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RESEARCH ARTICLE

β-xylosidase from *Selenomonas ruminantium*: Immobilization, stabilization, and application for xylooligosaccharide hydrolysis

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Abstract

The tetrameric β-xylosidase from *Selenomonas ruminantium* is very stable in alkaline pH allowing it to easily immobilize by multipoint covalent attachments on highly activated glyoxyl agarose gels. Initial immobilization resulted only in slight stabilization in relation to the free enzyme, since involvement of all subunits was not achieved. Coating the catalyst with aldehyde-dextran or polyethylenimine, fully stabilized the quaternary structure of the enzyme rendering much more stabilization to the biocatalyst. The catalyst coated with polyethylenimine of molecular weight 1300 is the most stable one exhibiting an interesting half-life of more than 10 days at pH 5.0 and 50 °C, being, therefore, 240-fold more stable than free enzyme. Optimum activity was observed in the pH range 4.0–6.0 and at 55 °C. The catalyst retained its side activity against *p*-nitrophenyl α-L-arabinofuranoside and it was inhibited by xylose and glucose. Kinetic parameters with *p*-nitrophenyl β-D-xylopyranoside as substrate were V_{max} 0.20 μmol.min^{-1} mg prot.$^{-1}$, K_m 0.45 mM, K_{cat} 0.82 s^{-1}, and K_{cat}/K_m 1.82 s^{-1} mM^{-1}. Xylose release was observed from the hydrolysis of xylooligosaccharides with a decrease in the rate of xylose release by increasing substrate chain-length. Due to the high thermostability and the complete stability after five reuse cycles, the applicability of this biocatalyst in biotechnological processes, such as for the degradation of lignocellulosic biomass, is highly increased.

Keywords: β-*xylosidase*, Selenomonas ruminantium, *enzyme immobilization*, *enzyme stabilization*, *xylooligosaccharide hydrolysis*

Introduction

Xylan is the second most abundant biopolymer in the plant cell walls and the main hemicellulosic polysaccharide. It is composed of a β(1 → 4)-D-xylopyranose backbone substituted, at varying degrees, by side chain residues as glucopyranosyl, 4-*O*-methyl-D-glucuronopyranosyl, α-L-arabinofuranosyl, acetyl, feruloyl, and *p*-coumaroyl (Saha 2003). The complete hydrolysis of xylan requires the synergistic action of various enzymes, known as a xylanolytic system (Biely 1985). Endo-β-1,4-xylanase (E.C. 3.2.1.8) and β-D-xylosidase (E.C. 3.2.1.37) are the main enzymes in the degradation of the polymer. Xylanases cleave internal β(1 → 4) bonds in the xylan backbone liberating xylooligosaccharides, and β-xylosidases are exoglycosidases that remove xylose from the non-reducing ends of these xylooligosaccharides.

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Finally, α-L-arabinofuranosidases, acetyl xylan- and ferulic or *p*-coumaric acid esterases are enzymes responsible for removing side chains of the polymer (Collins et al. 2005).

The interest and application of xylan degrading enzymes in different industries have increased over the last decades. β-xylosidase is used on industrial scale included in xylanases cocktails for the bioconversion of lignocellulosic material to sugars and other useful substances, clarification of juices and wines, biobleaching in the pulp and paper industries and in processing of fabrics. β-xylosidases are critical for the systems since they carry the greatest work load in terms of number of glycosidic bonds cleaved, as well as in relieving product inhibition of xylanases (Jordan and Wagschal 2010) and cellulases (Zhang and Viikari 2012). Thus, its performance is of primary importance especially when these enzymes are applied together in processes aiming the complete saccharification of lignocellulosic biomass.

The β-xylosidase from the ruminal anaerobic bacterium *Selenomonas ruminantium* is a very well characterized enzyme, corresponding to a bifunctional β-D-xylosidase/α-L-arabinofuranosidase glycoside hydrolase belonging to family 43 (GH 43) and structural clan F in the CAZy database. The enzyme is a homotetramer in solution, with MW of approximately 60 kDa of each subunit. It shows a p*I* of 4.4, pH stability in the range from 4.3 to 10.0 and stability up to 50 °C in pH 5.3 for 1 h (Jordan et al. 2007b; Jordan 2008; Jordan and Wagschal 2010).

Currently, the main concern is the rational use of β-xylosidases for the complete hydrolysis of xylooligosaccharides to the final product, D-xylose. Enzyme immobilization poses as a possibility to improve the characteristics of an enzyme in terms of stability, stabilization, and catalysis, as well as for processes improvement since it allows the reuse of the biocatalyst for many operational cycles (Mateo et al. 2007). Many β-xylosidases have been produced and characterized (Knob et al. 2010) and some studies have evaluated covalent immobilization of these enzymes as that from *Talaromyces thermophilus* (Guerfali et al. 2009) immobilized on chitosan or that from *Trichoderma reesei* QM 9414 immobilized on nylon powder (Dueñas and Estrada 1999). Nevertheless, to our knowledge, there is no report about the immobilization of β-xylosidases by multipoint covalent immobilization using agarose gels activated with glyoxyl groups as support.

The aim of this study was to immobilize the β-xylosidase from *Selenomonas ruminantium* by multipoint covalent immobilization, evaluate some physicochemical properties of the immobilized enzyme and also verify its performance on the hydrolysis of xylooligosaccharides.

Materials and methods

Materials

Agarose 4BCL was purchased from Agarose Bead Technologies (Madrid, Spain). *p*-nitrophenyl β-D-xylopyranoside (*p*NPX), *p*-nitrophenyl α-L-arabinofuranoside (*p*NPAra), xylose, glycidol, potassium tetraborate tetrahydrate, sodium borohydride, sodium periodate, ethanolamine, *Leuconostocc* spp dextran (MW 1500–70,000), polyethylenimine (PEI, MW 1300–1×10^6) and molecular weight standard proteins were obtained from Sigma-Aldrich Co. (St. Louis, MO). Cyanogen bromide (CNBr) Sepharose 4B and low molecular weight standard proteins were purchased from GE Healthcare (Uppsala, Sweden). *Selenomonas ruminantium* β-xylosidase (Lot 100301a) and standards xylooligosaccharides (DP 2-6) were purchased from Megazyme (Wicklow, Ireland). All reagents were of analytical grade. Xylobiose and xylooligosaccharides (xylotriose and larger than six residues xylooligosaccharides) from beechwood xylan (Sigma-Aldrich Co., St. Louis, MO) and from *Cannabis* spp xylan (obtained locally from textile industry) were prepared in our laboratory by enzymatic hydrolysis with the purified xylanase from *Humicola brevis* var. *thermoidea* (Masui et al. 2012).

Methods

All results represent the average of three experiments. The experimental error was lower than 5%.

Enzyme assay and protein determination

Standard β-xylosidase activity assays were carried out in a reaction mixture containing 5 mM *p*NPX in 0.05 M sodium acetate buffer pH 5.0 and appropriately diluted enzyme solution or catalyst suspension to 1 ml final volume. The reaction was stopped with 1 ml of a saturated potassium tetraborate solution and the absorbance was read at 405 nm (Kersters-Hilderson et al. 1982). 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 ($\varepsilon = 18{,}300\ M^{-1}\ cm^{-1}$).

Protein concentration was determined with the modified Bradford's method, with bovine serum albumin as standard (Sedmak and Grossberg 1977).

Preparation of glyoxyl-agarose support

A mass of 105 g of 4% cross-linked agarose beads was suspended in water to a final volume of 180 ml (0.7 g of agarose is roughly equivalent to 1 ml).

After mild homogenization, 50 ml of 1.7 M NaOH containing 1.425 g of $NaBH_4$ was slowly added. In an ice bath, 36 ml of glycidol was added drop-wise and the mixture was then gently stirred at room temperature for 18 h. After this period, the gel was finally washed with an excess of distilled water. Then, the wet glyceryl-activated gel was oxidized with aqueous $NaIO_4$ solution under very gentle stirring. After 2 h, the gel was washed with distilled water, vacuum dried (maintaining the pores of agarose gels filled with water) and stored at 4 °C (Guisán 1988).

Preparation of aldehyde-dextran

Leuconostoc spp dextran (MW 1500–70,000) was fully oxidized by adding 4.36 g of sodium periodate to 50 ml of an aqueous suspension containing 1.67 g dextran. The suspension was gently agitated by 2 h and then dialyzed (4×) against distilled water.

Stability of soluble enzyme at pH 10.0

The enzyme was 400-fold diluted in 0.1 M sodium bicarbonate buffer pH 10.0. The solution was incubated at 25 °C, and aliquots were withdrawn at different intervals and the activity was assayed as previously described.

Enzyme immobilization

Immobilization on glyoxyl agarose: the *S. ruminantium* β-xylosidase was 400-fold diluted in 10 ml of 0.1 M sodium bicarbonate buffer pH 10.0 at 4 °C. The solution (0.31 g prot./l) was added to 1 g of glyoxyl agarose support, and the suspension was gently stirred at 25 °C. The period of immobilization was determined after carrying out this procedure by 0.5 and 14 h. Both supernatant and suspension samples were withdrawn periodically and the enzyme activity was measured. After these periods, catalysts were reduced by adding 10 mg of sodium borohydride for 30 min, then washed with water and filtered under vacuum.

Dextran-coated catalysts: a mass of 0.5 g of the uncoated catalyst (immobilization carried out by 0.5 h, as described above) was added to 5 ml of aldehyde-dextran suspensions, pH adjusted to 7.0. Dextrans with MW of 1500, 6000, 25,000, and 75,000 Da were evaluated. The suspensions were gently agitated overnight, then 5 ml of 0.1 M sodium bicarbonate buffer pH 10.0 was added and the catalysts were reduced by adding 10 mg of sodium borohydride for 30 min, washed abundantly with water and filtered under vacuum.

PEI-coated catalysts: a mass of 0.5 g of the uncoated catalyst (immobilization carried out by 0.5 h, as described above) was added to 5 ml of 5% (w/v) PEI solutions, pH adjusted to 7.0. PEI with MW of 1300, 25,000, 75,000, and 600,000–1×10^6 Da was evaluated. The suspensions were gently agitated overnight, washed abundantly with water and filtered under vacuum.

Immobilization on CNBr-activated Sepharose: it was performed in very mild conditions by using a 100× diluted enzyme solution in 0.1 M sodium phosphate buffer pH 7.0 at 4 °C. After 15 min, the catalyst was filtered and suspended in 10 ml of a 1 M ethanolamine solution pH 8.0 for 2 h to block any remaining reactive group, according to the supplier.

The immobilization yield was defined as the ratio between the activities (or protein) in the supernatant compared with the activity (or protein) in the control. Expressed activity was defined as the ratio of the activity in the final suspension after the immobilization process and the initial activity of offered enzyme.

Optimum pH and temperature

Optimum pH was determined by assaying β-xylosidase activity at 25 °C at various pH in the range from 4.0 to 9.0. The following buffers were utilized: 0.05 M sodium acetate buffer pH 4.0 and 5.0, 0.05 M sodium phosphate buffer pH 6.0 and 7.0, and 0.05 M Tris HCl buffer pH 8.0 and 9.0.

Optimum temperature was determined by assaying β-xylosidase activity at temperatures ranging from 35 to 60 °C, with 5 °C intervals, in 0.05 M sodium acetate buffer pH 5.0.

Thermal stability

A mass of 0.1 g of each catalyst was suspended in 1.0 ml of 0.05 M acetate buffer pH 5.0 and incubated at 50 and 55 °C. In all cases, samples of the suspension were withdrawn at several intervals and the activity was assayed as previously described. Residual activity was calculated as the ratio between activity at a given time and the activity in the beginning of incubation.

pH stability

A mass of 0.1 g of each catalyst was suspended in 1.0 ml of different buffers to compose the pH range from 4.0 to 9.0. The following buffers were utilized: 0.05 M acetate buffer for pH 4.0 and 5.0, 0.05 M phosphate buffer for pH 6.0 and 7.0, and 0.05 M Tris HCl buffer pH and 8.0 and 9.0. The suspension was incubated at 50 °C. Samples were withdrawn at several intervals and the activity was assayed as

previously described. Residual activity was calculated as the ratio between activity at a given time and the activity in the beginning of incubation.

Specificity and kinetics

The specificity of the catalyst was verified by assaying the activity against 0.04 M *p*NPAra in 0.05 M sodium acetate buffer pH 5.0 at 25 °C. The results were expressed in relation to the activity on *p*NPX. Kinetics of the free and immobilized enzyme was carried out by assaying β-xylosidase activity with *p*NPX (0.1–3 mM) in 0.1 M succinate-NaOH buffer pH 5.3 at 25 °C. Maximum velocity (V_{max}) and Michaelis–Menten constant (K_m) were calculated with MMfit version 1.3.0 (2013, J.P.G. Malthouse). Protein concentration of 31 g l^{-1} and 0.95 mg g biocatalyst^{-1} for the free and immobilized enzyme, respectively, was used to calculate K_{cat}.

SDS-PAGE

SDS-PAGE was performed in 8% (w/v) gels (Laemmli 1970). The resolved protein bands were visualized after staining with 0.1% (w/v) Coomassie brilliant blue R-250 dissolved in methanol, acetic acid, and distilled water (4:1:5, v/v/v). Standard proteins were phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

Hydrolysis of xylooligosaccharides and analysis of the products

Solutions of xylotetraose, xylopentaose, xylotetrose, xylopentose, and xylohexaose (0.5 g/l) were prepared in 5 mM sodium acetate buffer pH 5.0. The PEI-coated glyoxyl catalyst was added (50 mg to 5 ml reaction medium) and hydrolysis of each substrate was carried out at 50 °C, under magnetic stirring (300 rpm), and samples were taken at several interval times.

The hydrolysis of beechwood and *Cannabis* spp xylans was carried out by *Humicola brevis* var. *thermoidea* xylanase immobilized on glyoxyl agarose. After 3 days, the medium was filtered and the supernatant containing xylobiose, xylotriose, xylooligosaccharides with DP >6, and non-hydrolyzed xylan (unpublished data) was submitted to a second enzymatic hydrolysis with *S. ruminantium* immobilized β-xylosidase (PEI-coated glyoxyl catalyst). The hydrolysis was carried out for 120 min in 0.05 M sodium acetate buffer pH 5.0 (200 mg catalyst to 10 ml reaction) at 25 °C.

Analysis of hydrolysis products by high-performance liquid chromatography

High-performance liquid chromatography (HPLC) was coupled with low-temperature evaporative light-scattering detection LT-ELSD using a detector Sedex75 (Sedere, Orleans, France). Analyses were carried out at 80 °C on a Rezec R50-oligosaccharide Ag^+ 4% column (200 × 10 mm) (Phenomenex, Torrance, CA). The elution was carried out with Milli-Q ultrapure apyrogenic water at a 0.3 ml/min flow rate using a TSP P100 pump and AS3000 auto sampler Spectra Series (Thermo Electron Co., San Jose, CA). Data acquisition and processing was performed using the Chromquest 4.1 software (Thermo Electron Co., San Jose, CA). The quantification of products was based on an external calibration using standard xylose, xylobiose, and XOS solutions (DP 3-6). Samples of each hydrolyzed substrate were previously centrifuged (10,000×*g*, 20 min), filtered (0.22 μm filters) and the adequately diluted supernatant was analyzed. Analyses were carried out in duplicate and data were expressed as mean value.

Reuse of the catalyst and storage

Reuse was verified by suspending the PEI-coated glyoxyl catalyst plus inert agarose in 3 mM *p*NPX (in 0.05 M sodium acetate buffer at pH 5.0) at the proportion 1:49:100 (w/w/v). Successive batch hydrolysis cycles were carried out for 1 h at 25 °C under magnetic stirring (300 rpm). After each cycle, the catalyst was washed three times with 0.05 M sodium acetate buffer pH 5.0, filtered under vacuum and re-suspended in the same buffer (1:10, w/v) to measure residual activity. Then it was filtered under vacuum again and new substrate was added for the following cycle. In order to verify the stability after long-term storage, the catalyst was maintained in sealed flasks at 4 °C. After 6 months, the catalyst was re-suspended in 0.05 M sodium acetate buffer pH 5.0 and the residual activity was assayed.

Results

Immobilization

The soluble β-xylosidase from *Selenomonas ruminantium* was very stable in pH 10.0, maintaining 100% of its activity up to 3 h-incubation in this condition. After 20 h, 57% of the activity was still observed (not shown). The high stability allowed the enzyme to be submitted to multipoint covalent immobilization at this pH, an ideal condition for the immobilization on glyoxyl. Initially, a catalyst was prepared by carrying

out immobilization for 0.5 h. No activity was detected in the final supernatant and the process presented 71.4% yield based on initial activity. Despite the 100% protein immobilization observed after this period, a second catalyst was prepared by carrying out immobilization during 15 h.

In a third stage, the catalyst prepared for 0.5 h immobilization was incubated with polymers, i.e., different MW dextran and PEI in order to coat the enzyme surface. The coating procedure and/or the second sodium borohydride reduction (dextran coated catalysts) caused some loss in the activity and the coated catalysts maintained 65% of the activity observed in the non-coated catalyst.

Soluble enzyme was the first control and the CNBr catalyst, corresponding to a one point immobilized enzyme, was a second control.

Catalysts characterization

Despite the possibility to obtain higher stability, the catalyst prepared with 15 h immobilization was less stable than that prepared by 0.5 h immobilization (Figure 1). In fact, the half-life of this catalyst (0.5h) was even lower than those observed for the free enzyme (1 h) and the CNBr catalyst (2 h) at 50 °C. At this temperature, the glyoxyl catalyst prepared by 0.5 h immobilization presented half-life of 7 h, and thus it was 7- and 3.5-fold stabilized in relation to the free enzyme and the CNBr catalyst, respectively. Due to the considerable stabilization obtained, this catalyst was chosen for the subsequent experiments.

The stability of the dextran-coated catalysts at 50 °C (Figure 2) was indirectly related to the MW of dextran, i.e., the lowest thermostability was observed for the catalyst coated with MW 70,000 dextran, presenting half-life lower than 30 min, while the highest thermostability was observed with MW 1500 dextran. This catalyst retained more than 50% of the activity after 5 days, indicating low MW dextrans to be better in adequately coating the enzyme subunits. Intermediate half-life values, between 1 and 2 h, were observed for the catalysts coated with MW 6000 and 25,000 dextrans.

The PEI-coated catalysts were all highly stabilized. The stabilization with PEI was similar to that obtained with the catalyst coated with MW 1500 dextran, although the size of the polymer had low influence on enzyme thermostability (Figure 3). Catalysts coated with PEI MW 1300 and 25,000 were more stable maintaining around 60% of the activity while the others maintained around 50% after 5 days. If considering these two latter catalysts maintained around 50% of the activity after 5 days of incubation at 50 °C, the enzyme was therefore 120- and 60-fold stabilized in relation to the free enzyme and the CNBr catalyst, respectively. The stabilization of the catalyst coated with PEI MW 1300 was much higher (more than 240-fold in relation to the free enzyme) since 52% of the activity was observed even after 10 days of incubation.

Comparing the stability at 55 °C, the catalyst coated with dextran MW 1500 was sparingly stable, while the catalyst coated with PEI MW 1300 presented half-life of 40 min (not shown). At this temperature and in different pH, the highest

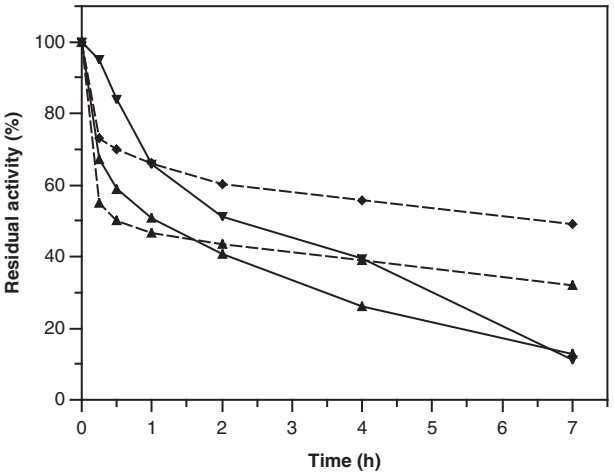

Figure 1. Thermostability of the free β-xylosidase from *S. ruminantium* and its catalysts. The incubation was carried out in 0.05 M sodium acetate buffer pH 5.0 at 50 °C and the activity was assayed in the same buffer at 25 °C. Dash lines: β-xylosidase activity of glyoxyl catalysts with immobilization carried out by 0.5 h (♦) and 15 h (▲). Solid lines: β-xylosidase activity of free enzyme (▲) and CNBr catalyst (▼).

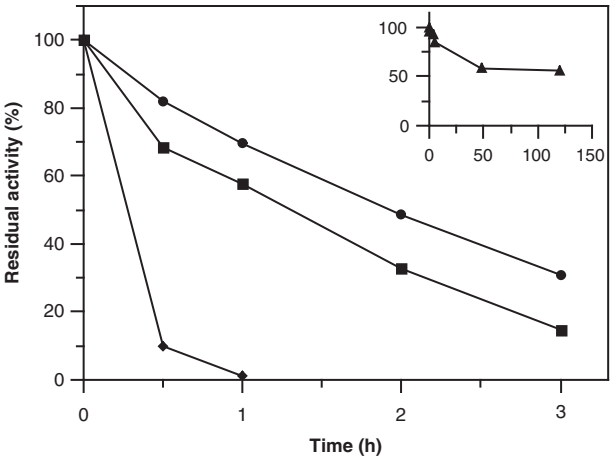

Figure 2. Thermostability of the dextran-coated glyoxyl catalysts of the β-xylosidase from *S. ruminantium*. The incubation was carried out in 0.05 M sodium acetate buffer pH 5.0 at 50 °C, and the activity was assayed in the same buffer at 25 °C. β-xylosidase activity of catalysts coated with dextrans of MW 1500 (▲, in detail), 6000 (●), 25,000 (■) and 70,000 (♦).

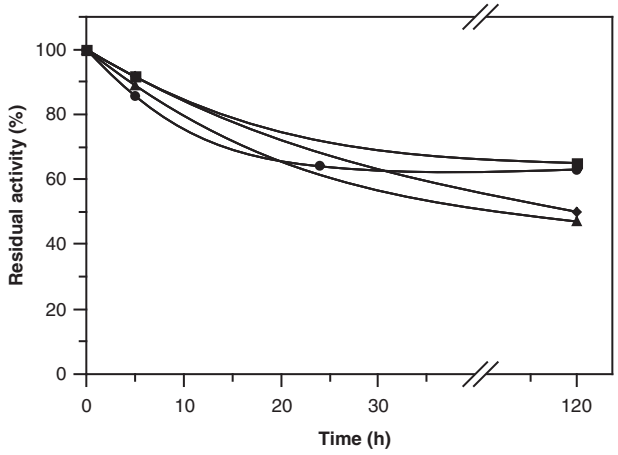

Figure 3. Thermostability of the PEI-coated glyoxyl catalyst of the β-xylosidase from *S. ruminantium*. The incubation was carried out in 0.05 M sodium acetate buffer pH 5.0 at 50 °C, and the activity was assayed in the same buffer at 25 °C. β-xylosidase activity of catalysts coated with PEI MW 1300 (●), 25,000 (■), 75,000 (♦), and 600,000–1 × 10^6 (▲).

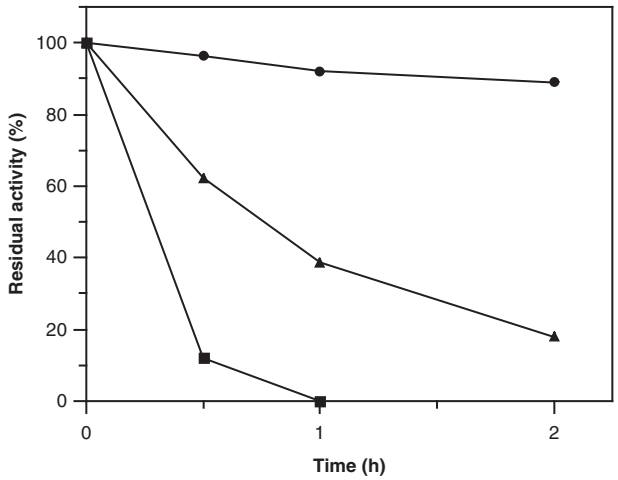

Figure 4. Stability at different pH values of the PEI-coated glyoxyl catalyst of the β-xylosidase from *S. ruminantium*. The incubation was carried out at 55 °C in 0.05 M sodium acetate buffer pH 4.0 (●) and 5.0 (▲), and 0.05 M sodium phosphate buffer pH 6.0 (■). The activity was assayed in 0.05 M sodium acetate buffer pH 5.0 at 25 °C.

thermostability of the PEI-coated glyoxyl catalyst was verified in pH 4.0, i.e. after 2 h the remaining activity still was around 90%. The lowest stability was verified in pH 6.0, while intermediate half-life value was observed in pH 5.0 (Figure 4).

When the catalysts were incubated under denaturing conditions, it can be observed that the enzyme was partially covalently immobilized since bands of the enzyme subunits were observed. Full stabilization of quaternary structure was only obtained after coating the surface of the biocatalyst with dextran (Figure 5). Considering this results

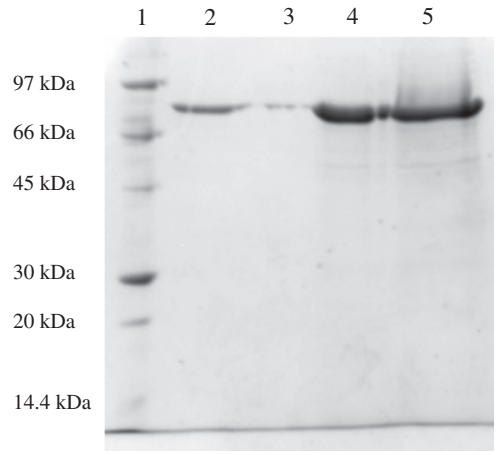

Figure 5. SDS-PAGE of the free β-xylosidase from *S. ruminantium* and its catalysts. Row 1: Standard proteins: phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa), row 2: uncoated glyoxyl catalyst (0.07 mg prot.), row 3: dextran-coated glyoxyl catalyst (0.07 mg prot.), row 4: CNBr catalyst (0.05 mg prot.), row 5: free enzyme (0.2 mg prot.).

and the higher stability indicate that low MW aldehyde-dextran and, similarly, different MW PEI are adequate to coat the immobilized enzyme, promoting the full stabilization of its quaternary structure.

Optimum activity of the catalysts was observed in the pH range from 4.0 to 6.0, while the free enzyme presented optimum activity in pH 4.0. Besides, elevated activity of the immobilized enzyme, higher than 80%, was observed in pH 7.0, while the activity of the free enzyme was considerably lower (Figure 6a). Optimal temperature for the free enzyme and for the catalysts was observed at 50–55 °C (Figure 6b). In addition, the side α-L-arabinofuranosidase activity was preserved in the catalyst corresponding to 18% of the β-xylosidase activity.

Table 1 shows the different values obtained for the kinetic constants on *p*NPX as substrate of the free and immobilized enzyme (PEI coated glyoxyl catalyst). The immobilized enzyme presented lower initial rates (Figure 7), resulting in V_{max} that is half of that observed for the free enzyme. K_m of 0.45 mM observed for the immobilized enzyme was lower than the 0.72 mM observed for the free enzyme, indicating the immobilized enzyme with higher affinity for the substrate.

The catalysts were also inhibited by xylose and glucose, being inhibition by xylose stronger than that by glucose. The PEI-coated catalyst and the free enzyme presented similar inhibition pattern and CNBr catalyst is a little more inhibited by xylose. In the presence of glucose, CNBr catalyst is a little more inhibited than the free enzyme and this effect is

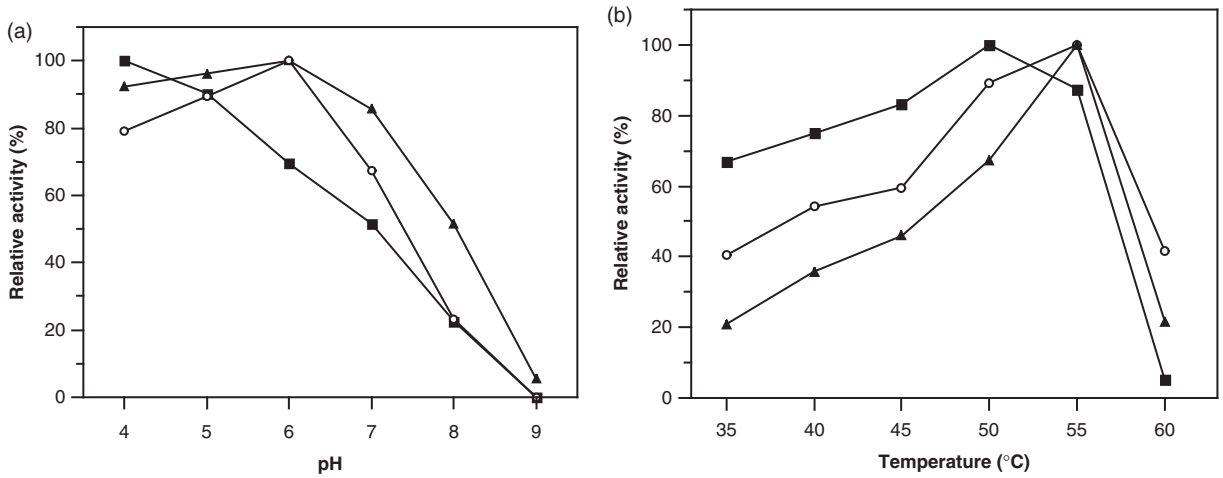

Figure 6. Activity profile at different pH values (a) and temperatures (b) of the free β-xylosidase from *S. ruminantium* and its catalysts. (a) The activity was assayed at 25 °C in the following buffers: 0.05 M sodium acetate buffer pH 4.0 and 5.0; sodium phosphate buffer pH 6.0 and 7.0; and Tris HCl buffer pH 8.0 and 9.0. (b) The activity was assayed in 0.05 M sodium acetate buffer pH 5.0 at 35, 40, 45, 50, 55, and 60 °C. PEI-coated glyoxyl catalyst (○), CNBr catalyst (▲), and free enzyme (■).

Table 1. Kinetic parameters for the free and immobilized β-xylosidase from *S. ruminantium*.

Enzyme	V_{max} (μmol min^{-1} mg prot.$^{-1}$)	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1} mM^{-1})
Free	0.43	0.72	1.83	2.47
PEI-coated glyoxyl catalyst	0.20	0.45	0.82	1.82

The activity was assayed at different *p*NPX concentrations in 0.1 M succinate-NaOH buffer pH 5.3 at 25 °C.

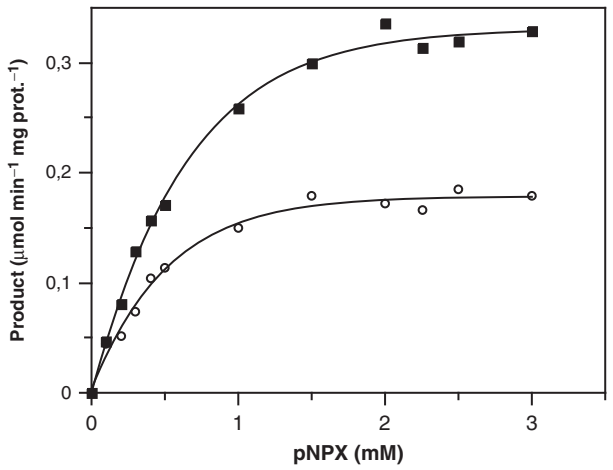

Figure 7. Saturation curves of initial velocity (V_0) with *p*-nitrophenyl β-D-xylopyranoside for the free and immobilized β-xylosidase from *S. ruminantium*. The activity was assayed at different concentrations of *p*NPX in 0.1 M succinate-NaOH buffer pH 5.3 at 25 °C. PEI-coated glyoxyl catalyst (○) and free enzyme (■).

Table 2. Influence of xylose and glucose on the free β-xylosidase from *S. ruminantium* and its catalysts.

	Relative activity (%)		
Monosaccharide (mM)	PEI catalyst	CNBr catalyst	Free enzyme
Xylose			
0	100	100	100
25	59.7	45.6	60.7
50	46.5	35.8	42.6
100	26.1	22.3	31.5
150	21.6	16.0	20.7
200	14.2	14.3	13.6
Glucose			
0	100	100	100
25	100	98.1	100
50	93.2	92.5	100
100	88.5	77.5	98.9
150	81.9	67.8	89.7
200	43.5	49.2	76.1

The activity was assayed in 0.05 M sodium acetate buffer pH 5.0 at 25 °C.

little more pronounced in the PEI-coated catalyst (Table 2). At 200 mM glucose, the enzyme in the PEI-coated catalyst was more than 50% inhibited while the free enzyme was only a little more than 20% inhibited when using *p*NPX at 5 mM.

Hydrolysis of xylooligosaccharides

The hydrolysis of various XOS by the PEI-coated glyoxyl catalyst was carried out in pH 5.0 at 50 °C, and the reaction products were analyzed by HPLC. Xylose release was observed from all substrates over time, including from natural XOS. Productions of 0.19 and 0.07 g/l were obtained from XOS derived

from beechwood and *Cannabis* xylans, respectively. Product profile at different periods of the hydrolyzed substrates is presented in Table 3. As observed, the enzyme presented an exo-type action as the main product formed after a short period was always one-xylosyl residue shorter than the incubated substrate. The rate of xylose release decreased with the substrate chain-length and, consequently, increased the period to obtain complete conversion of substrate to xylose. The conversion of each substrate to xylose decreased with time what can be attributed to inhibition caused by xylose accumulation.

Catalyst reuse and storage

The application of a biocatalyst in industry requires the stability of immobilized enzymes through many operational cycles. Long-term storage is also an important characteristic, since increased shelf life is a desired characteristic of bioproducts. The PEI-coated glyoxyl catalyst fully retained its activity after five re-use cycles; once again indicating the enzyme did not suffer subunit dissociation (Figure 8a). During these several hydrolysis–washing–dilution cycles, the curves of product were similar during five-hydrolysis cycles (Figure 8b). From the sixth cycle, the activity gradually decreased and consequently lower product release was observed specially in the beginning of the cycle. The biocatalyst also demonstrated to be very stable during long-term storage since after 6 months under refrigeration it fully retained the hydrolytic activity.

Discussion

The β-xylosidase from the ruminal anaerobic bacterium *Selenomonas ruminantium* is a very well characterized enzyme, and owing to its interesting properties, the enzyme presents high potential for application in industrial processes (Jordan et al. 2007a, 2007b; Jordan 2008; Jordan and Wagschal 2010). Due to its stability at alkaline pH, the enzyme could be easily immobilized on agarose highly activated with glyoxyl groups by carrying out immobilization for 0.5 h at pH 10 and 25 °C. Immobilization on glyoxyl occurs through the enzyme surface region having the highest density of lysine residues and has been the chosen protocol to obtain very intense enzyme-support multipoint covalent attachment for many enzymes. Besides, prolonged incubation period can increase protein-support attachments that could positively influence the catalyst properties in relation to its stability under different conditions (Mateo et al. 2006). Nevertheless, the catalyst prepared by carrying out

Table 3. Product composition of xylooligosaccharides hydrolysis by PEI-coated glyoxyl catalyst of the β-xylosidase from *S. ruminantium*.

	Product composition (%) at different hydrolysis periods																					
	5 min					10 min					30 min					60 min					120 min				
Substrate	X1	X2	X3	X4	X5	X1	X2	X3	X4	X5	X1	X2	X3	X4	X5	X1	X2	X3	X4	X5	X1	X2	X3	X4	X5
X2	36.1	–	–	–	–	62.1	–	–	–	–	94.0	–	–	–	–	98.0	–	–	–	–	100.0	–	–	–	–
X3	10.8	13.1	–	–	–	23.2	16.3	–	–	–	65.5	12.8	–	–	–	87.3	5.0	–	–	–	92.9	7.1	–	–	–
X4	9.0	–	14.1	–	–	13.2	10.3	21.4	–	–	53.0	7.6	18.5	–	–	71.0	6.1	13.3	–	–	85.8	3.7	4.1	–	–
X5	3.6	–	–	15.0	–	9.1	0.3	7.6	23.0	–	29.4	3.7	10.6	23.6	–	57.0	6.5	11.4	13.2	–	77.1	5.3	6.3	3.1	–
X6	3.7	–	–	–	76.9	6.9	ND	ND	2.7	19.3	20.0	1.5	6.8	9.9	35.5	44.5	1.0	6.4	11.9	16.8	68.3	6.8	7.1	15.1	2.7

Substrate solutions (0.5 mg/ml) were prepared in 5 mM sodium acetate buffer pH 5.0 and reactions were carried out at 50 °C, under magnetic stirring (300 rpm). X1: xylose; X2: xylobiose; X3: xylotriose; X4: xylotetraose; X5: xylopentaose; X6: xylohexaose; ND: not detected.

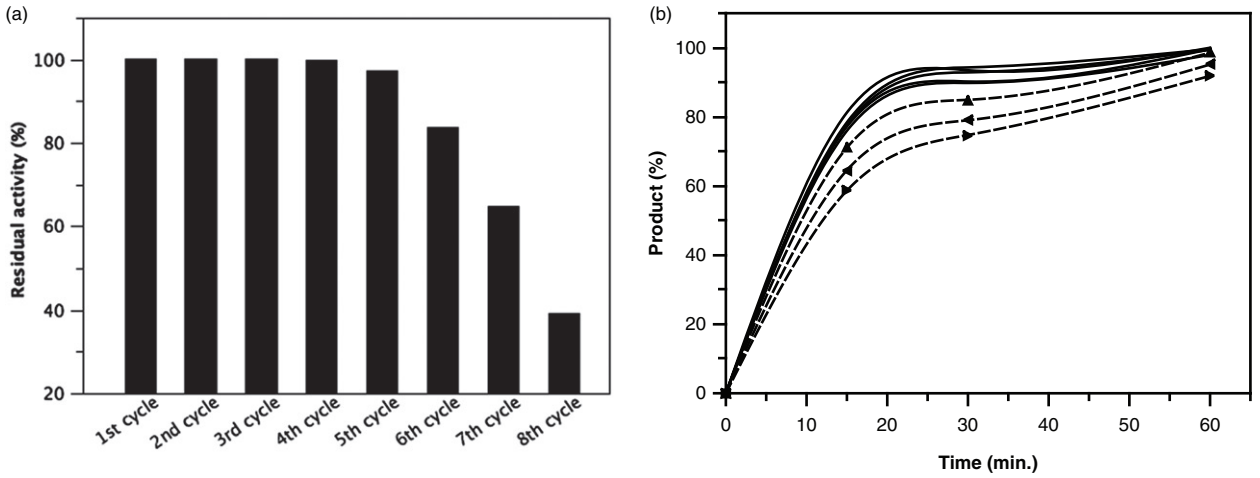

Figure 8. Operational stability (a) and product release (b) from *p*-nitrophenyl β-D-xylopyranoside hydrolysis by the PEI-coated glyoxyl catalyst of the β-xylosidase from *S. ruminantium*. The substrate was prepared in 0.05 M sodium acetate buffer pH 5.0 and reaction was carried out at 25 °C. Enzyme activity was assayed in the same buffer at 25 °C. (a) Residual β-xylosidase activity after consecutive cycles (■). (b) Full-lines: product release from the first to the fifth cycle; dashed lines: product release from the sixth (▲), seventh (◄), and eighth cycle (►).

immobilization for 15 h had lower stability than previously observed, that may be attributed to the distortion of the enzyme structure resulting from excessive attachments (Rodrigues et al. 2013). The marginal stability of biocatalysts has often prevented or delayed their implementation in industry. Consequently, there is great interest in understanding effects of reaction conditions on protein stability, as well as in developing strategies to improve protein stability (Polizzi et al. 2007). In the case of multimeric enzymes, as the tetrameric *S. ruminantium* β-xylosidase, the stabilization can be considered a special and complex problem in which the subunits dissociation can result in inactivation and even contamination of the product. In this sense, post-immobilization strategies such as coating the catalyst with polymers can stabilize the quaternary structure of immobilized enzymes rendering much more thermostability to the biocatalyst (Fernández-Lafuente et al. 1999; Bolivar et al. 2009). Thus, covalent immobilization of the β-xylosidase from *S. ruminantium* initially involves intense but controlled multipoint attachments and full stabilization is achieved by coating its surface with polyethylenimine and aldehyde dextran, resulting in a catalyst that is more than 240-fold more stable than the free enzyme. Free and immobilized enzyme presented similar characteristics as optimum temperature and pH. The catalyst also retained its side activity against *p*NPAra, a very important characteristic special considering hydrolysis of arabinoxylans, once the presence of side chains may hinder the action of other xylanolytic enzymes involved in xylan degradation (Rahman et al. 2003) The activity of different β-xylosidases can be associated to the substrate polymerization degree; for example, those from *Aspergillus phoenicis* (Rizzatti et al. 2001) and *Aspergillus versicolor* (Andrade et al. 2004) hydrolyze only up to xylotriose, while that from *Scytalidium thermophilum* (Zanoelo et al. 2004) hydrolyzed up to xylotetraose. Similarly to free enzyme (Jordan et al. 2007a), the immobilized β-xylosidase from *S. ruminantium* is able to hydrolyze up to xylohexaose, and increasing chain-length reduces hydrolysis rate of the substrate. After immobilization, kinetic parameters of the β-xylosidase from *S. ruminantium* were affected, i.e., K_m and V_{max} were 1.6- and 2-fold decreased, respectively. V_{max} values for other glyoxyl-immobilized enzymes are also reduced such as lactase from *Kluyveromyces lactis* (Mateo et al. 2004), invertase *Saccharomyces cerevisiae* (Goulart et al. 2008) and penicillin G acylase from *Escherichia coli* (Illanes et al. 2010). Despite the fact that immobilization on glyoxyl agarose is considered a very mild system due to the open pore structure and geometric congruence between enzyme and support (Mateo et al. 2006), mass transfer limitations are relevant in the case of immobilized enzymes; besides, factors other than diffusional restrictions, possibly conformational change as a consequence of immobilization, may also be playing a role in determining the catalytic properties of immobilized enzymes (Illanes et al. 2010). Changes in kinetic parameters after immobilization may also lead to changes in inhibition of enzyme (Garcia-Galán et al. 2001), as verified for the lactase from *Kluyveromyces lactis*, which inhibition by glucose was strongly decreased (Mateo et al. 2004). The immobilized β-xylosidase

from *S. ruminantium* is inhibited by xylose, similar to previous observations for the free enzyme (Jordan and Wagschal 2010). The free and immobilized enzyme is also inhibited by glucose, an important consideration particularly in the application of this catalyst for biomass saccharification (Kristensen et al. 2009).

Conclusions

The tetrameric β-xylosidase from *Selenomonas ruminantium* can be successfully immobilized on glyoxyl agarose by a simple protocol. Short period of immobilization is more effective in stabilizing the enzyme, although involvement of all subunits in the covalent immobilization is not achieved. Dextran and, specially, polyethylenimine adequately coat the enzyme subunits, fully maintaining its quaternary structure. The enzyme retained its native properties as optimum pH and temperature, the activity on xylooligosaccharides up to xylohexaose and its side activity against *p*NPAra. The immobilized enzyme demonstrated to be active against xylooligosaccharides originated from different xylans, such as commercial beechwood xylan and *Cannabis* sp xylan, a residue from textile industry. Besides, the prolonged thermostability, the full stability up to five reuse cycles and the prolonged storage stability improve the potential of this biocatalyst to be used in bioprocess to obtain the complete degradation of xylans.

Declaration of interest

The authors declare not having any conflict of interest.

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