PRODUCTION OF XYLO-OLIGOSACCHARIDES BY IMMOBILIZED-STABILIZED DERIVATIVES OF ENDO-XYLANASE FROM *Streptomyces halstedii*

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ABSTRACT

An endoxylanase from *Streptomyces halstedii* was stabilized by multipoint covalent immobilization on glyoxyl-agarose supports. The immobilized enzyme derivatives preserved 65 % of the catalytic activity corresponding to the one of soluble enzyme that had been immobilized. These immobilized derivatives were 200 times more stable 200 times more stable than the one-point covalently immobilized derivative in experiments involving thermal inactivation at 60 ºC. The activity and stability of the immobilized enzyme was higher at pH 5.0 than at pH 7.0. The optimal temperature for xylan hydrolysis was 10 ºC higher for the stabilized derivative than for the non-stabilized derivative. On the other hand, the highest loading capacity of activated 10 % agarose gels was 75 mg of enzyme per mL of support. To prevent diffusional limitations, low loaded derivatives (containing 0.2 mg of enzyme per mL of support) were used to study the hydrolysis of xylan at high concentration (close to 1 % w/v). 80 % of the reducing sugars were released after 3 hours at 55 ºC. After 80 % of enzymatic hydrolysis, a mixture of small xylo-oligosaccharides was obtained (from xylobiose to xylohexose) with a high percentage of xylobiose and minimal amounts of xylose. The immobilized-stabilized derivatives were used for 10 reaction cycles with no loss of catalytic activity.

Keywords: Multipoint covalent immobilization of enzymes, Thermo-stabilization of endoxylanases, Production of xylo-oligosaccharides, hydrolysis of xylan

INTRODUCTION

Xylo-oligosaccharides (XOS) are interesting prebiotics that are the subject of growing interest [1]. For example, a recent comparison of the prebiotic effect of several oligosaccharides concluded that XOS promotes an increase in the number of *Bifidobacteria* [2].

XOS can be obtained by chemical or enzymatic hydrolysis of xylan. Xylan (in the form of branched and modified arabinoxylan) is the major component of hemicellulose, one of the most abundant polysaccharides in the vegetal world [3]. Consequently, vegetal wastes can be converted to an important prebiotic ingredient. Enzymatic protocols that utilize endoxylanases for catalysis reactions are advantageous due to the absence of undesirable by-products (eg., furfural) [4-8].

The use of immobilized-stabilized derivatives of endoxylanases may be a very useful method for generating XOS. The biocatalyst could be reused for many reaction cycles at high temperatures (e.g. 50-60 □ C) High temperatures may be necessary to dissolve high concentrations of xylan, to prevent microbial contaminations and to increase the reaction rates. The utilization of immobilized derivatives also facilitates the careful design of reactor and control of the degree of hydrolysis to produce the most suitable mixture of different XOS (xylobiose, xylotriose, xylotetraose, etc.). In spite of these relevant advantages, protocols for immobilization and stabilization of endoxylanases have been hardly reported.

Enzymes under the name xylanase include proteins that break down the hemicellulose polysaccharide, beta-1,4-xylan, of the vegetal cell wall. In nature, these

enzymes are widely distributed as they function to aid in the growth of plants and microorganisms. For example, in fungi, xylanase enzymes degrade plant biomass to be utilized as a source of nutrients. Although humans do not produce xylanases, these enzymes are commercially utilized for a number of purposes; these processes include increasing the digestibility of animal feed [9], eliminating contaminant steps while obtaining white pulp [10] and improving the texture of bread dough [11].

Bacteria of the genus *Streptomyces* are saprophytic organisms that degrade a wide range of insoluble substrates using an arsenal of extracellular hydrolytic enzymes. Among these enzymes are the xylanases. The production of these xylanases has been reported in a number of *Streptomyces* strains that have been isolated from different sources. One such strain, originally isolated from agricultural waste, is *S. halstedii* JM8. *S. halstedii* JM8 produces an extracellular 45 kDa modular xylanase (Xys1 L) that contains a catalytic domain and a cellulose binding domain that is separated by a linker region. Extracellular serine proteases cleave the xylanase thus liberating the catalytic domain (Xys1S of 33.7 kDa). This catalytic domain has been shown to exhibit the same activity against xylan *in vitro* as than the complete protein [12]. The deletion of a Gly-rich like region located in the carboxy terminus of the Xys1S [13-14] generates a 32.6 kDa protein that has previously been utilized for microcalorimetric and crystallization studies [15] and has been modified with a hexa-His tag at its carboxy terminus.

In this paper, a poly-His tagged catalytic domain of *Streptomyces halstedii* JM8 endoxylanese was purified by using tailor-made immobilized metal chelates (IMAC

chromatography). The purified domain was then immobilized by multipoint covalent attachment on highly activated glyoxyl-agarose supports under alkaline conditions. This immobilization protocol involved the region of the enzyme surface containing the highest number of Lys residues. The formation of several bonds between each enzyme molecule and the support promotes the stabilization of the immobilized enzymes. [16].

The immobilized-stabilized derivatives of this endoxylanase were used to hydrolyze high concentrations of xylan (close to 1% w/v) at high temperature (55 C)The rate and yield of the release of reducing sugars was studied. To determine the composition of XOS, the reaction products were chromatographically analyzed at different stages of the hydrolysis reaction. The exact composition of oligosaccharides of different reaction products was studied.

Materials and Methods

Materials

Agarose 10BCL was purchased from Agarose Bead Technologies (Madrid, Spain). Beechwood xylan, glycidol, sodium borohydride, sodium periodate, ethanolamine and 3-5´-dinitrosalicylic acid were obtained from Sigma-Aldrich Co (St. Louis. MO). CNBr-activated Sepharose and low molecular weight standards were purchased from GE Healthcare (Uppsala, Sweden) and the xylo-oligosaccharides standards were obtained from Megazyme (Wicklow, Ireland.). All reagents were analytical grade.

A protein molecular weight standard consisting of 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) was obtained from Sigma Chem. Co.

Methods

All results represent the average of at least three experiments. The experimental error was never higher than 5 %.

Endo-1,4-β-xylanase production

S. lividans **JI66 [17] was used as the host for the multicopy plasmid pNX4 [18]. The production of the xylanase for this strain was carried out in YES medium (1 % yeast extract, 10.3 % sucrose, 5 mM $MgCl_2$, pH 7.2) supplemented with 1 % (w/v) xylose and 10 μg/mL of neomycin. Liquid cultures were carried out in 2 liters of YES medium aliquoted into 500 mL baffled flasks containing 150 mL of medium at 28 °C and shaken at 200 rpm. Culture supernatants were obtained after 6 days of growth and were utilized as the source of the enzymes for purification immobilization.**

Enzyme assay and protein determination

The quantity of reducing sugar released by the enzymatic hydrolysis of xylan was colorimetrically determined based on the reaction of the reaction mixture with 3-5´-dinitrosalicylic acid according to Miller [19] and by using xylose as the standard.

A mixture of 1 % (w/v) beechwood xylan in 100 mM sodium acetate buffer at pH 5.0 was stirred for 2 hours (under strong magnetic stirring) at 25 °C and then centrifuged for 20 minutes at 5000 *g*. The soluble fraction was used as the substrate. The assay was conducted at 25 °C under constant agitation (very mild magnetic stirring). One enzyme unit (U) was defined as the amount of enzyme able of producing 1 μmol of reducing sugar per minute.

The protein concentration was determined with the Bradford´s method with bovine serum albumin as the protein standard [20].

Purification of recombinant endoxylanase by adsorption on lowly activated Ni-IDA- 6 % agarose gels

Lowly activated Ni-IDA-agarose gels (containing 10 µEqs of chelates per mL of 6 % agarose gel) were prepared as previously described [21]. The crude endoxylanase extract was diluted 10-fold in 50 mM sodium phosphate buffer containing 150 mM NaCl and 20 mM of imidazole and adjusted to pH 7.0. Then, 150 mM NaCl was added to the binding buffer to prevent nonspecific ionic interactions between the non-recombinant proteins and the support.; 20 mM imidazol was used to minimize the adsorption of non-recombinant proteins on the lowly activated Ni-IDA-supports, and 50 mL of the diluted crude endoxylanase extract (0.8 mg/mL of protein concentration) were mixed with 1 mL of lowly activated Ni-IDA-agarose support [21]. The incubation was carried out at 25 °C and under constant gentle magnetic stirring. After 1 hour, the enzyme was completely adsorbed onto the chromatographic support. Then, the adsorbed enzyme was recovered by filtration and subsequently washed with 50 mL of 50 mM phosphate buffer at pH 7.0 containing 50 mM imidazole and 150 mM NaCl to remove the traces of non-recombinant proteins that were adsorbed onto the support. Finally, the desorption of endoxylanase was performed by incubating the chromatographic support for 30 min with 50 mL of 50 mM phosphate buffer at pH 7.0 containing 100 mM imidazole and 150 mM NaCl. The solution was dialyzed against distilled water and then lyophilized and stored in the refrigerator (it preserves fully active for 6 months).

164 ***Activation of supports: Preparation of glyoxyl-agarose support***

165 **105 g of 10 % agarose beads were suspended in water to a final volume of**
166 **180 mL (0.7 g of agarose is roughly equivalent to 1 mL). Following mild**
167 **homogenization, 50 mL of 1.7 M NaOH containing 1.425 g of $NaBH_4$ was slowly**
168 **added. In an ice bath, 36 mL of glycidol were added drop-wise. The mixture was then**
169 **gently stirred at room temperature for 18 hours, and the gel was finally washed with**
170 **excess distilled water. Then, 10 wet g of glyceryl-activated gel was oxidized with 50**
171 **mL of aqueous 100 mM $NaIO_4$ per mL of gel, and the oxidation was carried out under**
172 **very gently stirring. After 2 hours, the gel was washed with distilled water and**
173 **stored in the refrigerator at 4 ºC after vacuum drying (with the pores of agarose gels**
174 **filled with water) [22].**

175 ***Stability of soluble enzyme at pH 10***

176 **Multipoint covalent immobilization has to be carried out at pH 10. In order to**
177 **establish the temperature of immobilization the stability of soluble enzyme was**
178 **firstly studied. 0.2 mg of enzyme were dissolved in 10 mL of 100 mM sodium**
179 **bicarbonate buffer at pH 10.0. The enzyme was incubated at 25 and 4 º C and at**
180 **different times aliquots were assayed as described above. According to the stability**
181 **of soluble enzyme at pH 10.0 the immobilization protocol was designed.**

182 ***Enzyme immobilization***

183 **The immobilization on glyoxyl-agarose was performed by diluting up to 2 mg**
184 **of lyophilized xylanase to 50 mL of 100 mM sodium bicarbonate solution at pH 10.0**
185 **and 4 º C Then, enzyme was added to 10 g of support, and the suspension was**
186 **gently stirred at 4 º C Periodically, samples of the supernatant and suspension were**
187 **withdrawn, and the enzyme activity was measured. When the immobilization was**

completed (4 hours), the derivative was incubated at room temperature for 12 hours and finally it was reduced for 30 minutes with 1 mg/mL sodium borohydride.

On the other hand, very mild immobilization on CNBr-activated Sepharose was performed by using the same amount of enzyme diluted in a 100 mM sodium phosphate buffer at pH 7.0 and 4 °C. After 15 minutes, the derivative was filtered and suspended into 1 M ethanolamine solution at pH 8.0 for 2 hours to block any remaining reactive group [23].

The yield of the immobilization was defined as the ratio between the activities in the supernatant compared with the activity in the blank of soluble enzyme. 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.

Thermal stability studies

1 g of immobilized derivative was suspended in a 10 mL suspension containing 0.1 M of acetate buffer (pH 5.0) or 0.1 M of phosphate buffer (pH 7.0) at different temperatures. In all cases, at several time points, samples were withdrawn and their activity was tested as described above. The remaining activity was calculated as the ratio between activity at a given time and the activity at the start of the incubation.

SDS-PAGE

Samples underwent denaturing electrophoresis based on Laemmli´s method [24] using 12 % polyacrylamide gels. Gels were stained with Coomasie Blue.

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213 ***High-Performance Anion Exchange Chromatography with Pulsed Amperometric***
214 ***Detection (HPAEC-PAD) analysis***

215 **Xylo-oligosaccharides (XOS) were analyzed with HPAEC-PAD using an ICS2500**
216 **Dionex system (Dionex Corporation, Sunnyvale, CA) consisting of a GP50 gradient**
217 **pump, and ED50 electrochemical detector with a gold working electrode and**
218 **Ag/AgCl reference electrode. Data acquisition and processing was performed with**
219 **the Chromeleon software version 6.7 (Dionex Corporation). For eluents preparation,**
220 **MilliQ water (Milli-Q Synthesis A10 system; Millipore, Billerica, Mass., USA), NaOH**
221 **(50 %, w/v) and NaOAc (Fluka, Germany) were used. All eluents were degassed by**
222 **flushing with helium for 25 minutes.**

223 **Analyses were carried out at 25 °C on a CarboPac PA-1 column (250×4 mm) in**
224 **combination with a CarboPac PA-1 (50×4 mm) guard column. Separations were**
225 **performed at a flow rate of 1 mL/min. A gradient of 100 mM NaOH (eluent A) and**
226 **100 mM NaOH and 500 mM NaOAc (eluent B) was used (0-45 min, 0-70 % eluent B).**
227 **After each run, the column was washed for 10 min with 100 % of 100 mM NaOH and**
228 **1 M NaOAc (eluent C) and re-equilibrated for 15 min with the starting conditions of**
229 **the employed gradient.**

230 **Before injection (20 µL), samples and standard solutions were filtered**
231 **through a nylon Millipore FH membrane (0.22 µm) (Bedford, MA). The quantification**
232 **of XOS was based on an external calibration using standard solutions of XOS (degree**
233 **of polymerization from 1 to 6) and the calibration curve regression coefficients that**
234 **were higher than 0.99. All analyses were carried out in duplicate, and data were**
235 **expressed as the mean value. Standard deviation was never higher than 5 %.**

Results and Discussion

Purification of recombinant endoxylanase overexpressed in E. coli.

The recombinant endoxylanase was overexpressed and secreted to the culture medium. The crude extract was a fairly pure enzyme solution as analyzed with SDS-PAGE (Figure 1, lane 2). A very selective adsorption of the enzyme (approx. 90 % purity) on poorly activated nickel chelate supports in the presence of 20 mM imidazole. Contaminant proteins were only adsorbed in trace amounts (data not shown) and were easily desorbed with a first wash with 50 mM imidazole leaving the pure endoxylanase adsorbed onto the support (lane 3). Pure endoxylanase was eluted with 100 mM imidazole. The purification yield was 95 %, and the purification factor was greater than 2. The specific activity of the pure enzyme for the hydrolysis of xylan was 255 µmols of released reducing sugars per minute per mg of protein.

It was only possible to purify the His-tagged recombinant protein to homogeneity in one step. Moreover, the selective adsorption of the target enzyme facilitates the use of small volumes of chromatographic support and therefore makes the purification more cost-efficient. In fact, up to 0.9 grams of enzyme could be purified by using only 20 mL of chromatographic support.

Immobilization of endoxylanase

The pure enzyme was immobilized on CNBr-activated Sepharose. A very mild immobilization was performed at pH 7.0, 4 $^{\circ}$C for 15 minutes. In this way, only 30 % of the enzyme was immobilized, but any type of multipoint covalent attachment was

avoided. In fact, this mildly immobilized derivative preserves the 100 % activity that was immobilized, and this derivative exhibits the same thermal stability as the pure and the diluted soluble enzyme. Both enzyme preparations exhibit a half-life of 10 hours when incubated at 45 °C and pH 5.0. This mildly immobilized derivative, xyl-CNBr-agarose, with the molecules of the enzyme fully dispersed inside a porous support, was used as a blank representing the properties of the native enzyme in the absence of any artifact (aggregations, interaction with hydrophobic interfaces of air bubbles, etc.). Xylanase immobilized on CNBr-activated Sepharose and the soluble enzyme seem to be identical: e.g., showing the same activity and thermal stability. However the study of the behavior of native enzyme under more drastic experimental conditions (high temperatures, organic cosolvent, stirred tanks, etc.) is more accurate when using the mildly immobilized enzyme. Soluble enzyme may undergo artifacts: aggregations, interactions with hydrophobic interfaces and these artifacts are impossible with any enzyme immobilized on porous supports. When an enzyme is mildly immobilized on CNBr-activated Sepharose at pH 7.0, 4 °C and 15 minutes the multipoint immobilization is almost impossible and the derivative fairly represents the immobilized native enzyme.

The pure enzyme was also immobilized by multipoint covalent immobilization on glyoxyl-agarose under alkaline conditions. The stability of the soluble enzyme at pH 10 in bicarbonate buffer was evaluated at both 25 °C and 4 °C. At 25 °C, the soluble enzyme was fairly unstable (half-life time of 2 hours). In contrast, the enzyme was very stable at 4°C and retained 98 % activity after 2 hours. Very highly activated gels

with 150 µmols of glyoxyl (small aliphatic aldehyde groups) per mL of 10 %-agarose gel (1.4 of wet grams) were used, and immobilization was carried out at pH 10 and 4 °C. Furthermore, 95 % of the applied enzyme was immobilized in 2 hours and, after 4 hours of subsequent incubation at 4 °C, the immobilized derivative was incubated for 12 hours at 25 °C before borohydride reduction. The immobilized derivative (xyl-glyoxyl-agarose) retained 65 % activity when compared to the soluble enzyme that had been immobilized on the support. In the present paper soluble dextran is always used. Now, the behavior of soluble and immobilized enzyme is very similar (e.g., at low temperatures). At 55 °C the soluble enzyme could not be studied because of its very low stability. On the contrary Lin et al [6] have compared the behavior of soluble and immobilized enzyme on a mixture of soluble and insoluble xylan. Immobilized enzyme was only able to act on the soluble fraction of xylan (shorter chains) and the soluble enzyme is also able to act on insoluble xylan (longer chains). In this way, the behavior of both enzyme preparations was clearly different. As remarked out in Introduction, we propose the use of immobilized enzymes in order to simplify the reactor design: use of continuous reactors, use of stirred tanks with very easy end of the controlled hydrolysis.

To evaluate the activity-stability properties of immobilized endoxylanase in the absence of diffusional limitations, the immobilized derivatives were firstly prepared with a low enzymatic loading (50 IU/mL of support). On the other hand, the highest loading capacity of glyoxyl-10 % agarose gels was evaluated and 75 mg of the enzyme could be immobilized per mL of wet support (0.7 grams of wet agarose gels). This high loaded derivative exhibits an intrinsic activity of 12430 IU per mL of derivative. Intrinsic activity was analyzed after breaking the derivative under strong

magnetic stirring at 4 °C. This strong stirring promotes a very high reduction of particle size of immobilized enzyme.

Stabilization of Immobilized Endoxylanase

Both immobilized derivatives were incubated at 60 °C at pH 5.0. Xyl-glyoxyl-agarose exhibited a half-life of 15 days and xyl-CNBr-agarose had a half-life of less than 2 hours (Figure 2). Multipoint covalent immobilization promoted a stabilization that was more than 200-fold higher than that of the one-point immobilized derivative. Stabilization was also observed in experiments of activity versus temperature. The optimal temperature for stabilized derivative was 10 °C higher than one of the non-stabilized derivative (Figure 3). Immobilization on glyoxyl supports occurs directly via a multipoint covalent immobilization but the intensity of the enzyme-support multipoint attachment increases after incubation of immobilized derivatives at pH 10 for long times at room temperature. In fact, non incubated derivatives of this xylanase were 20-fold less stable than optimal derivatives obtained after a 12 hours incubation at 25 □ C.[25-26]. The stability at pH 7.0 and pH 5.0 was also evaluated. The stabilized derivatives were significantly more stable at pH 5.0 than at pH 7.0 (Figure 4). Further experiments of hydrolysis of xylan were carried out at pH 5.0.

Hydrolysis of xylan by immobilized-stabilized endoxylanase

The release of reducing sugars by enzymatic hydrolysis of xylan was studied at different temperatures. Xylan solutions are prepared by adding 1 gram of xylan to 100 ml of buffer at different temperatures. After 2 hours of vigorous magnetic

stirring the suspensions were centrifuged and the amount of pellet was measured. At 55 ºC the 50 % of xylan is dissolved. At lower temperatures the solubility of xylan is clearly lower: 30% at 25 ºC and 20% at 4 ºC. The results were very similar when solubility of xylan was measured by evaluating the decrease of "light scattering" at 650 nm as a consequence of the solubilization of xylan.

The rate of the hydrolysis and the final yields increase as temperature increases (Figure 5). The final yield achieved at 55 °C after 140 hours was considered to be 100 % of release; 80 % and 90 % release of the reducing sugars were achieved after 3 and 5 reaction hours, respectively. The highest yield (100 %) was achieved after 140 hours. Furthermore, 90 % hydrolysis was never achieved at 25 °C even after 140 hours. Both the hydrolysis rate and yield were decreased at 4 °C. In Figure 5 the final degrees of hydrolysis are calculated by taking as 100% the reducing sugars hydrolyzed at 55 ºC. The final yields of hydrolysis are very similar to the different solubilities. In this way, Figure 5 clearly shows the different rates of hydrolysis and the different xylan solubilities obtained at different temperatures.

Reuse of immobilized-stabilized xylanase

Because the immobilized-stabilized derivative was fairly stable at 60 °C, it seems that this derivative could be re-used for a number of reaction cycles at 50-55 °C. In Figure 6, we observe that enzymatic hydrolysis of xylan was unchanged after 10 reaction cycles (Figure 6). By using the high loaded derivative (75 mg of enzyme per mL of support), 80 % of hydrolysis could be achieved in less than 10 minutes.

Composition of XOS at different hydrolysis degrees

The composition of the XOS mixtures was studied at different conversion degrees. The enzyme seems to be an endoxylanase, and the release of xylose was minimal up to the release of 80 % of reducing sugars. However, at this conversion degree, 49 % of a XOS mixture (from xylobiose to xylohexose) was obtained (Table 1). This hydrolytic conversion was achieved after only 3 hours with the low loaded derivative, and the 80 % of xylan was hydrolyzed at this conversion. These results are quite interesting if we keep in mind that a significant part of the xylan chains are modified by arabinose, or other sugars, etc. and that these substituted chains are not hydrolyzed by endoxylanases. After 140 hours with a 100 % release of reducing sugars, the hydrolyzed xylan obtained was enriched in 56 % of xylobiose. Under these conditions, an exoxylanase activity was also observed and a 10 % of xylose was analyzed. Furthermore, 80 % of conversion seems to be the most adequate because of very short reaction times and a mixture of 50 % of prebiotics XOS. If xylobiose were the most important prebiotic, higher conversions should be achieved.

CONCLUSIONS

The over-expression of the catalytic domain of endoxylanase from *Streptomyces* with the insertion of a poly-His tail allows for the easy preparation of a large amount of a pure industrial enzyme. This enzyme was immobilized and highly stabilized (200-fold) by multipoint covalent attachment on glyoxyl-agarose. The maximal enzyme loading was 75 mg (12000 Units) per mL of support. The stabilized and low loaded enzyme derivative (0.2 mg per mL of support) could be used to catalyze the hydrolysis of xylan at 55 °C. In only 3 hours the hydrolysis of 80 % of 1 % (w/v) xylan

was achieved. After this hydrolysis a mixture of small prebiotic xylo-oligosaccharides (containing 50 % of XOS) was obtained. At higher conversion degrees a 56 % of xylobiose could be obtained.

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FIGURE LEGENDS

Fig. 1. SDS-PAGE gel of endo-1,4-β-xylanase from *Streptomyces halstedii.* Lanes: (1) molecular weight markers; (2) 10 μl of supernatant of *S. Lividans* carrying the pNX4 plasmid; (3) purified endo-1,4-β-xylanase. Experiments were performed as described in the Methods.

Fig. 2. Time-courses of thermal inactivation of the immobilized endoxylanase derivatives. A.- Enzyme immobilized on CNBr-activated Sepharose (♦)and B.- Enzyme Immobilized on glyoxyl-agarose 10BCL and on (■). Experiments were carried out at 60 °C. The activity was measured at 25 °C and at pH 5.0 as described in the Methods.

Fig. 3. Influence of temperature on the enzymatic activity of immobilized endoxylanase: glyoxyl-agarose 10BCL derivatives (■), CNBr-activated Sepharose derivatives (♦), soluble enzyme (◊). Activity assays were performed at pH 5.0.

Experiments were done by triplicate. Experimental error was lower than 5%.

Fig. 4. Time-courses of thermal inactivation of immobilized endoxylanase at different pH values. Glyoxyl-agarose 10BCL derivatives. (♦) pH 5.0; (■) pH 7.0. Experiments were carried out at 75 °C. The activity was measured at 25 °C and pH 5.0 as described In Methods.

Experiments were done by triplicate. Experimental error was lower than 5%.

Fig. 5. Time courses of hydrolysis of beechwood xylan by endoxylanase immobilized on glyoxyl-agarose 10BCL at different temperatures. 1 gram of xylan was added to 100 ml of buffer at different temperatures. The suspension was vigorously stirred for 2 hour and then centrifuged (♦) 4 ºC (20% of xylan dissolved) ; (■) 25 ºC (30% of xylan dissolved); (▲) 55 ºC (50% of xylan dissolved). Experiments were carried out at pH 5.0. 100% of reducing sugars are those measured at 55 ºC.

Fig. 6. Ten consecutive cycles of hydrolysis of 1 % (w/v) beechwood xylan by endoxylanase immobilized on glyoxyl-agarose 10BCL. Each reaction cycle was stopped when the immobilized derivative released 80 % of reducing sugars. Experiments were carried out at pH 5.0 and 55 ºC.

Experiments were done by triplicate. Experimental error was lower than 5%.

Table 1. XOS formation after hydrolysis of 1 % (w/v) beechwood xylan catalyzed by endoxylanase immobilized on glyoxyl-agarose 10BCL

Temperature (□C)	Reducing sugars (%)	% total carbohydrates (w/w)						
		X1	X2	X3	X4	X5	X6	Others XOS
-	4.4 (Control)	0.00	0.00	0.00	2.43	4.18	7.98	86
55	38	0.00	12.13	8.39	7.31	4.42	3.29	65
	64	0.21	17.18	11.17	7.32	4.46	3.01	57
	80	0.53	22.65	12.92	7.17	3.73	2.23	50
	100	10.82	56.07	0.96	0.40	0.23	0.24	30

Figure(s)

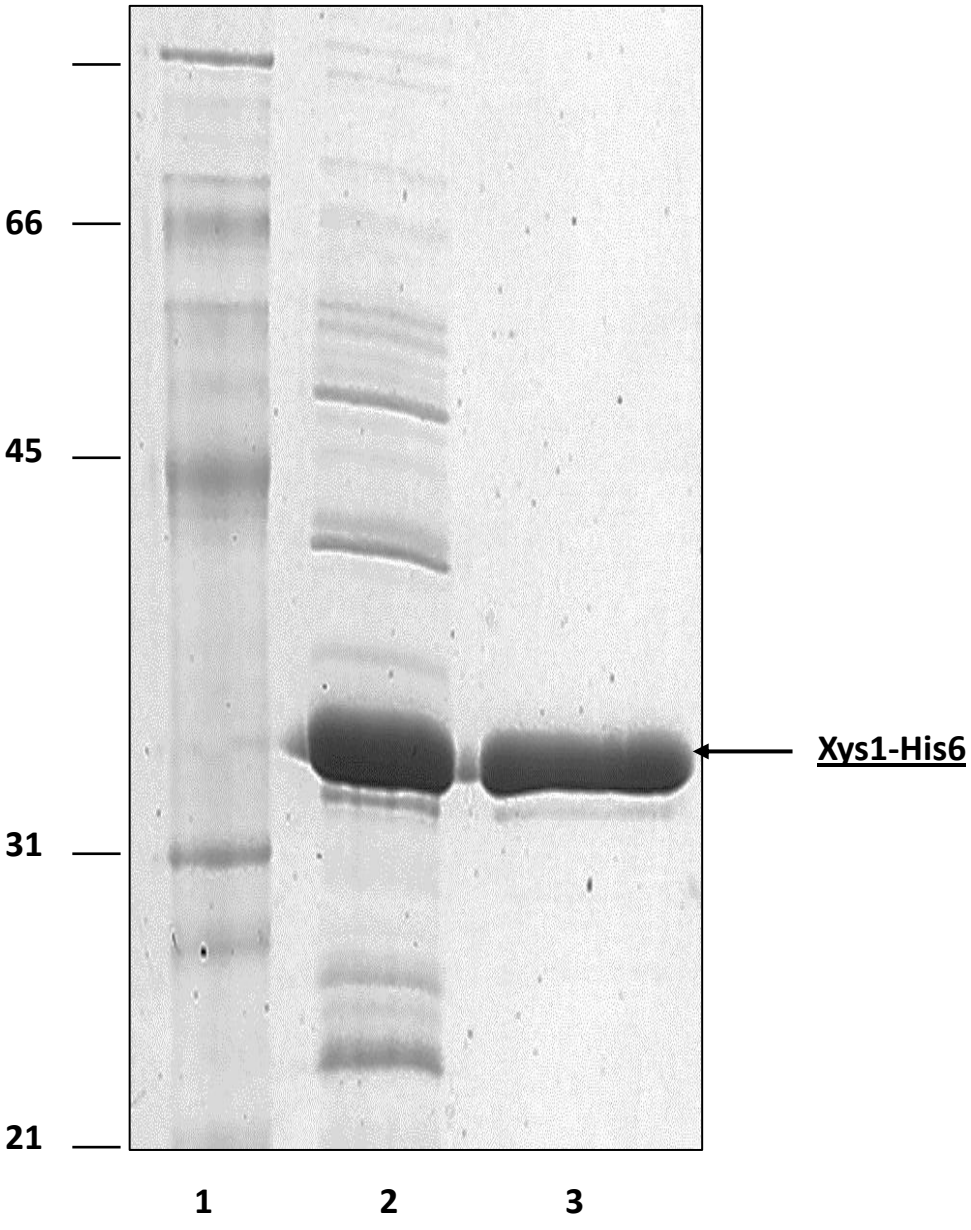
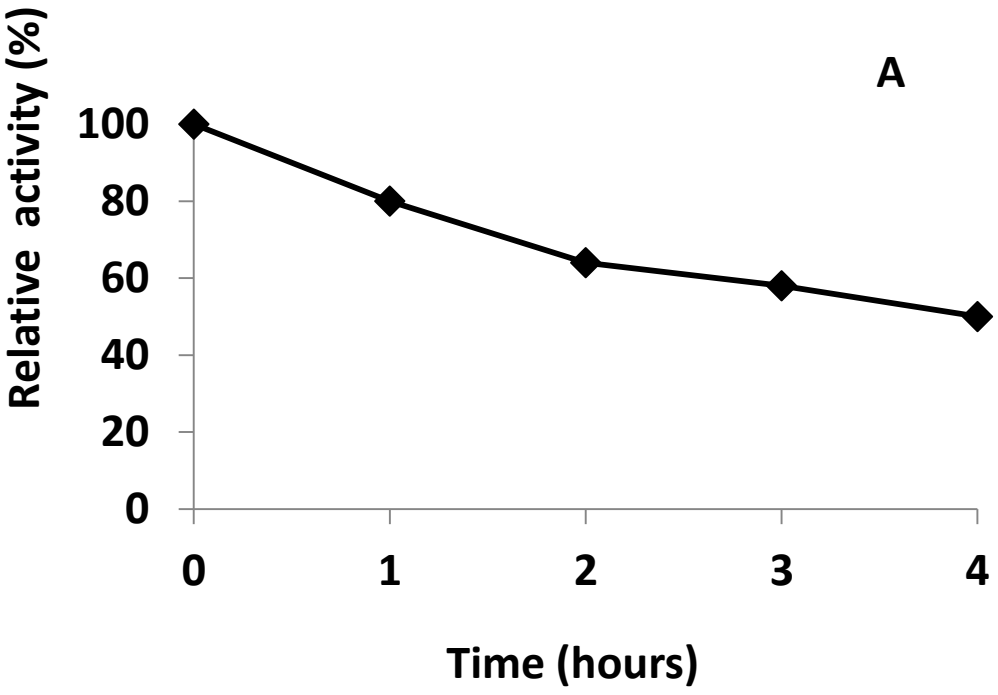


Figure 1


A
Relative activity (%)
100
80
60
40
20
0
0
1
2
3
4
Time (hours)

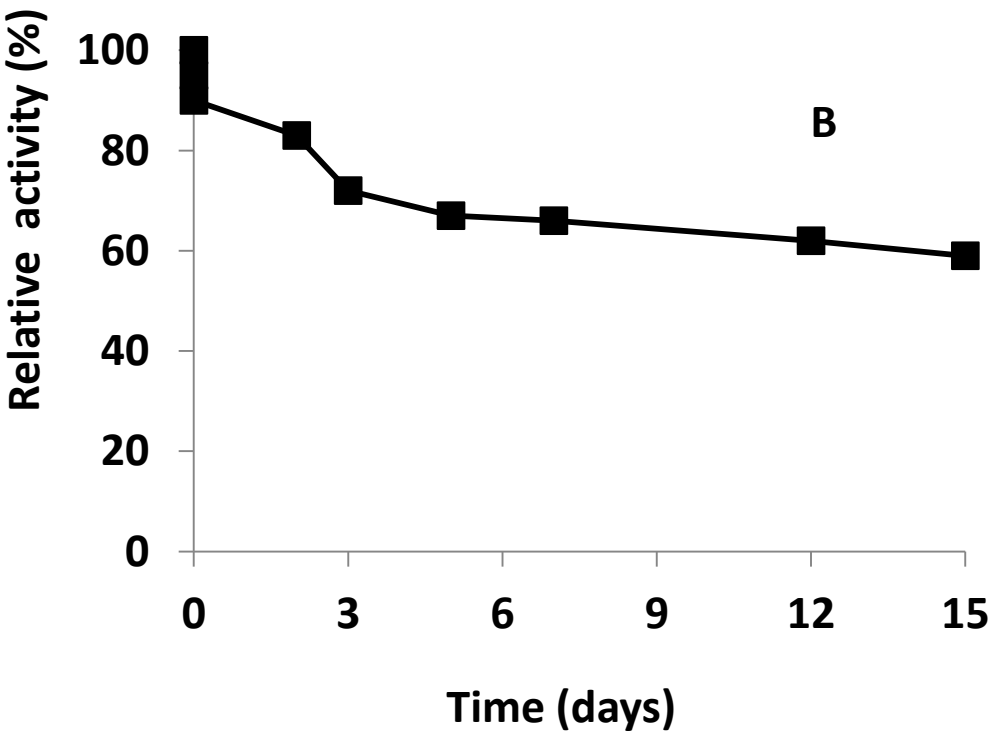
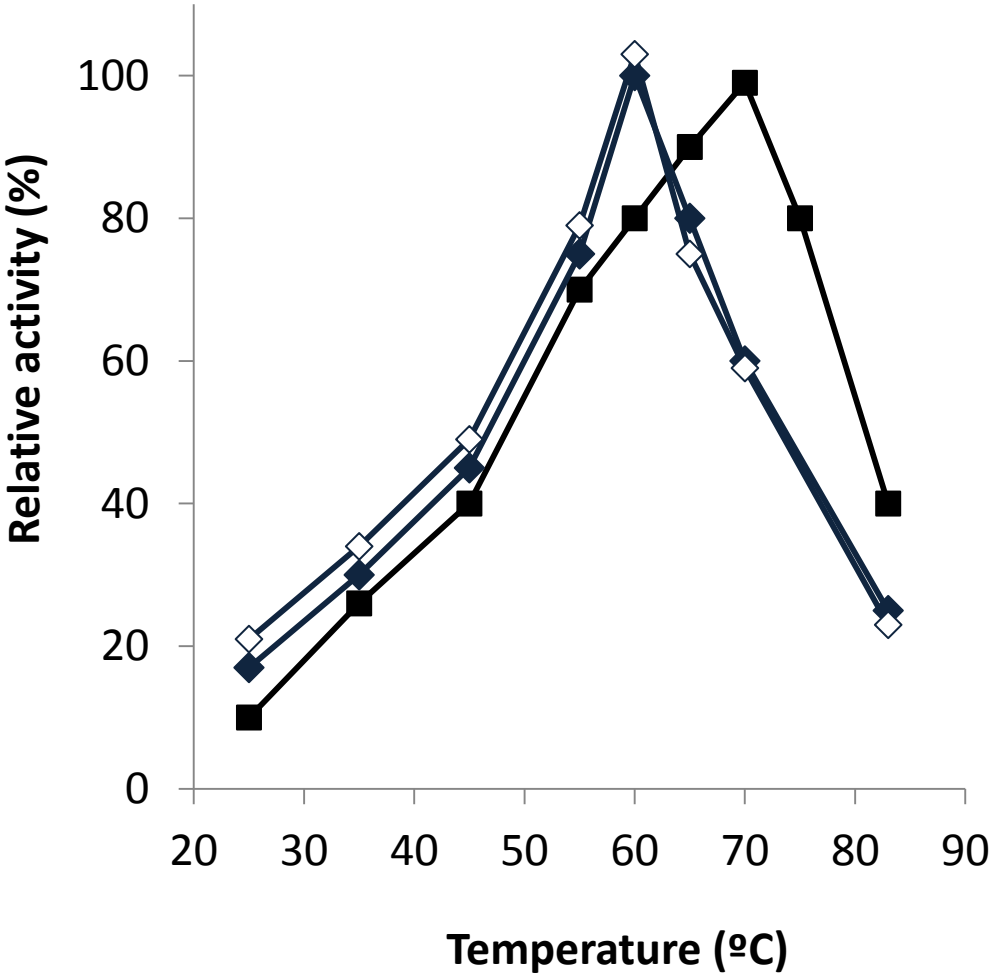

B
Relative activity (%)
100
80
60
40
20
0
0
3
6
9
12
15
Time (days)

Figure 2


Relative activity (%)
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80
60
40
20
0
20
30
40
50
60
70
80
90
Temperature (ºC)

Figure3

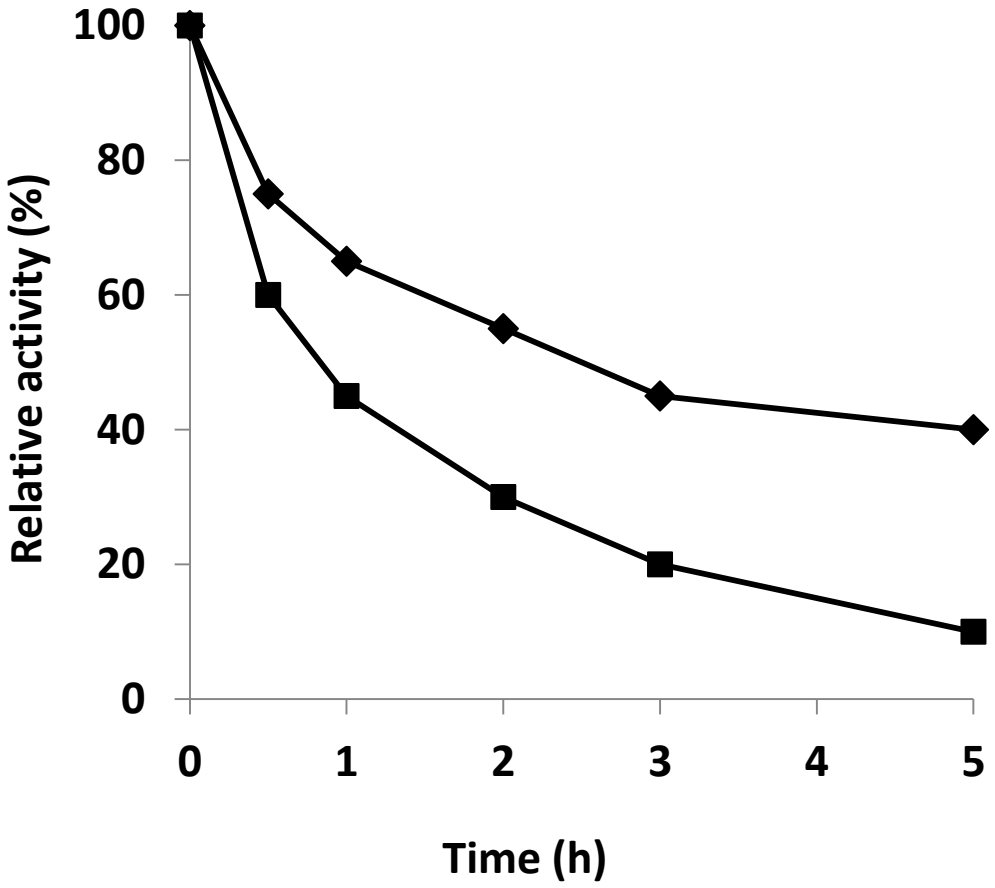
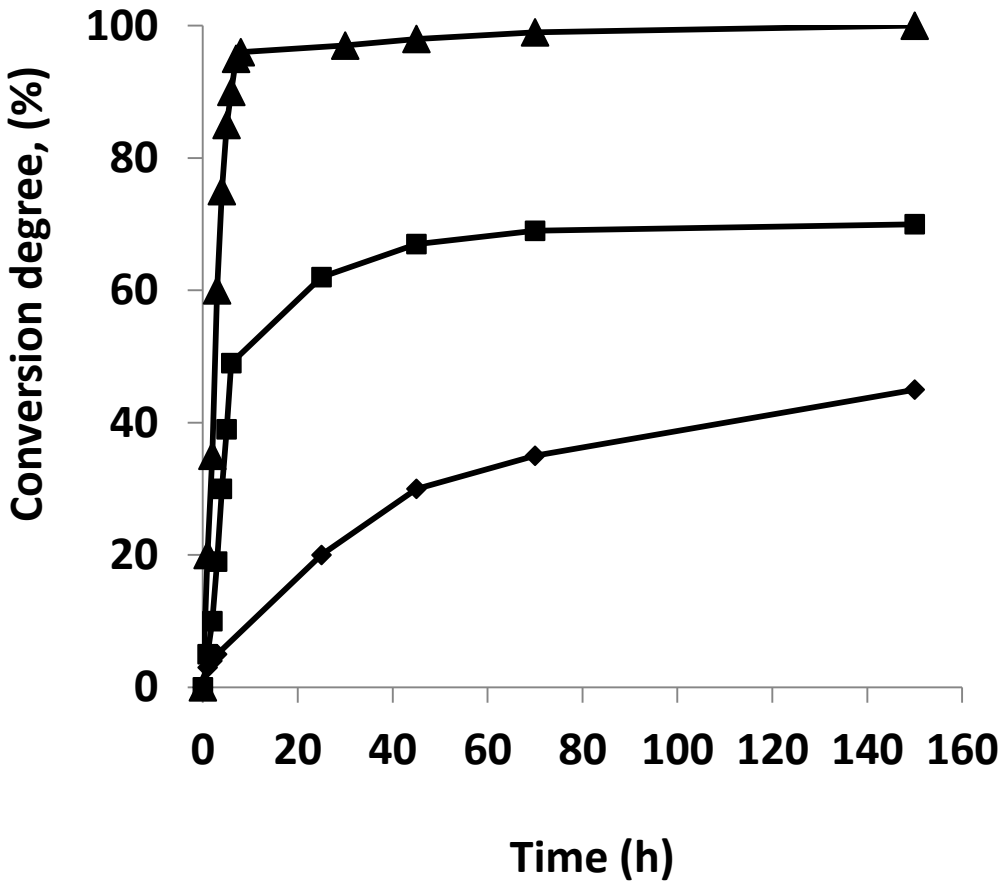


Figure 4


Conversion degree, (%)
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60
40
20
0
0
20
40
60
80
100
120
140
160
Time (h)

Figure 5

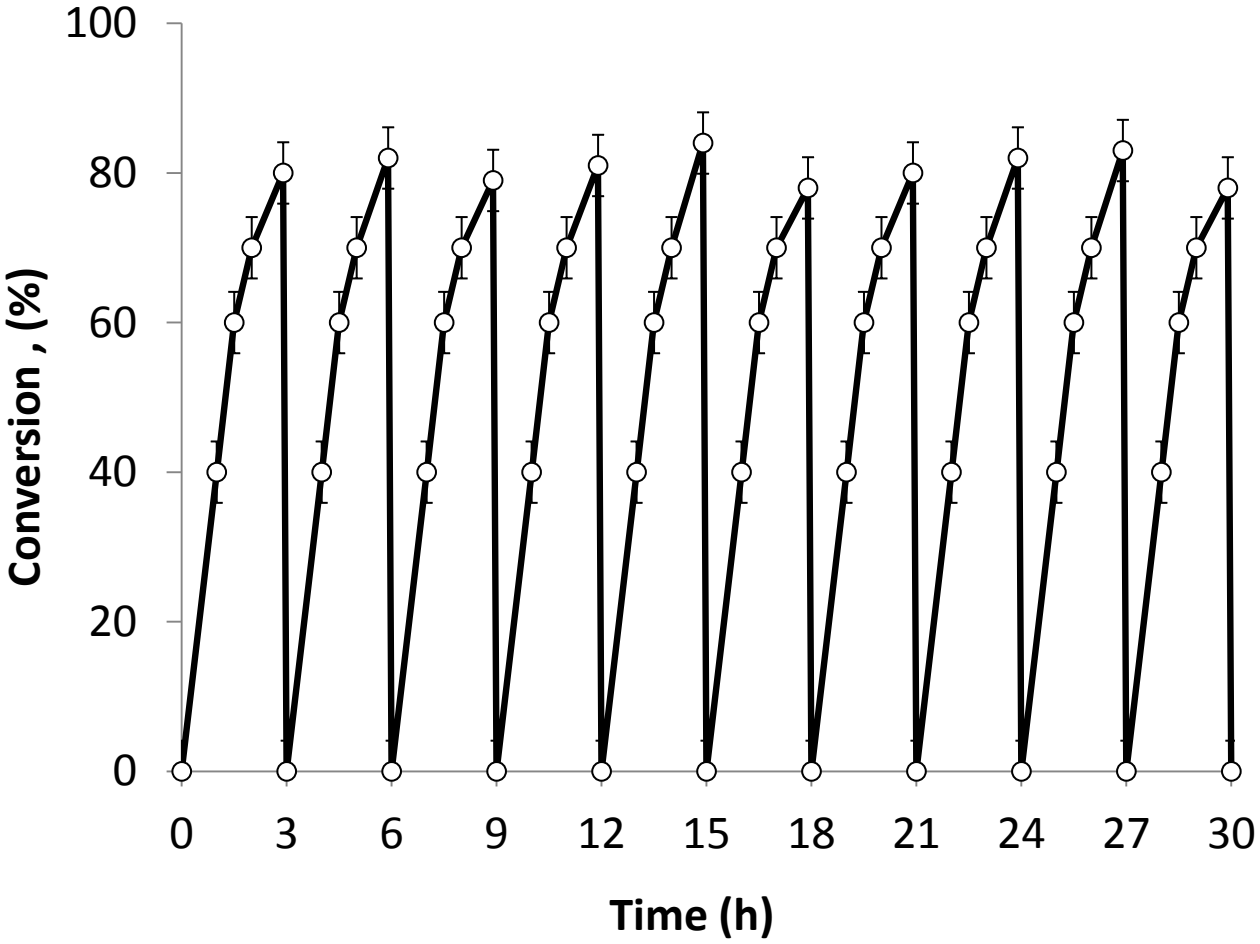

Figure 6