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Production of xylo-oligosaccharides from agro-industrial residues using immobilized *Talaromyces thermophilus* xylanase

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ABSTRACT

Talaromyces thermophilus stolk a thermophilic fungus isolated from soil samples collected in Tunisia. The single xylanase from this strain was purified to complete homogeneity. Several enzyme immobilization methods were then tested and compared for residual enzyme activity. The most efficient immobilization was achieved by gelatin (10%) using glutaraldehyde as a bifunctional agent. This method gave an immobilization yield of 98.8% and a xylanase activity recovery of 99.2%. The immobilized enzyme exhibited a shift in the optimal pH from 7 to 8 but the optimal temperature of activity was not affected. The immobilized enzyme retained about 94.0% of its initial catalytic activity even after being used during 13 successive cycles of hydrolysis at 50 °C. The main hydrolysis products yielded from xylan were xylobiose and other xylo-oligosaccharides such as xylotriose. The immobilized enzyme was then used for the large-scale continuous production of xylobiose, starting from the hemicellulose of lignocellulosic material waste as wheat bran. The co-immobilization of the β-xylosidase and the xylanase of *T. thermophilus* allowed the increase of xylan saccharification efficiency by liberation of xylose, proving the synergistic action of both enzymes.

1. Introduction

Hemicelluloses are plant heteropolysaccharides widely distributed in nature. Xylan, the most common component of hemicellulose is rich in xylose but contains also L-arabinose, acetyl, glucuronic, 4-0-methyl glucuronic and p-coumaric side chains [1]. The extensive degradation of the xylan into monosaccharides is often achieved by the synergistic action of several enzymes but the main ones are: endo β-1-4-xylanase (β-1,4-D-xylan xylanohydrolase; EC 3.2.1.8), and β-xylosidase (1,4-xylan xylohydrolase EC 3.2.1.37) [2]. Xylanases are mainly responsible for the hydrolysis of xylan and xylo-oligosaccharides.

Xylanases have been the focus of research owing to their industrial potential in many fields. They are, for instance, useful in pulp and paper industries particularly for the facilitative role they play in the bleaching of craft pulp in order to reduce the amount of chlorine required for target pulp brightness [3]. Xylanases are also useful in animal foodstuff industry for improving the nutritive quality of animal food. They increase lignin extractability and the release of chromophores from pulp. Xylanases have also found use in the baking industry for improving dough quality and bread volume. Furthermore, they are useful in the bioconversion of lignocellulosic materials to fuels and chemicals. A recent and exciting application of endoxylanases is the production of xylo-oligosaccharides with huge commercial value, because xylo-oligosaccharides, especially xylobiose, have been found to have a stimulatory effect on the selective growth of human intestinal *Bifidobacteria*, and are frequently defined as prebiotics [4–6].

The utilisation of thermostable enzymes might improve the technical and economic feasibility of all these biotechnological processes.

Because of the industrial potential of xylanases, a large number of studies have become interested in their immobilization for industrial application. Many advantages are gained through this technique and include the possibility of enzyme reuse, the enhancement of thermal stability, the easification of catalyst separation from the reaction mixture and the readiness application in automated continuous processes [7].

The covalent immobilization of enzymes can be achieved in various ways, depending on the carrier on which the bonding is intended. It generally involves the use of a reactive group already present in the polymer, for the enzyme bonding or the creation of a desired reactive group in the polymer. For instance, immobilization can be readily performed on poly-microspheres using the COOH-group [8]. Among the most commonly known carriers, gelatin and chitosan are often preferred. Both of them present high levels of biocompatibility and non-toxicity. Gelatin is a naturally occurring polymer produced by the partial hydrolysis of collagen. The reactive groups present in gelatine are primarily hydroxyl, carboxyl and

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amino functions. Their abundances are approximately 100, 75 and 30 mequiv. (per 100 g of gelatine), respectively [9,10]. Gelatin is available world wide at low cost. It has the biological properties of collagen [11].

Chitosan (D-glucosamine) is also a naturally occurring and readily available material. It is commercially produced from the waste products of the fishing industry. It is considered to be a suitable carrier for enzyme immobilization thanks to its high levels of non-toxicity, user-friendliness and protein affinity that make it appealing to a wide range of industries [12,13].

In fact, both chitosan and gelatine offer the advantage of a variety of reactive groups (amino groups) that can be useful for conducting successful immobilization of certain enzymes. The application of the immobilized enzyme, its technical setup and its assessment on several agro-industrial by-products are presented in this paper. The findings suggest that its application allows superseding several problems pertaining to enzyme stability, reuse, and continuous operation in various kinds of industries.

The present study aimed to immobilize xylanase from *Talaromyces thermophilus* and to engage in a comparative investigation that contrasts the reactive and catalytic of the free and immobilized enzymes. Application of the immobilized xylanase was assessed on several agro-industrial by-products for large scale use.

2. Materials and methods

2.1. Chemicals

Chitosan, chitin, birchwood xylan, oat spelt xylan, *p*-nitropenyl *β*-D-xylopyranoside (*p*NPX), glutaraldehyde (GA), acrylamide, and series of xylo-oligosaccharides, used as standards for exchange chromatography, were obtained from Sigma. The gelatin was provided by Amersham. DEAE-Sephadex was from Pharmacia. Column for high-pressure liquid chromatography (HPLC) was from Bio-Rad.

2.2. Methods

2.2.1. Microorganism and culture conditions

The present study reports on a newly isolated thermotolerant fungal strain from a soil sample collected in the thermal station of El Hamma in the south of Tunisia. The fungal isolate was identified as *T. thermophilus stolk* by CBS (Centraalbureau voor schimmelculturen, Holland) Code reference: detail 274-2003. The deposit number of *T. thermophilus* in the national strain bank of Tunisia (Tunisian Collection of Micro-organisms CTM 10.103) (Centre of Biotechnology of Sfax, Tunisia). The *T. thermophilus* was cultivated in a modified liquid Mandels medium [14]: KH_2PO_4, 1 g: K_2HPO_4, 2.5 g; $(NH_4)_2SO_4$, 1.4 g; $MgSO_4{\cdot}7H_2O$, 0.3 g; $CaCl_2$, 0.3 g; yeast extract, 1 g; urea, 0.7 g; Tween 80, 1 mL; water, 1 l and 2% wheat bran. The pH of the medium was 7.0 and was supplemented with 1 mL of an oligoelements solution with $MnSO_4$, 1.6 g/l; $ZnSO_4$, 1.4 g/l; $FeSO_4$, 5 g/l and $CoCl_2$, 2 g/l. The enzyme production was carried out in 500-mL flasks containing 100 mL of culture medium that was incubated at 50 °C and at an agitation rate of 150 rpm for 5 days.

2.2.2. Xylanase purification

The purification of the xylanase was carried out in three steps. The first step involved ammonium sulphate precipitation (80% saturation) of 1000 mL cell-free supernatant. The saturated solution was left stirring overnight at 4 °C, centrifuged and the precipitate was dissolved in the minimum volume of 20 mM phosphate buffer (pH 7). After dialysis against the same buffer, 20 mL of concentrated proteins were then loaded on an anion exchange DEAE-cellulose column (2.5 cm × 30 cm) which was pre-equilibrated with potassium phosphate buffer. The column was operated at a flow rate of 25 mL/h. A continuous NaCl gradient (0–1 M) was applied and fractions (3 mL each) were collected. The fractions showing xylanase activity were then pooled and dialyzed against the same buffer at 4 °C. The dialyzed enzyme preparation was loaded onto a Bio-Gel P-100 column (2.5 cm × 80 cm), previously equilibrated with the same buffer at a flow rate of 15.0 mL/h. The active fractions were pooled, dialyzed against distilled water and then concentrated by freeze-drying. In each step, the protein content and xylanase activity were determined.

2.2.3. Determination of xylanase and β-xylosidase activity

Xylanase activity was determined by measuring the reducing sugar released from birchwood xylan (Sigma) according to the method of Miller [15]. Unless specified otherwise, the assay mixture consisted of 0.5 mL xylan (1%, w/v) in 50 mM phosphate buffer pH 7.0 and 0.5 mL of enzyme solution or a corresponding weighted amount of the immobilized enzyme.

The reaction was incubated at 50 °C for 10 min. One unit of enzyme activity is defined as the amount of enzyme required to produce 1 μmol of xylose equivalent/min from xylan under assay conditions.

The activity of the soluble or immobilized form of β-xylosidase was determined according to Yanai and Sato [6] by the release of *p*-nitrophenol from 2 mM solution of *p*NPX (50 mM phosphate buffer (pH 7.0)). The activity of the soluble enzyme was assayed by the addition of 0.2 mL of enzyme solution to 0.2 mL of substrate. The reaction at 50 °C was terminated after 10 min by the addition of 1.6 mL of 1 M Na_2CO_3 solution. A blank containing buffer instead of enzyme solution was used to correct by accounting the thermal hydrolysis of *p*NPX. The determination of immobilized β-xylosidase activity was initiated by the addition of 0.1 g of support containing immobilized enzyme to 0.2 mL of the substrate (2 mM solution of *p*NPX in 50 mM phosphate buffer, pH 7.0). The absorbance at 405 nm due to the release of *p*-nitrophenol was measured. One unit of β-xylosidase activity is defined as the amount of enzyme required to liberate 1 μmol of *p*-nitrophenol from *p*NPX/min.

2.2.4. Immobilization methods

The purified xylanase from *T. thermophilus* culture was immobilised by different methods on different carriers. Immobilization steps and enzyme storage were carried out at 4 °C. Supernatants and washing volumes were pooled after each step and the non-immobilized activity was determined.

- Ionic binding (DEAE-Sephadex)

  1 g of DEAE-Sephadex resin was washed twice with a 50 mM phosphate buffer pH 7.0 and centrifuged for 2 min at 4600 × *g*. The resin was mixed with 0.5 mL of the enzyme preparation (67 U/mL) and 1.5 mL phosphate buffer during 20 min under agitation. The mixture was then washed twice with 25 mM phosphate buffer and centrifuged for 2 min at 4600 × *g*.
- Inclusion in polyacrylamide

  The immobilization in polyacrylamide gel was achieved by mixing 3 mL of a solution of acrylamide and bis acrylamide (30:1), 4 mL water, 2 mL of 100 mM Tris–HCl buffer pH 7.0 and 1 mL enzyme (67 U/mL) and the polymerization was achieved by the addition of 100 μL ammonium persulphate and 6 μL TEMED. The gel film was polymerized at 4 °C on a surface of 5 cm × 5 cm and cut into small blocks (1 cm × 1 cm). The gel pieces were washed twice with 25 mM phosphate buffer pH 7 before use.
- Covalent binding by glutaraldehyde to chitosan, chitin, Amberlite, Duolite, florisil and gelatin

  Chitosan (0.5 g) was dissolved in 50 mL of HCl 0.1 M containing 2.5% (v/v) glutaraldehyde for 2 h at 30 °C. The solubilized chitosan

was precipitated by the addition of 1 mL of 0.1 M NaOH. The precipitate was separated by centrifugation (10 min at 4000 × *g*) and washed with distilled water to eliminate the glutaraldehyde excess. The wet chitosan was mixed with 0.5 mL of the enzyme solution and stirred for a night at 4 °C. The unbound enzyme was removed by washing with distilled water until no protein or activity was detected [16]. Chitin (0.5 g) was shaken with 5 mL 2.5% (v/v) glutaraldehyde. Chitin was then collected by centrifugation (10 min at 4000 × *g*) and washed with distilled water to remove the glutaraldehyde excess. The wet chitin was mixed with 0.5 mL of the enzyme solution for a night at 4 °C. The unbound enzyme was removed by washing with distilled water as described early [16].

The gelatin powder (5–10%, w/v) used for immobilization of the xylanase enzyme was swelled in 5 mL potassium phosphate buffer 50 mM pH 7.0 and heated at 50 °C for 10 min till complete solubilization of gelatin. The mixture was cooled and the enzyme was added (67 U/mL). After mixing of enzyme, the required amount of organic cross-linker (0.6%, w/v), glutaraldehyde was added. The mixture was stirred constantly and poured on a 5 cm × 5 cm glass plate to prepare a thin film of the enzyme. The film was stored at 4 °C for complete cross-linking. The immobilized enzyme film was thoroughly washed with 50 mM phosphate buffer pH 7.0 and cut into small blocks (1 cm × 1 cm) before being eventually used in subsequent experiments.

The immobilization yield was expressed by the following equation:

$$\text{immobilization yield}\,(\%) = \frac{A-B}{A} \times 100.$$

And the activity yield was defined according to the following expression:

$$\text{activity yield}\,(\%) = \frac{C}{A} \times 100.$$

As the equations above indicate, various parameters were used in the estimation of immobilization: where *A* is the total enzyme activity used for immobilization; *B* is the unbound enzyme activity; $(A - B)$ is the theoretical immobilized enzyme activity; and *C* is the obtained immobilized enzyme activity. The total enzyme activity is the total number of units added to the support during the immobilization reaction; the non-immobilized activity is the number of units found in filtrates and washing volumes after immobilization; and the immobilized activity is the number of units detected in the support after immobilization and washing.

2.2.5. Properties of the free and immobilized xylanase

2.2.5.1. Effect of pH. The effect of pH on the free and immobilized xylanase was studied using sodium acetate buffer (pH 3–6, 50 mM), potassium phosphate buffer (pH 6–8, 50 mM) and Ampso buffer (pH 8–10, 50 mM).

2.2.5.2. Thermostability. The free enzyme as well as the immobilized one were incubated in phosphate buffer 50 mM pH 7.0 at different temperature (40–100 °C) for different periods of time. The residual xylanase activities were measured according to the standard assay method.

2.2.5.3. Optimum temperature. The optimum temperature for xylanase activity was determined by incubating the enzyme in 0.05 M phosphate buffer pH 7.0 at different temperature (40–100 °C). In each case, the substrate was pre-incubated at the required temperature before the addition of the enzyme.

2.2.5.4. Effect of additives. Various metals and others reagents at 10 mM were added to the standard enzymatic reaction. The relative activity was expressed as the percentage of the residual activity compared to 100% of activity observed in the absence of any compound.

2.2.5.5. Operational stability of the immobilized enzyme. A block of gelatin immobilized xylanase of the *T. thermophilus* containing about 2.7 U was incubated with 500 μL of 1% (w/v) birchwood xylan in phosphate buffer (50 mM, pH 7.0) at 50 °C for 10 min.

At the end of the reaction, the immobilized enzyme was collected, washed with distilled water and resuspended in 500 μL of freshly prepared substrate to start a new run. The supernatant was assayed for reducing sugars. The presented results are the average of three replicates of two separate experiments.

2.2.6. Hydrolysis of agro-industrial residues by immobilized enzyme

A block of 1 cm^2 of immobilized enzyme film corresponding to 2.7 U/mL was incubated with different agro-industrial residues during 30 min at 50 °C and pH 7.0. Aliquots were withdrawn and analyzed by measuring of the liberated reducing sugars by DNS method [15].

2.2.7. HPLC analysis of hydrolysis products of xylan

Xylan was hydrolyzed by three forms of enzymes (crude, purified free and immobilized enzyme) during 7 h at 50 °C and pH 7.0. At suitable time intervals, aliquots were withdrawn and analyzed by measuring the liberated sugars by DNS method and by HPLC (Aminex HPX-42A, column 7.8 mm × 300 mm). In the latter method the products were separated by elution with water at a flow rate of 0.6 mL/min, and detected with a refractive index detector (SHIMADZU, RID-10A). A solution of oligosaccharides (X_1, X_2, X_3), at 10 g/l each, was used as a standard.

2.2.8. Continuous hydrolysis of xylan by combined xylanase and β-xylosidase immobilized on chitosan

Chitosan containing the two co-immobilized enzymes (xylanase at 133 μg/g of support and β-xylosidase at 50 μg/g of support) was incubated in batch for 7 h at 50 °C in the presence of 3% of xylan Oat spelt that was pre-dissolved in 50 mM phosphate buffer.

The immobilized yield of xylanase and β-xylosidase was 89% and 87%, respectively.

At suitable time intervals, aliquots were analyzed by measuring the liberated reducing sugars.

2.2.8.1. Reproducibility. All the experiments were repeated at least four times and the results were reproducible. The data points represented the mean values within ±4.0% of the individual values.

3. Results and discussion

3.1. Selectivity of gelatine to bind xylanase

After purification [17], xylanase was immobilized on different carriers. Amberlite, DEAE-Sephadex, Duolite, chitin, chitosan, Florisil, polyacrylamide and gelatin were chosen for this study. Table 1 shows the activity and the immobilization yields using these polymers. Our results show that the yield of enzyme activity depended on the nature of the polymer used for immobilization. Gelatin at 10% retained the highest activity of the xylanase from *T. thermophilus* with the required amount of organic cross-linker (0.6%, w/v), glutaraldehyde was added. So, xylanase was covalently

Table 1
Xylanase immobilization and activity yields on different matrixes. DEAE-Sephadex, Amberlite, Duolite, Fluorosil, chitin, gelatine, polyacrylamide, chitosan.

Type of resin	DEAE-Sephadex	Amberlite	Duolite	Florisil	Chitin	Gelatin	Polyacrylamide	Chitosan
Activity yield (%)	85.0	95.2	98.2	55.8	100	100	77.0	96.8
Immobilization yield (%)	82.0	89.3	81.1	96.2	87.8	98.8	97.6	89.0

coupled on gelatin, chitosan through cross-linking mediated by glutaraldehyde after optimization of its concentration. It was noted that glutaraldehyde has an important role in the process of enzyme immobilization by covalent coupling. The number of covalent bonds between the support and the enzyme depended on the support activation degree (concentration of aldehyde groups in the support surface) and on the concentration of amine groups in the enzyme molecule [18].

With chitosan a high yield of xylanase activity was also observed after immobilization (96.8% and 89% of activity and immobilization yields, respectively) (Table 1). The decrease of activity with the other carriers could be attributed to diffusional limitation of the substrate and product [19]. These diffusional problems become more significant with macromolecular substrates, like xylan.

However, with more or less satisfactory activity and immobilization yields that reached up to 100% and 98.8%, respectively, a further characterization of the immobilized preparation was deemed necessary.

3.2. Characterization of the immobilized preparation

3.2.1. pH optimum and stability

To further characterize the immobilized preparation, pH and temperature dependence of enzymatic activities was investigated. Fig. 1 shows the activity of free and immobilized *T. thermophilus* xylanase at different pH values. The data indicate that the immobilized enzyme exhibited a shift of pH from 7 to 8 when compared to the free enzyme.

An improvement of pH stability upon immobilization was also observed. Such improvement has been obtained especially at low pH values (acidic conditions). This characteristic is particularly required in paper and agro-industrial fields. The residual immobilized xylanase activity is 90% at pH 6 and 9 and 70% at pH 4 and 10. However, at pH 11, 50% of xylanase activity was observed. These results show that immobilized xylanase can act at different pH values, ranging from 4 to 11, exhibiting more than 50% of the initial activity.

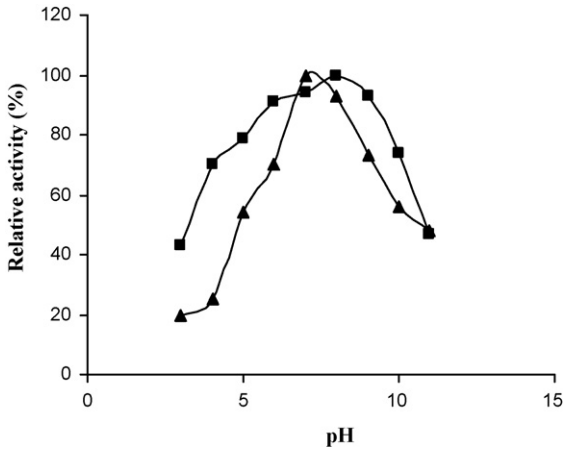

Fig. 1. Effect of pH on free and gelatin-immobilized xylanase. Enzyme activity was measured at 50 °C with 1% xylan buffered at different pH. Values reported in the figure are the means of determination performed in triplicate. Free enzyme (▲); immobilized enzyme (■).

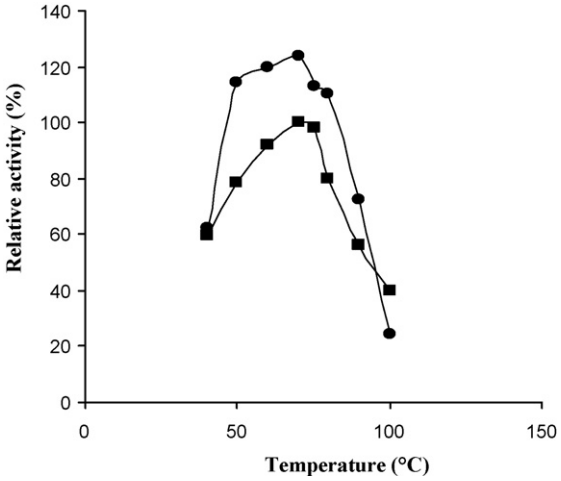

Fig. 2. Effect of temperature on free and immobilized xylanase. Enzyme activity was measured at different temperatures with 1% xylan dissolved in 50 mM phosphate buffer, pH 7.0. Values reported in the figure are the means of determination performed in triplicate. Free enzyme (■); immobilized enzyme (●).

3.2.2. Effect of temperature on immobilized enzyme activity and thermostability

This high thermostability is of special interest in biotechnological and pharmaceutical applications. The dependence of the soluble and immobilized xylanase activities on temperature was studied in 0.05 M phosphate buffer pH 7.0. The results indicated that the free and immobilized enzymes tented to have similar optimal temperatures for activity of about 75 °C (Fig. 2). The highest stability was observed at temperatures ranging between 50 and 80 °C.

The *T. thermophilus* xylanase had higher stability (100% of activity for 10 days at 50 °C) (data not shown) than other xylanases of thermophilic fungi [17,20,21]. The thermostability of free and immobilized xylanase was assessed in the temperature range of 65–100 °C at pH 7.0 during 10 h (Fig. 3). The immobilized xylanase activity was more stable than that of the free enzyme and retained 50% of its activity after 3 h incubation at 100 °C, whereas the free enzyme retained only 50% of activity after 2 h incubation at the same temperature.

The free and immobilized xylanase retained 45% and 80% of their optimum activities respectively after a 3 h incubation at 90 °C. These

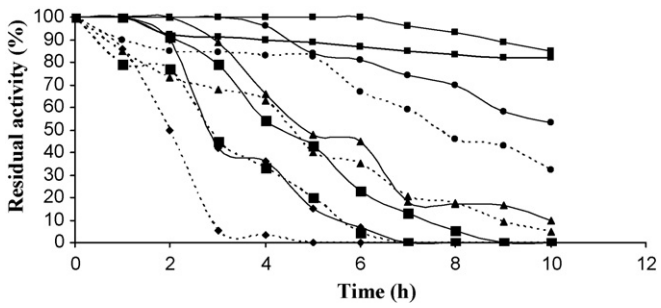

Fig. 3. Thermostability of the free and immobilized enzyme. Free and gelatin-immobilized enzyme at 65 (■); 75 (●); 80 (▲); 90 (■) and 100 °C (♦). Residual enzyme activity was determined in the periodically withdrawn samples using xylan as substrate. Free xylanase (- - -); gelatin-immobilized xylanase (—).

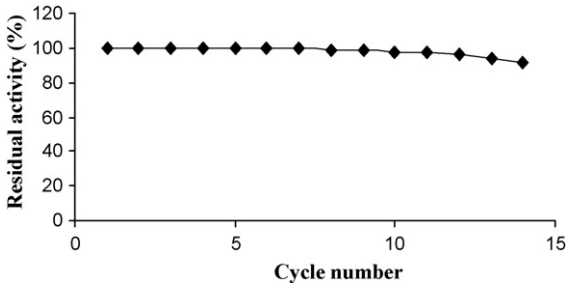

Fig. 4. Cycle number of the immobilized enzyme. The immobilized preparation was reused consecutively for 13 cycles.

Table 2
Effect of additives on immobilized and free enzyme.

Reagents 10 mM	Relative activity (%) free enzyme	Relative activity (%) immobilized enzyme
Control	100	100
$CaCl_2$	103 ± 0.03	74.5 ± 0.02
$ZnCl_2$	80.0 ± 0.03	79.0 ± 0.04
$CoCl_2$	112 ± 0.04	136 ± 0.01
$FeSO_4$	80.0 ± 0.00	80.0 ± 0.00
$CuSO_4$	105 ± 0.01	78.4 ± 0.03
$MgSO_4$	80.0 ± 0.02	77.5 ± 0.02
$MnSO_4$	65.0 ± 0.01	33.0 ± 0.02
DTT	85.0 ± 0.03	85.0 ± 0.02
Urea	89.0 ± 0.03	90.0 ± 0.04
EDTA	90.2 ± 0.03	66.4 ± 0.02
SDS	109 ± 0.04	71.0 ± 0.04

results clearly demonstrate the effectiveness and potential of the immobilized xylanase of *T. thermophilus*.

The increase in the thermostability of the immobilized xylanase could be attributed to the fact that the latter is less susceptible to conformational changes caused by temperature after immobilization into gelatin system while the quaternary structure of the free enzyme can be easily disaggregated [22].

3.2.3. Reusability of the immobilized enzyme

The reusability of the immobilized preparation was evaluated in repeated batch processes. The immobilized enzyme exhibited more than 94% of the initial catalytic activity after 13 cycles of use. Each enzyme activity cycle lasted for 10 min (Fig. 4). The operational stability of the immobilized *T. thermophilus* xylanase was much higher than that reported by Gouda and Abdel-Naby [23] for *Aspergillus tamarii* xylanase immobilized on Duolite A147 which retained only 73% of the initial activity after 8 cycles of use. It was also higher than that of the xylanase of *Thermomyces lanuginosus* immobilized on Eudragit S-100 which maintained only 62% of its activity after 6 cycles of use [24] and than the xylanase of *Thermotoga maritime* immobilized on nickel-chelated Eupergit C which retained 92.5% after 10 cycles of use [25].

3.2.4. Effect of additives on free and immobilized xylanase

The effect of various additives on free and immobilized *T. thermophilus* xylanase was determined by measuring the residual activity in the presence of 10 mM of each compound (Table 2).

Co^{2+} activated the free and immobilized enzyme but Mn^{2+}, Mg^{2+}, urea and EDTA adversely affected the activity of both enzyme preparations. DTT had no effect on xylanase activity. However, it was observed that the inhibitory effects of Mn^{2+}, Cu^{2+}, Ca^{2+} and SDS were higher with the immobilized enzyme.

3.2.5. Kinetics study

In order to study the kinetic parameters of immobilized activity, the rates of xylan hydrolysis by the free and immobilized xylanase were measured at various xylan concentrations (0.05–2.5 mM). The K_m values of free and immobilized xylanases for xylan were calculated using hyper 32 programm and were found to be 22.5 and 25.6 mg/mL at 50 °C, respectively (Table 3). Several researchers reported that the K_m could change slightly after the immobilization of xylanases on Eudragit S-100 or Eudragit L-100 [26,27].

Moderate increases in K_m value upon immobilization, however, have been frequently reported when the matrix was insoluble or at solid form [23,28,7].

The V_m values for the free and immobilized enzyme were found to be 1.2 and 0.96 μmol/min at 50 °C, respectively. The slight increase of the K_m value and the slight decrease of the V_m after xylanase immobilization may be partially due to the limited accessibility of xylan molecules to the active sites of the immobilized xylanase as a result of the spatial distribution of the xylanase molecules in the polymer layer and conformational changes of xylanase caused by the immobilization [19,7].

Gelatin is a protein which contains hydrophilic and hydrophobic groups, unlike chitosan which belongs to polysaccharides. The presence of the hydrophilic groups in the gelatine layer could have lead to the adsorption of the hydrophobic xylo-oligosaccharides on the membrane through hydrophobic interaction and, thus, could have influenced the kinetic parameters of the enzyme [29].

3.3. Hydrolysis of xylan and agro-industrial residues

Xylan was hydrolyzed by the free and immobilized enzyme. The hydrolysis products were analyzed quantitatively by measuring the released reducing sugars by DNS method and by determining the sugar composition by HPLC.

The amount of xylose generated by the immobilized enzyme was less important compared to that of the free enzyme. This decrease could be due to the low accessibility of the substrate to the active site of the fixed enzyme (Fig. 5). At the beginning of the reaction, the quantity of reducing sugars released by the crude preparation and the free enzyme was higher than that of the immobilized enzyme. This quantity was fivefold higher at 50 °C (Fig. 5aA) and threefold higher at 70 °C (Fig. 5Ba).

After 6 h of incubation, the quantity of reducing sugars released by the fixed enzyme increased and exceeded that of the free enzyme and the crude preparation. This could be explained by the better thermostability of the immobilized enzyme.

The released sugars were analyzed by HPLC, using mainly xylose, xylobiose and xylotriose as standards. During the hydrolysis reaction, the free and immobilized enzymes released mainly xylobiose (Fig. 5bA and bB), which is the major product of xylan degradation by xylanase enzymes.

However, the crude xylanase preparation released mainly xylose (data not shown), which is probably due to the presence of other enzymes that contributed to the degradation of the oligosaccharides, particularly the β-xylosidase which degrades xylobiose to

Table 3
Kinetic parameters of immobilized and free *Talaromyces thermophilus* xylanase.

Substrate	Immobilized enzyme		Free enzyme	
	K_m (mg/mL)	V_{max} (μmol/(mL min))	K_m (mg/mL)	V_{max} (μmol/(mL min))
Birchwood xylan at 50 °C	25.6	0.96	22.5	1.2

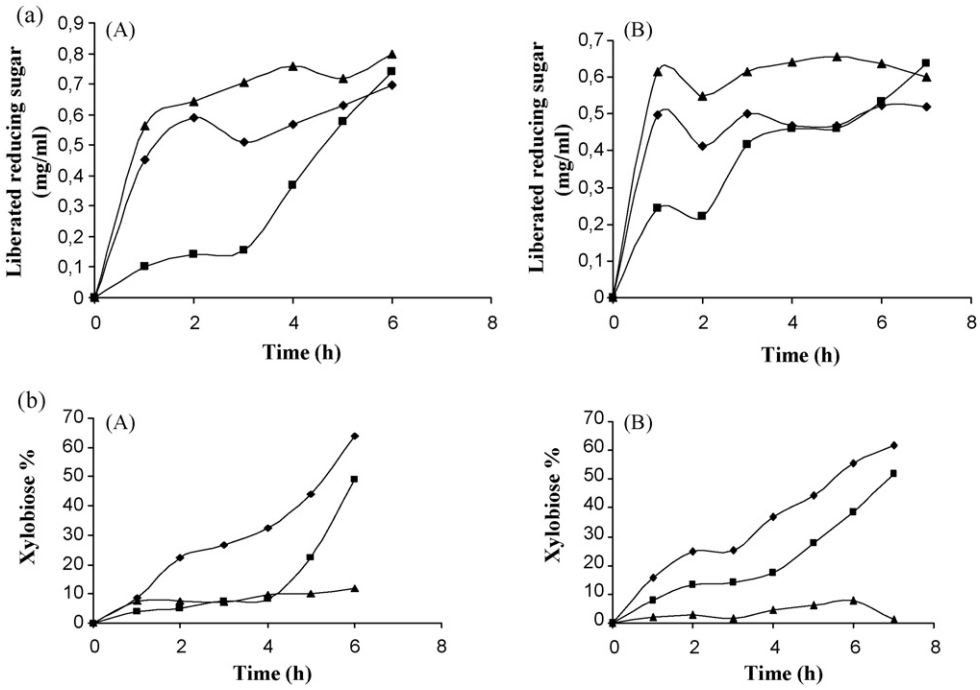

Fig. 5. Hydrolysis of xylan by the free, immobilized and crude enzyme at 50 °C (A) and at 70 °C (B). (a) Measuring of liberated reducing sugar; (b) HPLC analysis of hydrolysis products. The free enzyme (●); immobilized enzyme (■) and crude enzyme (▲).

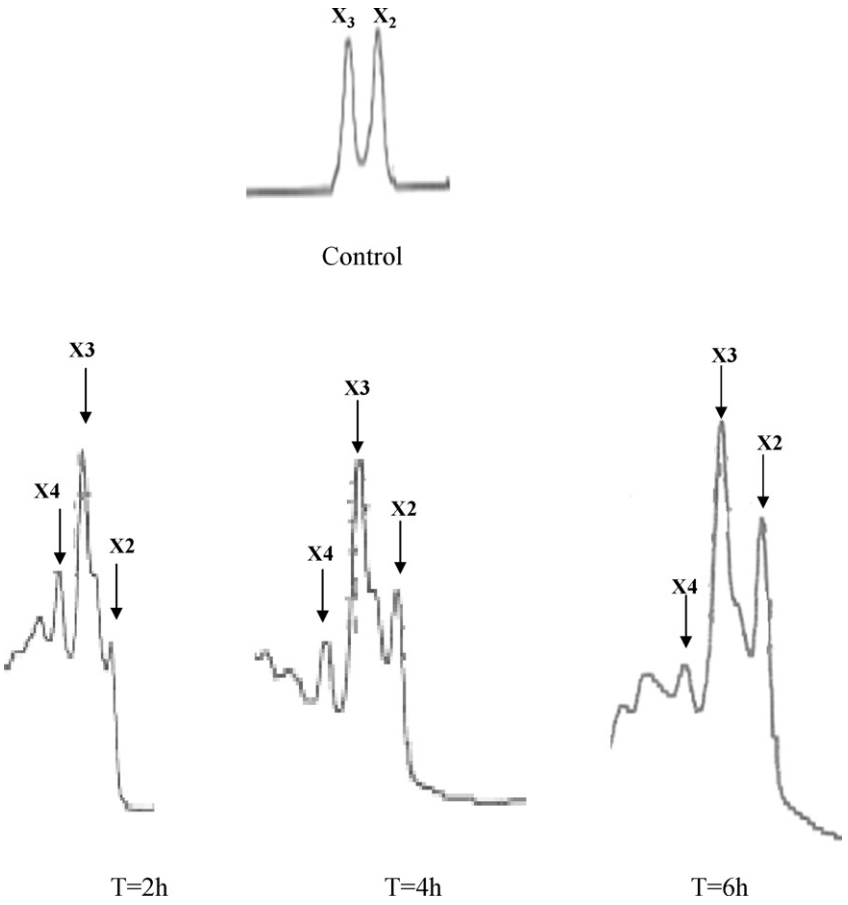

Fig. 6. Chromatographic response analyses of xylo-oligosaccharide oat spelt xylan hydrolysis by the gelatin-immobilization xylanase in different times (2, 4, and 6 h). X_2: xylobiose, X_3: xylotriose, X_4: xylotetraose.

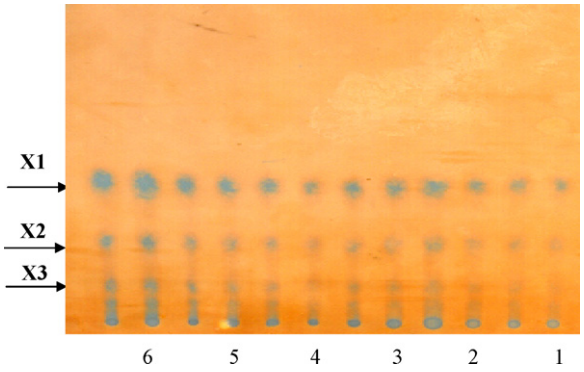

Fig. 7. TLC analysis of the hydrolyzed mixture derived from continuous hydrolysis of oat spelt xylan by the immobilized xylanase. X_1: xylose; X_2: xylobiose and X_3: xylotriose. Reaction time (h) is indicated.

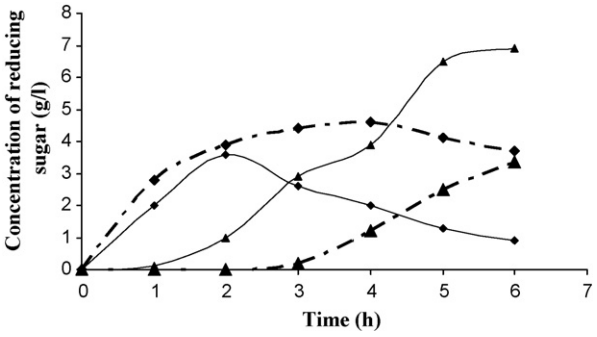

Fig. 8. Hydrolysis of the xylan by the co-immobilization of xylanase and β-xylosidase. The co-immobilization of xylanase and β-xylosidase (—); gelatin-immobilized xylanase alone (- - -). The hydrolysis products were detected X_1 (▲) and X_2 (♦).

xylose [30]. This explains the presence of a small quantity of xylobiose detected after the hydrolysis by the crude enzyme preparation (Fig. 5bA and bB).

During the initial incubation of xylan and agro-industrial residues hydrolysis, xylotriose was the major product liberated by the free and immobilized enzyme. After a long-term incubation period of more than 6 h, however, the end-products advanced a xylobiose rich content, indicating that a part of the xylotriose or xylotetrose was converted to xylobiose (Fig. 6). The hydrolysis products of xylan by the immobilized xylanase in a continuous packed-bed reactor were also analyzed by TLC (Fig. 7). It should be noted that xylobiose has a stimulative effect on the selective growth of *Bifidobacteria* of the human intestine, which is important for maintaining a healthy intestinal microbial flora [31].

The immobilized xylanase has been widely used to produce xylo-oligosaccharides. Results obtained (Fig. 7) show that oligosaccharides with comparatively high polymerization formed in the initial stage of reaction are progressively degraded to oligosaccharides with lower DP. In the late reaction stages, the xylo-oligosaccharides with low DP tend to be hydrolyzed into xylose.

Thus, the immobilized xylanase of *T. thermophilus* can advantageously be applied for the production of xylo-oligosaccharides, mainly xylobiose from xylan, and other substrates such as wheat bran, barley, corn hull and rice straw.

In the present study, the immobilized xylanase released 197.5 mg of reducing sugars per gram of xylan, which was used as a control.

Agro-industrial residues hydrolysis by immobilized *T. thermophilus* xylanase gave different amount of reducing sugars: 88.4 mg/g of wheat bran, 62 mg/g of wheat kernel and 26 mg/g of rabbit food (Table 4).

The increasing interest in biotechnological processes employing lignocellulosic residues is quite justifiable because these materials are cheap, renewable and readily available sources of sugars [32].

Table 4
Hydrolysis of agro-industrial residues by the free and immobilized enzyme.

Sample	Amount of liberated sugar (mg/g of substrate) by immobilized enzyme	Amount of liberated sugar (mg/g of substrate) by free enzyme
Grinded wheat	9.0 ± 0.005	4.0 ± 0.01
Grinded corn	2.0 ± 0.005	1.8 ± 0.005
Grinded barley	16 ± 0.01	8.2 ± 0.01
Wheat kernel	62 ± 0.03	40 ± 0.03
Rabbit food	26 ± 0.02	27 ± 0.03
Rice straw	4.9 ± 0.005	3.2 ± 0.005
Wheat bran	88.4 ± 0.04	78 ± 0.03
Xylan	197 ± 0.03	131 ± 0.04

These conversion abilities of agro-industrial residues are very important since inexpensive hemicelluloses rich by-products are the major potential substrates for useful xylo-oligosaccharides production [33].

Most of free and immobilized xylanases can hydrolyze xylans to yield xylo-oligosaccharides with DP 2–5 [34].

3.4. Synergistic hydrolysis of xylan by the co-immobilized β-xylosidase and xylanase

Xylose yields as a major product of xylan hydrolysis can be improved when β-xylosidase was added to a purified xylanase reaction mixture (data not shown) or when both were immobilized simultaneously on the same carrier which was the chitosan. Fig. 8 shows that after co-immobilization, 7 g/l of xylose was obtained. This amount was 10 times higher than that achieved with the free xylanase (Fig. 5aA) and was 2 times higher compared to that containing gelatin-immobilized xylanase alone. This is due to the presence of the β-xylosidase which can act on oligomers of small degree of polymerization (DP) and consequently xylobiose, liberated by xylanase, was immediately transformed into xylose. On the other hand, the use of β-xylosidase alone, even for a long-time, did not catalyze any xylose release from xylan, while the addition of xylanase resulted in a significant increase in xylobiose and xylotriose liberation during reaction at pH 7 and at 50 °C. These findings suggest that the degradation of xylan to xylose requires the synergistic action of β-xylosidase and xylanase. The action of xylanase and β-xylosidase of *Bacillus thermantarcticus* on xylan gave only xylose [35]. The co-immobilization of both xylanase and β-xylosidase was reported in other works and the synergy of these two enzymes is very important, since the oligosaccharides production is consistently improved [26,36].

4. Conclusions

The xylanase from *T. thermophilus* can be immobilized on gelatine with a high immobilization yield 98.8%. This strategy combines the excellent properties of gelatine as a carrier for covalent immobilization of enzymes with an advantageous low cost source for industrial use. The immobilized enzyme was stable and could be used many times without considerable loss of activity. Compared to the soluble form of the protein, the immobilized enzyme was more resistant to deactivation caused by changes in pH or temperatures.

The optimal temperature was not affected by immobilization but the optimal pH shifted from 7 to 8. Moreover, the hydrolysis of oat spelt xylan by the immobilized xylanase yielded xylobiose as a major end-product. This certainly prompts further investigations

on the continuous production of xylobiose on a large scale starting from lignocellulosic materials wastes.

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