drastic conditions such as high ionic strength and very low pH values.

On the other hand, the addition of a PEI layer to the Ag-G D333MDP biocatalyst surface led to a destabilization of this conjugate. This negative effect was independent of the size of the polymer used. As shown in Figure 3B, Ag-G D333MDP coated with PEI 1.3 kDa, 10 kDa and

25 kDa retained 29, 26 and 19% of activity after 4 hours of incubation at pH 7.0 and 60 ºC, respectively. In this particular case, the strong ionic interaction between the large cationic polymer with the anionic groups of the enzyme surface could promote the destabilization of the regions covered by the polymer. In contrast, the chemically aminated Ag-G D333MDP biocatalyst (Ag-G D333MDP AMN) has been shown to be more stable and active than the physically aminated catalyst retaining 40% of its initial activity after 90 hours under the same incubation conditions [23].

The different properties presented by the D333MDP and BIO biocatalysts regarding stability and activity highlight the different composition of the two enzymatic cocktails. Thus, physically coating with PEI had an adverse effect on the thermal stability of rigidified D333MDP (mainly composed of Xyn II from *T. reesei*) enzyme molecules by multipoint covalent immobilization. In contrast, chemically aminated Ag-G D333MDP was between 45 and 90-fold more stable than the same physically aminated modified biocatalyst. On the contrary, physically coating of rigidified BIO enzymes (a more complex cocktail) by multipoint covalent immobilization with PEI had a positive effect on the enzyme stability and activity.

4. 5. Xylan hydrolysis reaction and reuse of the immobilized biocatalysts

The optimal immobilized biocatalysts of BIO (Ag-G BIO-PEI 10) was evaluated for their capability to release reducing sugars from beechwood xylan hydrolysis reaction at 50 ºC and pH 5.0. Figure 4 shows the results of the kinetic of beechwood xylan hydrolysis performed by Ag-G BIO-PEI 10 and Ag-G BIO. After 2 hours of hydrolysis, Ag-G BIO-PEI 10 and Ag-G BIO reached the release of 54% and 52% of reducing sugars, respectively. Compared to the optimal biocatalyst of D333MDP (Ag-G D333MDP AMN) reached 36% of conversion at the same time [23]. Biocatalyst Ag-G BIO-PEI 10 hydrolyzed the substrate more efficiently than

the unmodified Ag-G BIO and Ag-G D333MDP AMN, obtaining 100% of hydrolysis after only 12 hours. While, Ag-G BIO and Ag-G D333MDP AMN required 15 and 24 hours to achieve 100% of conversion, respectively.

The composition of the XOS mixture was analyzed by HPAEC at the end of the xylan hydrolysis reaction (Table 1). Both cocktails seem to be composed mostly of endoxylanases, since the release of xylose was minimal (5%). The product composition after 100% of xylan hydrolysis consisted mainly in xylobiose (90%) with both immobilized biocatalysts, although Ag-G BIO PEI-10 reached complete conversion in half of the time compared to Ag-G D333MDP AMN.

In order to evaluate the immobilized biocatalysts for their industrial application, they were used for several reaction cycles of beechwood xylan hydrolysis at pH 5.0 and 50 ºC. Taking into account the results described above, we set 12 and 24 hours as the duration of each cycle for Ag-G BIO-PEI 10 and Ag-G D333MDP AMN, respectively. After 10 reaction cycles (hydrolysis-washing-hydrolysis sequential steps), Ag-G BIO PEI 10 achieved 80% of hydrolysis (Figure 5). Over six reaction cycles, Ag-G Bio-PEI 10 reached more than 90% of the maximum reducing sugars release. On the contrary, after 3 reaction cycles, the Ag-G D333MDP AMN biocatalyst reached more than 90% of the release of reducing sugars. This degree of xylan conversion into xylobiose is very interesting in terms of applicability at industrial scale due to its importance in different sectors but mainly in the food industry [3, 5, 8]. The lower conversion rate and the lower thermal stability of the immobilized and aminated D333MDP compared to Ag-G BIO-PEI 10 limit the applicability of this biocatalyst for XOS production. Therefore, the physicochemical modification with PEI 10 kDa of the Ag-G BIO led to obtain a more robust biocatalyst, allowing to perform at least 10 reaction cycles with high

conversion degrees (>80%). Furthermore, 90% of conversion was reached in only 8 hours, so this biocatalyst could be used in very short reaction times, which would extend its useful life.

5. Conclusions

The immobilization techniques are considered interesting alternatives to overcome important obstacles for the application in industrial processes such as stability and reuse of the biocatalyst. The immobilization of BIO preparation by multipoint covalent attachment on agarose highly activated with aldehyde groups yielded highly active and stable biocatalyst. Surfaces of Ag-G BIO and Ag-G D333MDP were also physically coated with PEI. The addition of a PEI layer did not improve the stability of Ag-G D333MDP. Results point out that Ag-G BIO covered with a layer of PEI was >100-fold more stable than the soluble preparation. This result reveals the different characteristics of the enzymes included in the xylanolytic cocktails from *T. reesei*. Thus, the physical amination with a 10 KDa PEI polymer of the Ag-G BIO led to obtain a more stable and active biocatalyst, allowing to perform 10 reaction cycles with high conversion degrees (> 80%). On the contrary, the Ag-G D333MDP AMN biocatalyst reached more than 50% release of reducing sugars after 10 reaction cycles. Moreover, Ag-G BIO-PEI 10 could be used in short reaction times, which would extend its useful life, because 90% of conversion was reached in only 8 hours of reaction. Finally, this improvement of the enzyme properties will allow us to optimize the enzymatic hydrolysis of xylan from different sources and further studies on a larger scale.

Declaration of interest

The authors declare not having any conflict of interest.

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Figure captions

Figure 1. SDS–PAGE analysis of the D333MDP and BIO enzymatic cocktails. Lane 1: molecular weight protein standards were α-lactalbumin (14.4 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), phosphorylase b (97 kDa); Lane 2: Soluble D333MDP (20 µg); Lane 3: soluble BIO (10 µg).

Figure 2. Stability of the xylanases from different BIO preparations at pH 10 and 25 ºC **(A)** and pH 7.0 and 60ºC **(B)**. Symbols: soluble BIO (▲) and Ag-G BIO (×). BIO preparations were diluted/suspended in 0.025 M sodium phosphate pH 7.0 or 0.1 M sodium bicarbonate buffer pH 10.0 in aproportion 1:10 (w/v).

Figure 3. (A) Effect of physical amination on the stability of the immobilized BIO at pH 7.0 and 60 ºC. Symbols: Ag-G BIO (Δ); Ag-G BIO coated with PEI 1.3 kDa (∗); Ag-G BIO coated with PEI 10 kDa (◊); and Ag-G BIO coated with PEI 25 kDa (+). **(B)** Effect of physical amination on the stability of the immobilized D333MDP at pH 7.0 and 60 ºC. Symbols: Ag-G D333MDP (□); Ag-G D333MDP coated with PEI 1.3 kDa (-); Ag-G D333MDP coated with PEI 10 kDa (○); and Ag-G D333MDP coated with PEI 25 kDa (●). The different biocatalysts were suspended in 0.025 M sodium phosphate buffer pH 7.0 in proportion 1:10 (w/v).

Figure 4. Kinetic of beechwood xylan hydrolysis by immobilized BIO biocatalysts. Hydrolysis reactions were performed using 0.1 g of immobilized biocatalyst in sodium acetate buffer containing 4% (w/v) of solubilized beechwood xylan. Reaction conditions were pH 5.0, 50 ºC and 150 rpm. Symbols: Ag-G BIO (×) and Ag-G BIO-PEI 10 (◊).

Figure 5. Operational stability of Ag-G BIO-PEI 10 (filled bars) and Ag-G D333MDP AMN (unfilled bars) biocatalysts. Each hydrolysis cycle reaction was performed using 0.1 g of immobilized biocatalyst in sodium acetate buffer containing 4% (w/v) of solubilized beechwood xylan. Reaction conditions were pH 5.0, 50 ºC and 150 rpm.

Figure 1

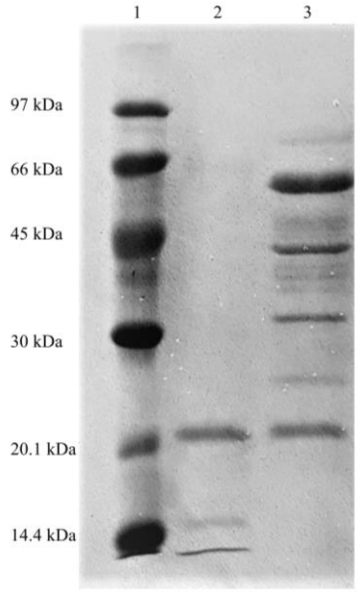


Figure 2

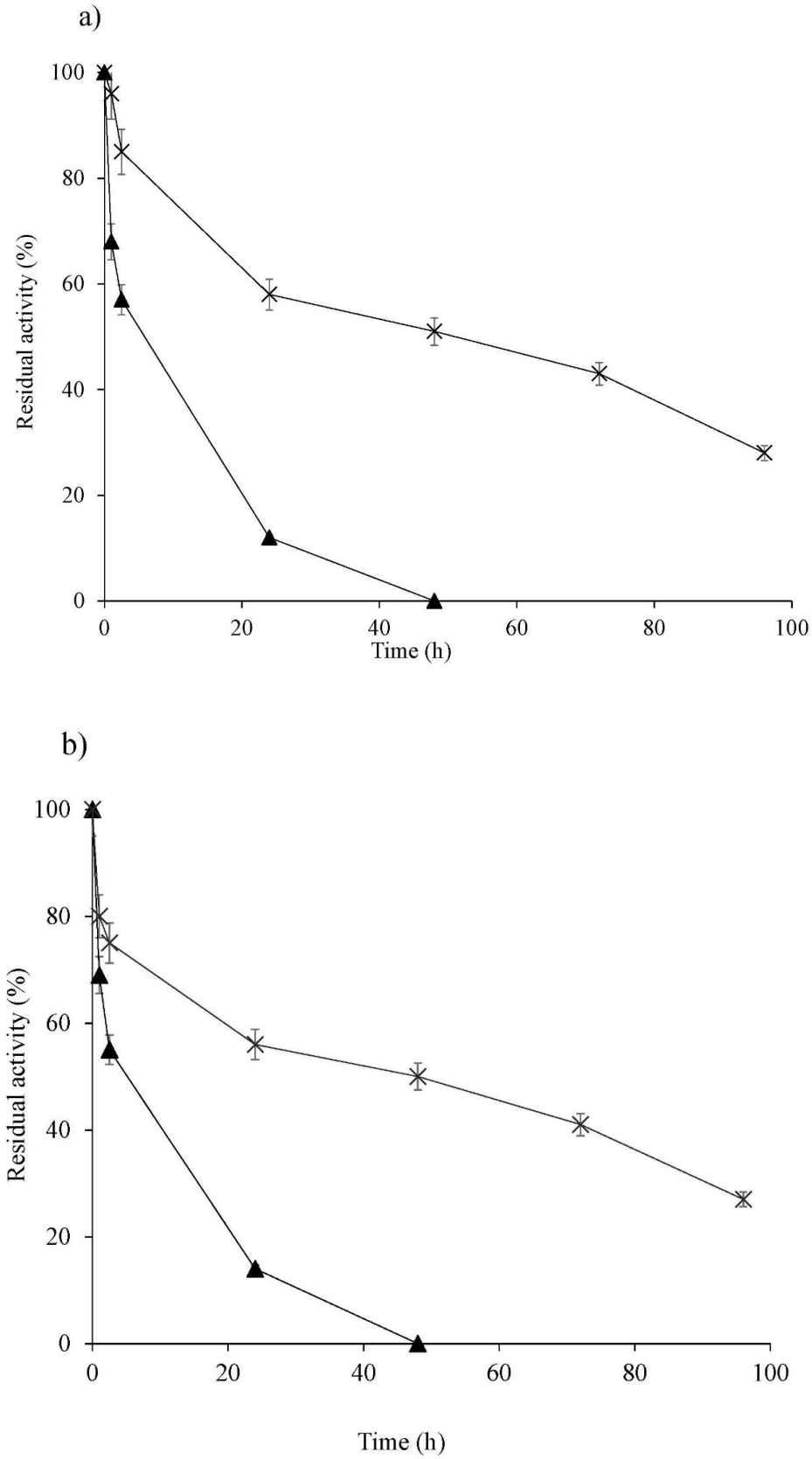
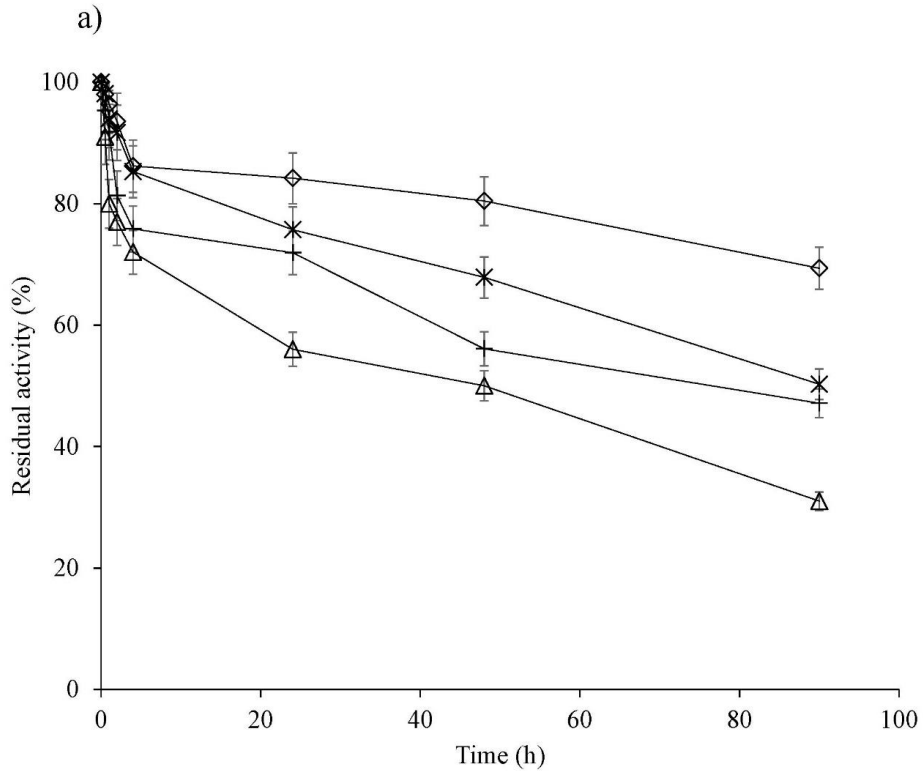
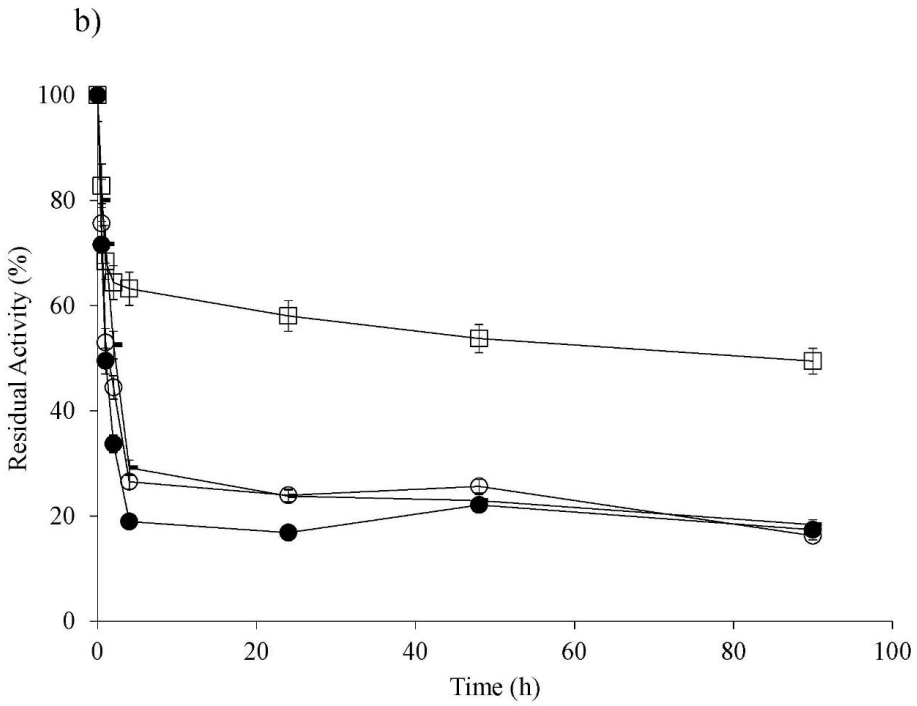


Figure 3





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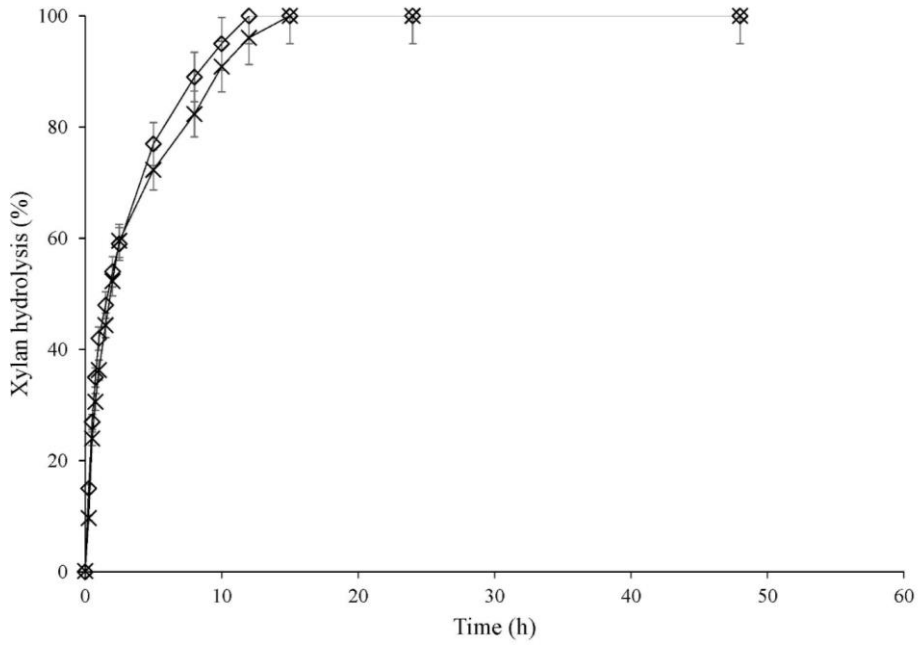

Figure 5

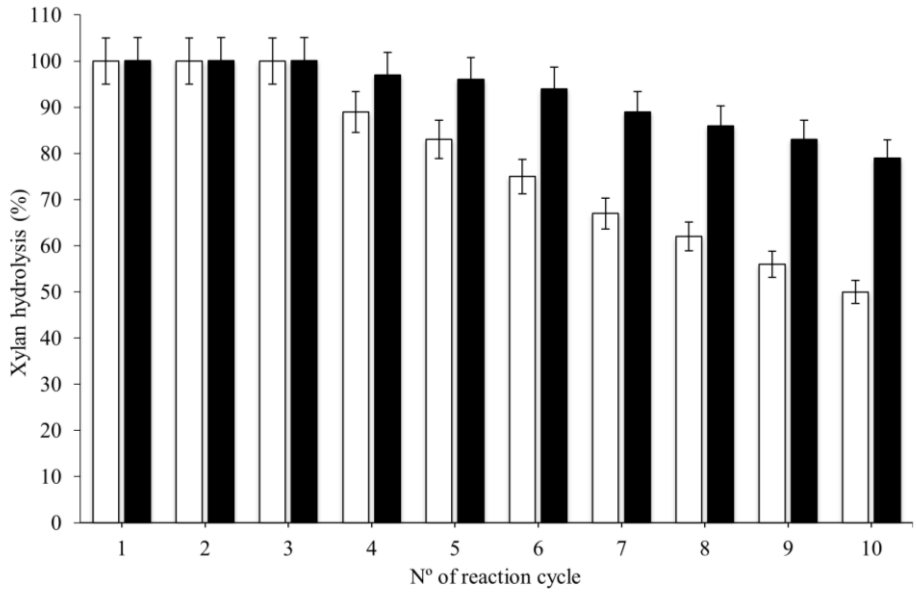

Table 1. Composition of the XOS mixture obtained from beechwood xylan hydrolysis using immobilized BIO and D333MDP biocatalysts.

Biocatalyst	Reaction time (h)	Xylooligosaccharide (%)		
		Xylose	Xylobiose	Xylotriose
Ag-G BIO-PEI 10	12	4	90	6
Ag-G D333MDP AMN [a]	24	5	90	5

Analysis was performed at the end of the hydrolysis reaction by HPAEC-PAD. Each hydrolysis cycle was carried out at 150 rpm, pH 5.0 and 50 ºC. [a] Results obtained from [23].