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Catalytic properties of the immobilized *Talaromyces thermophilus* β-xylosidase and its use for xylose and xylooligosaccharides production

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ABSTRACT

The present study explores the efficiency of *Talaromyces thermophilus* β-xylosidase, in the production of xylose and xylooligosaccharides. The β-xylosidase was immobilized by different methods namely ionic binding, entrapment and covalent coupling and using various carriers. Chitosan, pre-treated with glutaraldehyde, was selected as the best support material for β-xylosidase immobilization; it gave the highest immobilization and activity yields (94%, 87%, respectively) of initial activity, and also provided the highest stability, retaining 94% of its initial activity even after being recycled 25 times. Shifts in the optimal temperature and pH were observed for the immobilized β-xylosidase when compared to the free enzyme. The maximal activity obtained for the immobilized enzyme was achieved at pH 8.0 and 53 °C, whereas that for the free enzyme was obtained at pH 7.0 and 50 °C. The immobilized enzyme was more thermostable than the free β-xylosidase. We observed an increase of the K_m values of the free enzyme from 2.37 to 3.42 mM at the immobilized state. Native and immobilized β-xylosidase were found to be stimulated by Ca^{2+}, Mn^{2+} and Co^{2+} and to be inhibited by Zn^{2+}, Cu^{2+}, Hg^{2+}, Fe^{2+}, EDTA and SDS. Immobilized enzyme was found to catalyze the reverse hydrolysis reaction, forming xylooligosaccharides in the presence of a high concentration of xylose. In order to examine the synergistic action of xylanase and β-xylosidase of *T. thermophilus*, these two enzymes were co-immobilized on chitosan. A continuous hydrolysis of 3% Oat spelt xylan at 50 °C was performed and better hydrolysis yields and higher amount of xylose was obtained.

1. Introduction

Enzymes are biocatalysts with high specificity, catalytic efficiency and bio-degradability and are becoming popular in diverse industrial and medical applications particularly for their abilities to speed up the rate of chemical reactions by lowering the activation energy [1]. However, the effective use of enzymes has often been restricted because of certain shortenings, such as their non-reusability, instability and sensitivity to denaturation. Their use has also been deterred by the high cost associated with their isolation, purification and characterization, as well as with the difficult recovery of active enzymes from reaction mixtures.

These restrictions, which remain a challenge for the application of free enzymes in biotransformation and chemical processes, have actually been eased by the use of immobilized enzymes. More succinctly, the immobilization of enzymes on various water-insoluble supports have been reported to improve biocatalysts long-term stability, to allow enzyme reusability and application in continuous operations, and to minimize the time and cost burdens associated with those activities [2].

This immobilization can be accomplished by physical or chemical methods [3]. Based on their characteristics, the currently used protein immobilization techniques can be classified into five broad categories; physical adsorption [4], entrapment in a matrix [5], ionic binding [6], covalent binding [7] and cross-linking [8]. The later involves the formation of covalent bonds between the support material and enzyme molecules through the employment of a bi- or multifunctional reagent.

Enzyme immobilization is often accompanied by a number of changes that affect enzymatic activity, optimum pH, affinity to the substrate, etc. The extent of these changes is known to depend upon the type of the enzyme and carrier support as well as on the conditions in which immobilization is performed [9].

β-Xylosidase (EC 3.2.1.37) is one of the component enzymes of the hemicellulase complex. It catalyzes the hydrolysis of xylooligosaccharides, such as xylobiose and xylotriose, to xylose by recognizing the xylosyl residue at the non-reducing end and by cleaving the β-1,4 glycosidic bonds [10]. β-Xylosidase is, therefore, a key enzyme in the xylanolytic system with a great potential in many biotechnological applications, particularly in paper pulp,

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food, beverage, and animal foodstuff bioconversion industries [11]. β-Xylosidases are often classified into five families: 3, 39, 43, 52, and 54, based on amino acid sequence similarities [12,13]. These enzymes, as well as other glycosidases, have recently been proven to be powerful glycosynthases that can be used efficiently in the synthesis of oligosaccharides [14].

β-Xylosidases are particularly important as they exhibit the dual function of catalyzing both the hydrolysis and synthesis reactions. The immobilized enzyme used for the hydrolysis of xylo-oligosaccharides prepared from xylan [15,16] can also be used in the synthesis of xylo-oligosaccharides and alkyl-xylosides by transglucosylation reaction [17].

Only few studies have reported that, using a high xylose concentration, this enzyme can catalyze a condensation reaction and produce xylobiose, including β-xylosidase of *Aspergillus niger* and *Sporotrichum thermophile* [18,19].

The present study was undertaken to explore the immobilization of the β-xylosidase of *Talaromyces thermophilus* and to evaluate its hydrolytic efficiency for xylose production when compared to the free enzyme. The first step involved the immobilization of this β-xylosidase of *T. thermophilus* on different supports and the subsequent identification of its properties (kinetic parameters, temperature and pH optimum, thermal stability and reusability) compared to those of the free enzyme. The second step involved the application and subsequent evaluation of the immobilized enzyme for xylose production and xylooligosaccharide synthesis by oat spelt xylan hydrolysis and condensation reaction, respectively.

2. Materials and methods

2.1. Chemicals

Chitosan, chitin, xylan (beechwood, oat spelt), *p*-nitrophenyl β-D-xylopyranoside (*p*NPX), *p*-nitrophenol, glutaraldehyde, acrylamide, and series of oligosaccharides used as standard for (HPLC) chromatography were obtained from Sigma. Gelatin was from Amersham. DEAE-cellulose and DEAE-sephadex were from Pharmacia.

2.2. 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). The *T. thermophilus* was cultivated in a modified liquid Mandels medium [20]: 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 160 rpm for 5 days.

2.3. Enzyme preparation protocol

The production of β-xylosidase by *T. thermophilus* strain in submerged fermentation was examined. The strain was grown on optimized nutrient medium containing wheat bran as a carbon source. Cultivation was performed at 50 °C for 5 days. The extracellular proteins were recovered by centrifugation and the supernatant was treated with ammonium sulfate (80% saturation). The precipitate was collected by centrifugation at 9000 rpm for 15 min, dissolved in 20 mM phosphate buffer, pH 6.0, and then dialyzed overnight against the same buffer. The dialyzed enzyme solution was loaded on a DEAE-Cellulose column (1.25 × 28 cm) pre-equilibrated with 20 mM phosphate buffer, pH 8. The column was extensively washed with the same buffer. β-xylosidase activity was eluted with a gradient of 0–1 M NaCl in the same buffer, at a flow rate of 24 ml/h. The active fractions were pooled, concentrated using PEG 6000 and dialyzed overnight against 20 mM phosphate buffer, pH 6.0. The β-xylosidase obtained from the ion exchanger was further purified by gel filtration HPLC (Shodex, Protein kw P 802.5, 8 mm × 300 mm) equilibrated and eluted by 50 mM phosphate buffer, pH 6.0 at a flow rate of 0.8 ml/min. The highly active β-xylosidase fractions were pooled, concentrated and used as purified enzyme for subsequent studies. The purified enzyme has a high specific activity of 147.5 U/mg proteins.

2.4. Enzyme immobilization

T. thermophilus β-xylosidase was immobilized by different methods on different supports. The immobilization steps and enzyme storage were carried out at 4 °C. The supernatants and washing volumes were pooled after each step and non-immobilized activity was determined.

2.4.1. Ionic binding (DEAE-cellulose and DEAE-sephadex)

1 g resin DEAE-cellulose or DEAE-sephadex was washed twice with 50 mM phosphate pH 7.0 and centrifuged for 2 min at 4600 × *g*. The resin was mixed with 1 ml of the enzyme preparation (1.5 U/ml, 46 μg/ml) and 2 ml phosphate buffer for 20 min under agitation. The mixture was then washed twice with 25 mM phosphate buffer and centrifuged for 2 min at 4600 rpm.

2.4.2. Entrapment in polyacrylamide gel

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 100 mM Tris–HCl buffer pH 7 and 1 ml enzyme (1.5 U/ml). Polymerization was achieved by the addition of 100 μl ammonium persulfate at 1% 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. The gel pieces were washed twice with 25 mM phosphate buffer, pH 7.0, before use.

2.4.3. Covalent coupling (chitosan, chitin and gelatin)

Chitosan (0.5 g) was dissolved in 50 ml of 0.1 M HCl containing 2.5% (v/v) glutaraldehyde (GA) at 30 °C for 2 h. The solublized chitosan was precipitated by the addition of 1 ml NaOH (1.0 mol/l). The precipitate was separated by centrifugation (10 min at 6000 rpm) and washed with distilled water to remove excess of GA. The wet chitosan was mixed with 2.0 ml (1.5 U/ml) of the enzyme solution and stirred at 4 °C for 24 h. The unbound enzyme was removed by washing with phosphate buffer 20 mM until no protein or activity was detected [21]. This protocol was repeated in the presence of different GA concentrations (0.5–4%) to investigate the latter's effect on the immobilization and activity yields of the enzyme.

Chitin (0.5 g) was shaken with 5 ml 2.5% (v/v) GA. It was then collected by centrifugation (10 min at 6000 rpm) and washed with distilled water to remove excess of GA. The wet chitin was mixed with 2.0 ml of the enzyme solution at 4 °C for 24 h. The unbound enzyme was removed by washing with distilled water as described earlier [21].

The gelatin powder (5%, w/v) was swelled in 5 ml (50 mM) phosphate buffer (pH 7.0) and heated at 50 °C for 10 min to ensure its complete solubilization. The mixture was then cooled and the enzyme was added (1.5 U/ml). After the thorough mixing of the enzyme, the required amount of organic cross-linker (0.6% w/v) glutaraldehyde was added. The mixture was constantly stirred and

then 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 before being used in subsequent experiments.

The immobilization yield was expressed by the following equation:

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

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

$$\text{activity yield}\,(\%) = \left[\frac{C}{A}\right] \times 100$$

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; $A-B$ the theoretical immobilized enzyme activity; and C is the obtained immobilized enzyme activity [15]. 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.

2.5. Enzymes assay

The activity of the soluble and the immobilized form of β-xylosidase was determined according to Yanai and Sato [22] by the release of *p*-nitrophenol from *p*NPX in 50 mM phosphate buffer (pH 7.0). The activity of the soluble enzyme was assayed at 50 °C by the addition of 0.2 ml of the enzyme solution to 0.2 ml of the substrate. The reaction was terminated after 10 min by the addition of 1.6 ml of 1 M Na_2CO_3 solution. The determination of immobilized β-xylosidase activity was initiated by the addition of 0.1 g of support containing the 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 detected and measured. One unit of β-xylosidase activity was defined as the amount of enzyme required to liberate 1 μmol of *p*-nitrophenol from *p*NPX per minute.

Xylanase activity was assayed by measuring the reducing groups released from Birchwood xylan [23]. The reaction mixture consisted of 500 μl of 1% xylan solution, 400 μl of 50 mM phosphate buffer, pH 7.0, and 100 μl of enzyme solution. After incubation at 50 °C for 10 min, the liberated reducing sugars were determined by DNS method. [24]. One unit was defined as the amount of enzyme that released 1 μmol xylose/min under the assay conditions. In immobilization condition, the mixture reaction contained 0.1 g of support, 0.4 ml of phosphate buffer, pH 7.0, and 0.5 ml 1% of xylan solution.

2.6. Effect of pH and temperature on activity and stability

The activity of the free and the immobilized β-xylosidase was assayed at different temperatures (40–70 °C) and pH values (50 mM citrate buffer pH: 3, 4 and 5; 50 mM phosphate pH: 6, 7 and 8; AMPSO pH: 8 and 9; and 50 mM glycine pH: 10, 11). The effect of temperature on enzyme stability was checked at 45, 50, 55 and 60 °C from 30 min to 10 h. Both free and immobilized enzyme preparations (equal amounts in terms of proteins) were incubated in 50 mM phosphate buffer, pH 7.0, at the desired temperature. The residual activity was measured at specific time intervals under the standard assay conditions described above. The free and immobilized enzyme was incubated at various pH values at 50 °C for 10 h and their residual activities were determined at the optimum pH (7.0 for the free enzyme and 8.0 for the immobilized enzyme).

2.7. Determination of kinetic parameters

As far as the kinetic experiments were concerned, the *p*NPX substrate was prepared in nine different concentrations (from 0.5 to 20 mM) using 50 mM phosphate buffer (pH 7.0 for the immobilized and the free enzyme, respectively) and incubated with the free or immobilized β-xylosidase at 50 °C for 10 min. The amount of released *p*-nitrophenol was measured at 405 nm. The K_m and V_{max} values were calculated from the kinetics data using the "hyper 32" software (exe program, version 1.0.0, 2003).

2.8. Effect of various reagents on the activity of free and immobilized enzyme

To investigate the effect of some reagents and metal ions on the activity of the free and the immobilized β-xylosidase, the same amounts (in terms of proteins) were pre-incubated for 15 min at room temperature in the presence of different reagents (5 or 10 mM). The residual activities were measured by standard assay procedures.

2.9. Operational stability of the immobilized enzyme

0.1 g of different immobilized enzyme supports were incubated with 200 μl of 2 mM *p*NPX solution (50 mM phosphate buffer pH 8.0) at 50 °C for 10 min. At the end of the reaction, the immobilized enzyme was collected by centrifugation at 5000 rpm for 5 min, washed with phosphate buffer, and then reused with 200 μl of freshly prepared substrate to start a new run.

2.10. High-performance liquid chromatography (HPLC)

The oligosaccharide synthesis or hydrolysis was monitored by HPLC (Aminex HPX-42A), column 7.8 × 300 mm. 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, X_4), at 10 g/l each, was used as a standard.

2.11. Xylooligosaccharide synthesis

Xylooligosaccharides (XOS) syntheses were tested in batches by using free β-xylosidase and chitosan immobilized enzyme at the same concentration (0.1 mg/ml) in the presence of D-xylose (40%) in phosphate buffer (50 mM, pH 7.0) and after incubation at 50 °C for different periods. The XOS were analyzed by HPLC (Aminex HPX-42A), column 7.8 × 300 nm. All results were expressed as milligrams of oligomers produced per milliliter of reaction medium. In all the assays, blank tests were conducted with and without heat-denatured enzyme, and no reaction was observed in both cases.

2.12. Saccharification of Oat spelt xylan by co-immobilization of β-xylosidase and xylanase

The xylanase and β-xylosidase of *T. thermophilus* were co-immobilized simultaneously, using chitosan support, for the saccharification of Oat spelt xylan and the production of xylose. 4 g of chitosan were pre-incubated overnight at 4 °C with (133 μg/g of support) xylanase and (50 μg/g of support) β-xylosidase, giving immobilization yields of 89% and 87%, respectively. Chitosan containing the two enzymes was incubated in batch for 7 h at 50 °C

Table 1
Variation in immobilization and activity yields of β-xylosidase as function of resin used.

Type of resin	Polyacrylamide	DEAE-cellulose	DEAE-sephadex	Chitin	Chitosan	Gelatin
Activity yield (%)	51.6	97.1	82.2	76.5	94	62.5
Immobilization yield (%)	44.1	75.3	60.0	54	87.0	57.3
Immobilized proteins (%)	36.2	71.0	51.2	60.5	65	46

in the presence of 3% of xylan Oat spelt that was pre-dissolved in 50 mM phosphate buffer. At suitable time intervals, aliquots were withdrawn and analyzed by HPLC; and the reducing sugars were estimated by dinitrosalicilic acid [24]. The extent of xylan hydrolysis was calculated as follows:

$$\text{xylan hydrolysis}\,(\%) = \frac{\text{mg xylose produced} \times 0.9}{\text{mg initial xylan}} \times 100$$

The coefficient 0.9 estimates water elimination from xylose molecules for the formation of glycoside bonds in xylan. The saccharification yield and xylose production achieved through the co-immobilized xylanase and β-xylosidase enzyme were compared to those obtained by xylanase being immobilized alone.

3. Results and discussion

3.1. Study of immobilization support

The β-xylosidase from *T. thermophilus* was immobilized on different matrixes and by varied methods, including ionic binding (DEAE-cellulose and DEAE-sephadex), entrapment in polyacrylamide gel, and covalent coupling (chitosan, chitin and gelatin). Table 1 shows the immobilization and activity yields of the used matrix and the percentages of the bound protein. All the matrixes fixed β-xylosidase but the binding percentage was low with most types of supports.

DEAE-cellulose provided high yields in terms of activity and immobilization yields (97.11%, 75.3%, respectively). Adsorption is a simple method but is often reported to cause problems of leaching. Covalent coupling can help to overcome this problem, but, at the same time, harsh conditions can lead to an undesirable loss of activity [25], which also holds true for DEAE-Sephadex. Entrapment in polyacrylamide support actually showed the lowest activity and immobilization yields (51.66%, 44.15%, respectively). Due to diffusion limitations, this method can be applied only to low molecular weight substrates and products [26]. This was therefore not attempted in this particular phase of the study wherein the formation of high molecular weight was obtained by xylan hydrolysis or synthesized by condensation reaction.

β-Xylosidase was covalently coupled on chitin, chitosan and gelatin, through cross-linking mediated by glutaraldehyde. Compared to chitin and gelatin, chitosan provided the highest activity and immobilization yields (87.06%, 94%, respectively). Chitosan is a natural cationic polysaccharide derived from chitin and is known as a good support for enzyme immobilization because of its hydrophilicity, biocompatibility and biodegradability [27]. Due to its porous structure, chitosan lowers the diffusion limitation caused by high substrate size and offers a good stability for the immobilized enzyme [27]. In the present work, chitosan was selected for further β-xylosidase application in either xylan degradation or oligosaccharides synthesis. In fact, chitosan has often been used to immobilize other β-xylosidases [28] and many other enzymes, such as α-L-arabinofuranosidase [29], α-amylase [30] and *endo*-1,4-β-xylanase [28].

3.2. Influence of glutaraldehyde concentration on the immobilization and activity yields of β-xylosidase

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 [31]. The immobilization yield was also affected by the glutaraldehyde concentration and the support activation period. Therefore, the effects of glutaraldehyde concentration on the enzyme's immobilization and activity yields were investigated (Table 2). The chitosan support was prepared in the presence of different glutaraldehyde concentrations (0.5–4%) and the immobilization and activity yields were measured. The maximum immobilization and activity yields were observed, in the presence of 2% or 2.5% of glutaraldehyde corresponding to 92.8%, 78.9% and 93.4%, 84.6%, respectively. It was noted that while a low glutaraldehyde concentration (0.5%) slightly affected the immobilization and activity yields (72.25% and 64.54%, respectively); a strong concentration (4%) drastically decreased the activity yield to 43.24% and had no effect on the immobilized yield (77.2%). This latter fact could be attributed to the steric hindrance occasioned by the increased level of polymerization in the enzyme-support matrix which impedes the access of the substrate to the immobilized enzyme. This inhibitory effect leads to the reduction in the interaction between the substrate and the active site of the immobilized enzyme [32].

3.3. Effect of pH and temperature on the activity and stability of immobilized β-xylosidase

Fig. 1 shows the activity of the free and chitosan immobilized *T. thermophilus* β-xylosidase at different pH values. The optimum pH of the immobilized enzyme shifted from pH 7.0, which was the optimum for the free enzyme, to alkaline range (pH 8.0).

Compared to the free enzyme, the immobilized β-xylosidase exhibited a higher activity at pH above 7.0 and lost only 13% of

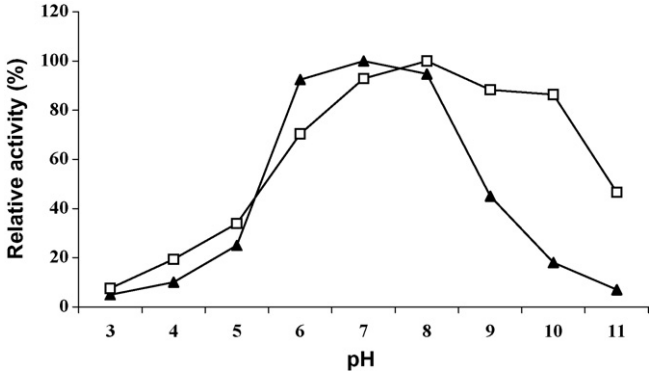

Fig. 1. Effect of pH on free and chitosan-immobilized β-xylosidase. Enzyme activity was measured at 50 °C with 2 mM *p*NPX buffered at different pH. Values reported in the figure are the means of determination performed in triplicate. Free enzyme (▲); Immobilized enzyme (□).

Table 2
Influence of glutaraldehyde concentration on immobilization and activity yields of β-xylosidase.

Glutaraldehyde (GA) concentration (%)	0.5	1	1.5	2	2.5	3	4
Activity yield (%)	72.2	77.3	87.4	92.8	93.4	81.2	43.2
Immobilization yield (%)	64.5	68.1	72.8	78.9	84.6	82.5	77.2

its activity at pH 9 and 10. In its free state, however, this loss increased to 55% and 82% at pH 9 and 10, respectively. The alkaline shift of catalytic activity can be accounted for by the increase of negative charges [33]. This being so, the concentration of H^+ in the micro-environment of the immobilized enzyme increased. This means that the pH surrounding the immobilized enzyme became more acidic than that of the external solution. The effect of pH on the enzyme activity was the direct result of external pH solution since the latter increased in this particular case [33]. The pH stability of the free and immobilized *T. thermophilus* β-xylosidase were compared at pH ranging between 5.0 and 11.0 at 4 °C during 10 h of incubation. At different pH conditions the immobilized β-xylosidase was more stable than the free enzyme (Fig. 2).

At extreme pH (5.0 and 11.0), the immobilized β-xylosidase lost only 20% of its residual activity whereas the free state, this loss was twice superior. This could be attributed to the ability of the micro-environment, created between the support and the enzyme, to protect the latter from the denaturation caused by the change in pH and to make it more stable. This stabilizing effect in acidic and alkaline media through carriers having an anionic character has not been previously reported for other immobilized β-xylosidases. This lends the chitosan support to the special importance of this immobilization technique in the stabilization of β-xylosidase at different ranges of pH.

The temperature dependence of the activity of the soluble and immobilized β-xylosidase was studied in 50 mM phosphate buffer (pH 7.0 for the free enzyme and pH 8 for the immobilized enzyme) (Fig. 3). The optimal reaction temperature shifted from 50 to 53 °C. Compared to other immobilized β-xylosidases, this shift was low; it ranged from 5 °C for *Sulfolobus solfataricus* β-xylosidase [16] up to 25 °C for *Aspergillus niger* B 03 β-xylosidase [15].

The temperature stabilities of the free and immobilized β-xylosidase were compared at temperatures ranging between 45 and 60 °C at pH 7.0 during incubation periods of 10 h (Fig. 4). The activity of the immobilized enzyme was more stable than that of the free one, particularly when the temperature exceeded 50 °C. It was observed that the immobilization process on chitosan protected the enzyme against heat inactivation. The immobilized β-xylosidase, for instance, retained 50% of its activity at 55 °C after 3 h, whereas the free enzyme retained 60% of its activity after 1 h. The immobilized and free β-xylosidase retained 40% and 15% of their activities, respectively, and after 1 h incubation at 60 °C. These results clearly demonstrate the efficiency and, therefore, the importance of this immobilization method. The difference in the temperature stability profiles between the free and immobilized β-xylosidase lies in the fact that the latter is less sensitive to temperature. Gottschalk and Jaenicke [34] reported on this difference by stipulating that the covalent binding of immobilized enzyme (multiple-point attachment) resulted in an increase in enzyme rigidity, which is commonly reflected by an increase in stability toward denaturation.

3.4. Determination of kinetic parameters

The K_m and V_{max} values of the free and immobilized β-xylosidase enzyme were determined using different *p*NPX concentrations (0.5–20 mM). Lineweaver-Burk plots of the free and

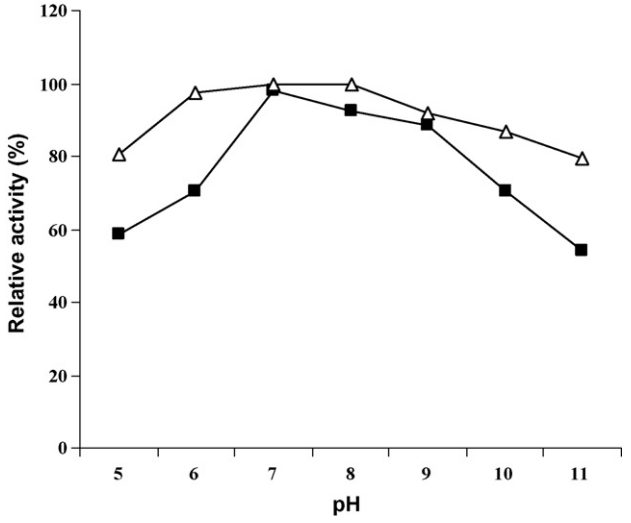

Fig. 2. pH stability of free (■) and chitosan immobilized (△) *Talaromyces thermophilus* β-xylosidase.

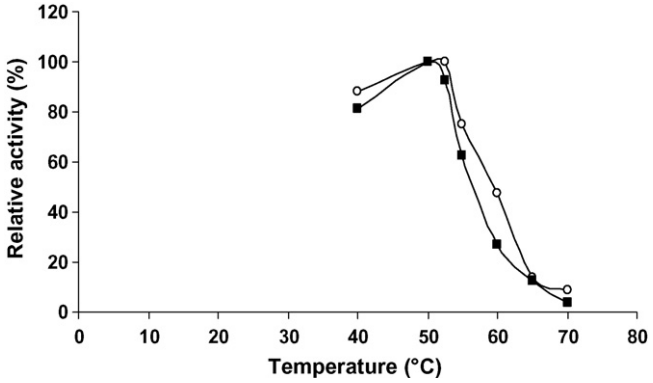

Fig. 3. Effect of temperature on free and chitosan-immobilized β-xylosidase. Enzyme activity was measured at different Temperatures with 2 mM *p*NPX dissolved in 50 mM phosphate buffer, pH 7.0. Values reported in the figure are the means of determination performed in triplicate. Free enzyme (■); immobilized enzyme (○).

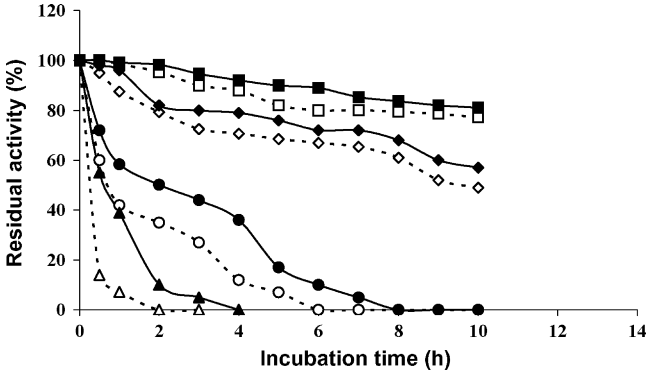

Fig. 4. Thermal stability of *T. thermophilus* β-xylosidase. Free and chitosan-immobilized enzyme at 45 °C (■); 50 °C (♦); 55 °C (○) and 60 °C (▲). Residual enzyme activity was determined in the periodically withdrawn samples using *p*NPX as substrate. Free β-xylosidase (- - -); chitosan-immobilized β-xylosidase (—).

Table 3
Effect of various reagents on the activity of free and chitosan immobilized *Talaromyces thermophilus* β-xylosidase. The data presented are an averages and standard errors of two independent experiments.

Reagents	Concentration (mM)	Relative activity (%) free enzyme	Relative activity (%) immobilized enzyme
Control	–	100	100
$CaCl_2$	5	145 ± 3	120 ± 5
$BaCl_2$	5	100 ± 1	98 ± 1
$CoCl_2$	5	122 ± 0	111 ± 3
$MnSO_4$	5	130 ± 3	128 ± 4
$MgSO_4$	5	101 ± 2	98 ± 2
$ZnCl_2$	5	34 ± 2	56 ± 2
$FeSO_4$	5	70 ± 2	96 ± 1
$CuSO_4$	5	49 ± 2	64 ± 2
$HgCl_2$	5	20 ± 1	42 ± 1
DTT	10	111 ± 1	99 ± 3
EDTA	10	55 ± 2	63 ± 4
SDS	10	44 ± 0	65 ± 2
Urea	10	95 ± 0	95 ± 2
Triton X-100	10	93 ± 1	100 ± 3

immobilized *T. thermophilus* β-xylosidase gave the K_m values of 2.37 and 3.42 mM, respectively. The V_{max} of the free and immobilized β-xylosidase were 0.049 and 0.035 μmol min^{-1} (mg protein)$^{-1}$, respectively. An increase in K_m value was observed after enzyme immobilization. This is most likely due to either the structural changes of the enzyme which are introduced by the applied immobilization procedure and/or to the lower accessibility of the substrate to the catalytic site of the immobilized enzyme [28]. On the other hand, the multiple fixation of the enzyme by covalent binding (as in the present case) would also lead to a decrease in enzyme flexibility, which resulted in a decrease in catalytic activity [34]. The increase in K_m value and decrease in V_{max} have also been previously reported in the literature [28,35].

3.5. Effect of various reagents on the activity of free and chitosan immobilized enzyme

The effects of various ions and detergents on the activity of the immobilized β-xylosidase compared with that of the native enzyme are listed in Table 3. The Ca^{2+}, Co^{2+} and Mn^{2+} ions were found to be interesting activators for the free and immobilized enzyme. DTT slightly activated the free enzyme (111%) but did not have any effect on the immobilized enzyme (99%). Other reagents, such as Ba^{2+}, Mg^{2+}, urea and Triton X-100, did not have any significant effect on enzyme activity on both states of the enzyme. On the other hand, Zn^{2+}, Cu^{2+}, Hg^{2+}, Fe^{2+}, EDTA and SDS adversely affected the free and immobilized enzyme activity. However, it was observed that the inhibitory effect of these reagents was less pronounced in the case of the immobilized enzyme. This may be due to the fact that the enzyme has a fixed structure and that the carrier provides a protective role to the immobilized enzyme. Similar results were previously reported for other immobilized enzymes [36,37].

3.6. Operational stability of the immobilized enzyme

The operational stability of an immobilized preparation is one of the most important factors in impairing its utilization in a bioconversion process. The operational stability of chitosan immobilized β-xylosidase was, therefore, extensively investigated. The immobilized β-xylosidase enzyme was reused repeatedly in 25 consecutive batch cycles at pH 8.0 and at 50 °C. Each enzyme activity cycle lasted for 10 min (Fig. 5). The immobilized chitosan preparation exhibited a very high operational stability as it retained about 94% of its total immobilized activity after being reused 25 times using 2 mM *p*NPX as a substrate. When using the other supports the operational stability was relatively low as shown in Fig. 5, supporting our selection of chitosan as immobilization support. In addition, and as previously reported [28], the chitosan support is endowed with a high capacity for reusability. Moreover, the operational stability of the immobilized β-xylosidase of *T. thermophilus* seems to be superior to that reported by Delcheva et al. [15]. Indeed, *A. niger* β-xylosidase, immobilized on polyacrylamide membrane, retained about 50% of its total immobilized activity after 16 cycles. These findings are of a great interest, as they would allow the set-up of a bioreactor for continuous application.

3.7. Enzymic synthesis of xylooligosaccharides

The synthesis of oligosaccharides is carried by enzymes that have high specificity for their substrates and mild reaction conditions. Glycoside hydrolases can be used to catalyze the reverse hydrolysis reaction for oligosaccharides synthesis. These enzymes have the particular advantages of being inexpensive and easily available and of having remarkable low specificity towards glycon acceptors [38]. To test its ability to synthesize xylooligosaccharides in the presence of high xylose concentration and at high temperature, the β-xylosidase from *T. thermophilus* was immobilized on chitosan by covalent binding. In the presence of 40% of xylose and at 50 °C, the free *T. thermophilus* β-xylosidase synthesized 5.6 mg/ml of xylobiose after 6 days, corresponding to a 1.4% of xylobiose production yield. No other forms of xylooligosaccharides have been detected during the condensation reaction (data not shown). Fig. 6A shows the chromatogram of the synthesized xylooligosaccharides by the immobilized β-xylosidase at different times. After 48 h, the immobilized enzyme produced 1.6 g/l xylobiose, 3.2 g/l xylotriose and 0.7 g/l xylotetraose. The synthesis evolution was not the same for di-, tri- and tetra-saccharides (Fig. 6B). After 96 h, the xylobiose concentration, reached 4 g/l that corresponded to 1% of the conversion yield of xylose. The synthesis of xylotriose, however, begun after 24 h and reached a maximum of 3.2 g/l after 48 h. It then decreased to disappear after 96 h. The decrease of xylotriose might reflect its concomitant transformation into xylotetraose. The latter reached an 8.2 g/l at 96 h, corresponding to 2.05% conversion yield. These results show that the immobilization process greatly improved the xylooligosaccharides synthesis activity of *T. thermophilus* β-xylosidase.

The xylooligosacharides were synthesized with the immobilized enzyme and not with the free enzyme. This might be attributed to the immobilization process wherein a hydrophobic microenvironment was created at the active site, which favoured the synthetic activity [39].

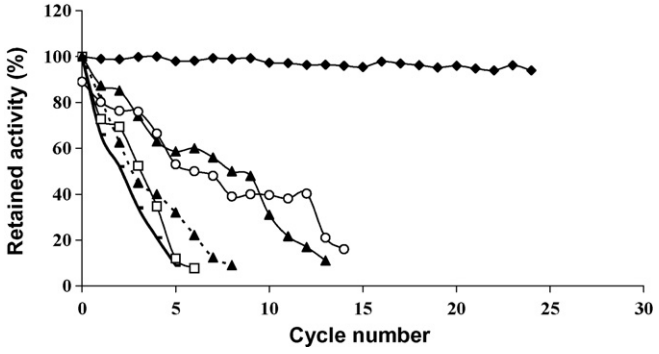

Fig. 5. Reusability of different supports-immobilized enzyme, chitosan (♦), polyacrelamide (□), chitin (▲), DEAE-cellulose (—), DEAE-sephadex (▲) and gelatin (○). The immobilized preparation was used consecutively. The reaction mixture is described in the text.

The nature of the support, and most particularly glutaraldehyde used for the immobilization process, also increased the polymerization degree and the total oligomer production. Nakanishi and co-workers [40] have reported the increase in the yield of oligosaccharides synthesis by enzymes after immobilization, to the some modifications caused by the glutaraldehyde in the active site. This fact makes the enzyme more suitable for synthesizing activity and catalysis the reverse hydrolysis. The immobilization of *T. thermophilus* β-xylosidase can, therefore, be considered as a potential strong alternative for the eventual production of xylooligomers using a simple protocol.

3.8. Saccharification of Oat spelt xylan by co-immobilization of β-xylosidase and xylanase

Enzyme saccharification seems to offer significant advantages and interesting possibilities for industrial applications over the currently used chemical hydrolyses processes. The technique, however, has a number of problems and shortcomings pertaining to enzyme stability, cost, reusability, and the like. Enzyme immobilization provides an effective approach to ease those difficulties. In order to examine the synergistic action of the two immobilized enzymes, i.e., xylanase and β-xylosidase, in the degradation of xylan, a continuous enzyme hydrolysis of 3% Oat spelt xylan at 50 °C was performed (Fig. 7). The extent of hydrolysis was estimated by measuring the amount of reducing sugar using DNS method [24] and by determining the concentration of xylose and xylobiose produced at different time intervals using HPLC. Fig. 7A shows that the efficiency of xylan saccharification increased when both xylanase and β-xylosidase from the *T. thermophilus* strains were co-immobilized. This was demonstrated by the high amount of xylose (7.3 g/l) produced after 7 h compared to the xylose produced (2.03 g/l) when xylanase was immobilized alone on chitosan corresponding to 22%, and 6.1% to saccharification yields, respectively. These yields can be increased by using a low concentration of xylan and by reducing the problems associated to diffusion limitations of subtract as for example by physical and chemical pretreatment of xylan. The xylobiose liberated by xylanase, however, decreased in the presence of β-xylosidase which immediately transformed it to xylose. On the other hand, the treatment with β-xylosidase alone for 6 h at 50 °C did not lead to xylose release from xylan.

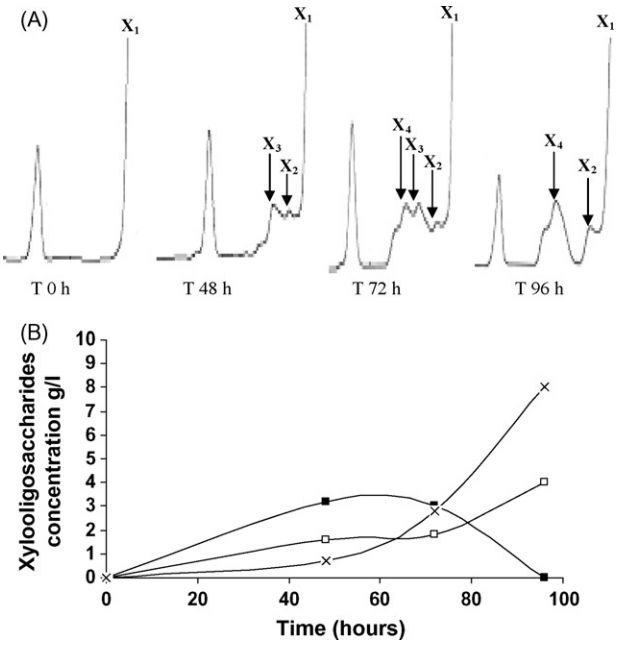

Fig. 6. Chromatographic response analyses of xylo-oligosaccharide synthetics by immobilization β-xylosidase in different times. X_1: Xylose; X_2: xylobiose; X_3: xylotriose; X_4: xylotetraose (A). Quantities of xylooligosacharide produced at different times to express in g/l (B). Xylobiose (□); xylotriose (■); xylotetraose (×).

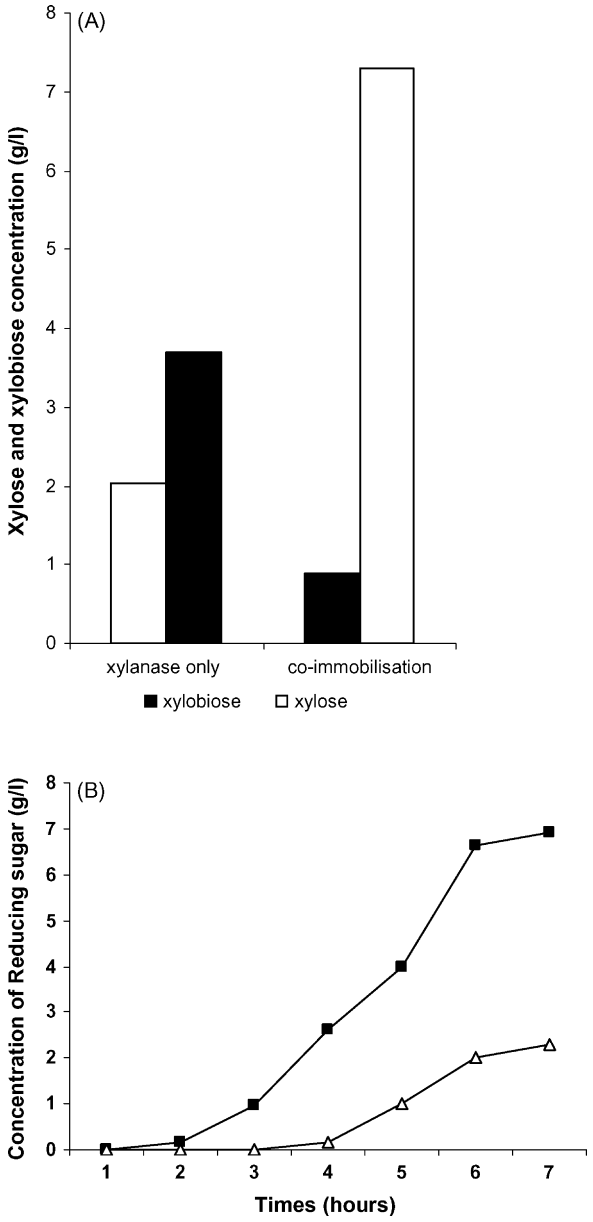

Fig. 7. Xylose and xylobiose produced after 7 h to Oat spelt xylan saccharification with the immobilization of xylanase alone and co-immobilization of xylanase and β-xylosidase (A). Kinetic of reducing sugars produced during Oat spelt xylan saccharification with the immobilization of only xylanase (△) and co-immobilization of xylanase and β-xylosidase (■) (B).

The production kinetic of reducing sugars was also activated when xylanase and β-xylosidase were co-immobilized. In this condition, xylose was found to be produced immediately and to reach a plateau after 6 h of hydrolysis. The immobilized xylanase alone, in contrast, begun to liberate xylose only after 4 h to reach a maximum at the end of the reaction (Fig. 7B).

These results suggest that the enzymatic degradation of xylan to xylose requires the synergistic action of β-xylosidase and xylanase. The addition of β-xylosidase to endoxylanases has also been reported to enhance the release of reducing sugars from

xylan, probably by avoiding the relief of the end-product inhibition [41,42]. Therefore, a large part of the synergistic effect can be attributed to the formation of xylooligosaccharides by endoxylanase, which can be further hydrolysed by the β-xylosidase. The co-immobilization of xylanase and β-xylosidase using enzymes was previously reported for *Aspergillus* sp. [42] and *A. niger* B 03 [15].

4. Conclusion

Considering the advantages of immobilized enzymes, especially the possibility for their recovery and reuse, we decided to immobilize the thermostable β-xylosidase of *T. thermophilus* fungus by varied methods. We have shown that the covalent binding to chitosan through glutaraldehyde was the best method for β-xylosidase immobilization and, therefore, a potential candidate for further industrial applications. This technique exhibited high loading efficiency, interesting operational stability, and good thermal and pH stability. According to Abdel-Naby [28], the immobilization on chitosan offers also various interesting advantages, namely its relatively inexpensive cost and easiness to prepare and regenerate.

In addition, we found that *T. thermophilus* β-xylosidase catalyzes the reverse hydrolysis reaction for oligosaccharides synthesis. Furthermore, after immobilization the synthetic activity was more accented and contributed to the oligomers production. The xylanase and β-xylosidase of *T. thermophilus* were also co-immobilized on chitosan support that increase the saccharification yield of xylan and show the synergistic action of the two enzymes.

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