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Hydrolytic Potential of *Talaromyces thermophilus* β-Xylosidase and Its Use for Continuous Xylose Production

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Summary

We report here the enhanced hemicellulase production by a *Talaromyces thermophilus* strain in a fed-batch fermentation using 3.6-litre laboratory-controlled bioreactor. When grown on wheat bran, this fungus produces a wide spectrum of polysaccharide-hydrolysing enzymes, mainly endo-β-1,4-xylanase (27 U/mL), β-xylosidase (1.4 U/mL), α-L-arabinofuranosidase (1.05 U/mL) and β-D-mannosidase (0.78 U/mL). The β-xylosidase was purified and shown to hydrolyse xylobiose and short xylooligosaccharides, but it was inactive on xylan. It released xylose from xylooligosaccharides with a degree of polymerisation ranging from 2 to 5. *Talaromyces thermophilus* β-xylosidase activity was unaffected by high glucose or arabinose concentration (0.5 M) and retained 75 % of its original activity in the presence of 133 mM xylose. Chitosan-immobilised β-xylosidase was used in a continuous process of conversion of wheat bran hydrolysate to xylose in a packed bed reactor. Xylose production of 18.6 mg/g was reached after six hours in the bioreactor and was twofold higher than that produced by the free enzyme. The produced xylose was further converted into xylitol using the crude intracellular enzyme of *Talaromyces thermophilus.*

Key words: hemicellulases, β-xylosidase, xylose, fed-batch fermentation, xylitol

Introduction

Xylan is the major constituent of hemicellulosic polysaccharides in the cell wall of land plants, representing up to 30–35 % of the total dry mass (*1*). It is the second most abundant natural resource and represents potentially renewable energy supply that could be utilised to improve the technology of plant biomass bioconversion into useful products (*2*). Xylan has a relatively complex structure based on a β-(1,4)-linked D-xylose backbone substituted to various extent with acetyl, L-arabinofuranosyl, D-galactosyl, D-glucuronyl or 4-*O*-methylglucuronyl groups (*3*).

In nature, the complete degradation of xylan requires the synergistic action of several enzymes, mainly endo-β-1,4-xylanases (EC 3.2.1.8), which cleave the β-1,4 glycosidic bond between xylose residues to produce xylooligosaccharides of low polymerisation degree, and β-xylosidase (EC 3.2.1.37), which hydrolyses short xylooligomers and xylobiose from the non-reducing end, liberating xylose (*4*). The affinity of xylooligosaccharides towards β-xylosidase activity showed a tendency to decrease with the increase in the degree of polymerisation. β-Xylosidase is almost completely inactive against xylan (*5*). In combination with other enzymes, especially cellulases, xylanolytic enzymes have opened new possibilities for food industry and for the bioconversion of agricultural wastes into easily fermentable products (*6*). The fermentation of xylose or xylose-containing hydrolysate for ethanol production or bioconversion into xylitol has recently been reviewed (*7*). The β-xylosidase is an essential enzyme of the microbial xylanolytic system, as it contributes to the decrease in the inhibition of xylanases by the end-product of xylan hydrolysis (*8*). This enzyme is cell-associated in most bacteria (*9*) and yeast, but is found free in the culture media of wide majority of fungi (*10*).

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Although many bacterial and fungal β-xylosidases have been purified and characterised, only few β-xylosidases from thermophilic fungi have been reported in the literature (*11*).

Researchers have recently realized that effective β--xylosidase production is important for a wide range of applications. It is, for instance, of particular importance for the effective conversion of hemicellulosic biomass into fuels and chemicals, for efficient delignification of pulp, the proper use of plant materials for animal feed (*11*) and for the improvement of grape monoterpenyl glycoside hydrolysis during wine fermentation (*12*). Therefore, there is a need to develop suitable β-xylosidases for the conversion of hemicelluloses to fermentable sugars that could be used in the subsequent production of bioethanol and other value-added chemicals (*13*). In this context, we have used a new *Talaromyces thermophilus* strain, which proved to be efficient in the production of large amounts of thermostable xylanolytic enzymes (*14*). In a previous report, we studied the biochemical properties of the unique β-xylosidase purified from *T. thermophilus* (*15*). The optimum temperature and pH of this enzyme were 50 °C and 7.0, respectively. Among all fungal β--xylosidases studied until now, the β-xylosidase from *T. thermophilus* and that from *Sporotrichum thermophilum* displayed optimal activity at neutral pH (*16*).

In the present study, we investigated the hemicellulase production by *T. thermophilus* strain in fed-batch fermentation, and we also studied the hydrolytic potential of the purified *T. thermophilus* β-xylosidase and its use for continuous xylose production from wheat bran hydrolysate. The produced xylose was further bioconverted by the crude intracellular *T. thermophilus* enzyme into xylitol.

Materials and Methods

Chemicals

Xylan (oat spelt, beechwood and birchwood), *p*-nitrophenyl-β-D-xylopyranoside (*p*NPX), *p*-nitrophenyl-α--L-arabinofuranoside (*p*NPA), *p*-nitrophenyl-β-D-mannopyranoside (*p*NPM), xylobiose, xylose, xylitol and 3,5-dinitrosalicylic acid were purchased from Sigma-Aldrich (Steinheim, Germany).

Microorganism and fermentation conditions

The thermotolerant fungal strain was identified as *Talaromyces thermophilus* Stolk by CBS (Central Bureau of Fungal Cultures, Utrecht, The Netherlands), code reference: 274-2003. The deposit number of *T. thermophilus* in the Tunisian Collection of Microorganisms is CTM10.103 (Centre of Biotechnology of Sfax, Sfax, Tunisia). Fungal cultivation was carried out in a 3.6-litre stirred tank fermentor (Infors, AG GH-4103 Bottmingen, Switzerland) equipped with the instrumentation for measurement and/or control of agitation, temperature, pH and dissolved oxygen concentration. The agitator was equipped with two 6-bladed Rushton impellers of 45 mm in diameter, positioned 14 cm from each other. The cultivation temperature was maintained at 50 °C and dissolved oxygen was kept above 20 % of medium saturation. The pH was controlled at 6.7±0.1 by automatic addition of ammonium hydroxide (4 M) and phosphoric acid (2 M). *T. thermophilus* was cultivated in the optimised liquid medium (*17*) containing (in g): KH_2PO_4 1, K_2HPO_4 2.5, $MgSO_4$ 1.2, $CaCl_2$ 0.3, yeast extract 1, and Tween 80 1 mL; distilled water was added to 1 L, supplemented with 1 mL of an oligoelement solution (in g/L): $MnSO_4$ 1.6, $ZnSO_4$ 1.4, $FeSO_4$ 5 and $CoCl_2$ 2. The initial working volume was 1.6 L, containing 1.5 % of wheat bran as the only carbon source. The medium was sterilised by autoclaving at 120 °C for 20 min. Ten milliliters of broth samples were taken regularly during the course of fermentation and examined for growth, contamination and hemicellulase activity.

The feeding started after three days with one feed every 24 h (200 mL of culture medium containing 0.5 % wheat bran for each feed except for the last, which was 0.7 %). In total, four feeds were completed to reach a final volume of 2.4 L and 3.7 % of wheat bran (the same conditions of optimized substrate ratio as in batch culture).

Enzyme assay

β-Xylosidase, α-L-arabinofuranosidase, and β-mannosidase activities were determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl-α-L-arabinofuranoside, *p*-nitrophenol-β-D-xylopyranoside and *p*-nitrophenol-β-D--mannopyranoside, respectively. A volume of 200 μL of each substrate (2 mM) was mixed in 50 mM potassium phosphate buffer (pH=7.0) with 200 μL of enzyme solution, incubated for 10 min at 50 °C, after which 1.6 mL of 1 M Na_2CO_3 were added to stop the reaction (*18*). The absorbance resulting from the release of *p*-nitrophenol was measured at 405 nm. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol per min in the reaction mixture under the assay conditions.

The xylanase activity was assayed by measuring the reducing saccharides released from birchwood xylan (*19*). 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 released reducing sugars were determined by the dinitrosalicylic acid (DNS) method (*20*). One unit of enzyme activity was defined as the amount of enzyme that releases 1 μmol of xylose equivalents per min under the assay conditions.

The filter paper activity, which describes the overall cellulolytic activity, was determined by the release of reducing sugars produced in 60 min from a mixture of 0.5 mL of appropriately diluted enzyme, 2 mL of 0.1 M acetate buffer, pH=4.8, and 50 mg of Whatman no. 1 disk filter paper incubated at 50 °C. One international filter paper unity (FPU) was defined as the amount of enzyme that releases 1 mmol of glucose per min. Glucose generated during the assay was estimated by using the DNS method (*20*).

β-xylosidase preparation protocol

At the end of the fermentation, the mycelium was removed by centrifugation at 4500×*g* for 10 min at 4 °C. The supernatant was precipitated with ammonium sul-

phate (80 % saturation) and the β-xylosidase activity was purified to homogeneity by sequential operation of DEAE--cellulose column chromatography and gel filtration on HPLC column (*15*). Purified enzyme was used for all subsequent studies.

Sugar tolerance of the purified β-xylosidase

The effect of three monosaccharides on the β-xylosidase activity was determined as follows: 100 μL of purified enzyme (0.65 U/mL) were incubated at 50 °C for 10 min in 2 mM of *p*NPX dissolved in 50 mM phosphate buffer (pH=7.0) containing various amounts of xylose, glucose or arabinose ranging from 1 to 10 %. Then, the residual β-xylosidase activity was measured according to the standard assay method.

Hydrolytic conversion of xylobiose to xylose

Purified β-xylosidase (0.65 U/mL) was added to a solution of 10 g/L of xylobiose prepared in 50 mM phosphate buffer, pH=7.0. The mixture was incubated at 50 °C for 1 h. Aliquots were taken at various intervals and analysed by HPLC column (300×7.8 mm; Aminex® HPX 42A, Bio-Rad, Hercules, CA, USA). The products were separated by elution with water at a flow rate of 0.6 mL/min, and detected with a refractive index detector (RID-10A, Shimadzu, Kyoto, Japan). Xylose and xylobiose, at 1 g/L each, were used as standards. The peak areas were used for hydrolytic product quantification.

Preparation of xylooligosaccharides (xylan hydrolysate)

A mixture of xylooligosaccharides was prepared by partial hydrolysis of birchwood xylan in hydrochloric acid. Xylan (3 %) was first incubated at 100 °C for 10 min in HCl (200 mM). Then, the pH of the mixture was neutralised by adding NaOH solution (1 M) and concentrated by evaporation. The liberated xylooligosaccharides were treated with *T. thermophilus* β-xylosidase at 50 °C for 24 h. The hydrolysis was monitored using thin layer chromatography (TLC) on Kieselgel 60 plates (Merck, Darmstadt, Germany) with a 1-butanol/acetic acid/water (2:1:1 by volume) solvent system. The plates were developed with two runs followed by heating for a few minutes at 120 °C in an oven after spraying the plates with a methanol-sulphuric acid mixture (95:5 by volume). After separation, the chromatogram was scanned and analysed by the ImageJ software (v. 1.46r) for the quantification of xylooligosaccharide spots (*21*).

β-xylosidase immobilisation

The β-xylosidase was immobilised by covalent coupling using chitosan as carrier. A mass of 0.5 g of chitosan was dissolved in 50 mL of 0.1 M HCl containing 2.5 % (by volume) glutaraldehyde (GA) at 30 °C for 2 h. The solubilised chitosan was precipitated by the addition of 1 mL of 1 M NaOH. The precipitate was separated by centrifugation (10 min at 3000×*g*) and washing with distilled water to remove the excess GA. 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 20 mM phosphate buffer until no protein or activity was detected. The immobilisation yield was expressed by the following equation:

$$\text{Immobilisation yield} = \frac{(A-B)}{A} \times 100 \qquad /1/$$

The activity yield was defined according to the following equation:

$$\text{Activity yield} = \left(\frac{C}{A}\right) \times 100 \qquad /2/$$

As the equations above indicate, various parameters were used in the estimation of immobilisation: *A* is the total enzyme activity used for immobilisation, *B* is the unbound enzyme activity, (*A-B*) is the theoretical immobilised enzyme activity and *C* is the obtained immobilised enzyme activity (*22*). The total enzyme activity is the total number of units added to the support during the immobilisation reaction, the non-immobilised activity is the number of units found in filtrates and washing volumes after immobilisation; and the immobilised activity is the number of units detected in the support after immobilisation and washing.

Wheat bran hydrolysis

Wheat bran was first crushed using electric chopper (rotary shear shredder, Waring, Stamford, CT, USA) to obtain a particle size smaller than 1 mm in diameter and washed with sodium hydroxide solution at low concentration (100 mM), followed by intensive washing with deionized water. Secondly, xylooligosaccharides were extracted according to the method of Herrera *et al.* (*23*). This method consists of incubating 20 g of the prepared wheat bran at 100 °C for 30 min in diluted acidic solution (HCl, 200 mM). The supernatant was recovered by filtration (Whatman GF/A glass microfibre filters), neutralised and concentrated using a SpeedVac Concentrator (Savant SpeedVac SC210A, Thermo Fischer Scientific, Waltham, MA, USA). This pool of oligosaccharides was then treated by chitosan-immobilised β-xylosidase.

Column reactor

For continuous hydrolysis of wheat bran hydrolysate, a packed bed reactor was prepared using 10 g (9.5 IU) of chitosan-immobilised β-xylosidase. To ensure a constant reaction temperature, the reactor was prepared in a jacketed column (23 cm in length and 1.5 cm i.d.) and temperature was maintained at 50 °C using a circulating water bath. The continuous flow of the substrate (total volume of 30 mL), through fixed bed reactor, was insured using a peristaltic pump that allowed relatively fine control of the flow rate (0.5 mL/min). Analysis and quantification of products were performed by TLC and HPLC as described above. The hydrolysis of WBH was monitored by discontinuous evaluation of free xylose and the efficiency was calculated by comparing free xylose to the theoretical xylose content in original wheat bran.

Intracellular enzyme extraction and xylitol production

A mass of 3 g of mycelium recovered by centrifugation from the culture of *T. thermophilus* was first washed with 50 mM phosphate buffer (pH=7.0) and then crushed

by adding aluminium oxide (3 g) in 5 mL of the same buffer, using a mortar. The supernatant containing crude intracellular enzyme was incubated in the presence of 5 % of xylose at 40 °C during 24 h. The produced xylitol was quantified by HPLC analysis.

Results and Discussion

Hemicellulase production by T. thermophilus in fed-batch fermentation

Hemicellulase production by *T. thermophilus* was studied in fed-batch fermentation using previously optimised culture medium (*17*). The cultivation was monitored following the secretion of reducing sugars and proteins (Fig. 1a). When grown on wheat bran as the only carbon source, this filamentous fungus produces a wide spectrum of hemicellulases (Fig. 1b). In contrast, no cellulase activity was detected in the culture filtrate. Compared to the *T. thermophilus* batch culture (*14*), the fed-batch fermentation led to an improvement of the production rate of all hemicellulosic hydrolases. The xylanase activity was predominant with a production rate of 27 U/mL after 192 h of culture (corresponding to 60 U/mg of specific activity), compared to the rate obtained by batch culture (49 U/mg) (*14*). Wheat bran contains many nutritional compounds that serve not only as a carbon source and provide trace nutrients for cell growth, but also as an inducer of hemicellulase production due to its high arabinoxylan content (up to 40 %) (*24*). In fact, this substrate induced high titres of β-xylosidase (1.4 U/mL), α-L-arabinofuranosidase (1.05 U/mL) and β-D-mannosidase (0.78 U/mL), which are usually poorly produced by the majority of fungal cultures (*25*). Table 1 (*10,15, 16,25–30*) exhibits a comparative study of some culture conditions for different fungal species with the corresponding production rate of β-xylosidase activity. Except for the hypercellulolytic mutant of *Trichoderma reesei* (6.3 U/mL) (*30*), all other fungi produce a low level of β--xylosidase activity (generally <1 U/mL). The time course of β-xylosidase production began after 24 h of cultivation and reached a maximum after 168 h. The end of cultivation (at 192 h) was marked by a drastic decrease of the concentration of reducing sugars and an increase of dissolved oxygen (72 %). This means that the fungus has consumed all the sugar released from the substrate and can no longer assimilate the dissolved oxygen, which is essential for growth. Under these conditions the culture was immediately stopped. The absence of cellulase activity in the culture filtrate of *T. thermophilus* is of great interest in the pulp and paper industries, which require the use of cellulase-free xylanases. They are used primarily as bleaching agents to reduce the amount of chlorine required to achieve desirable levels of paper brightness (*31*).

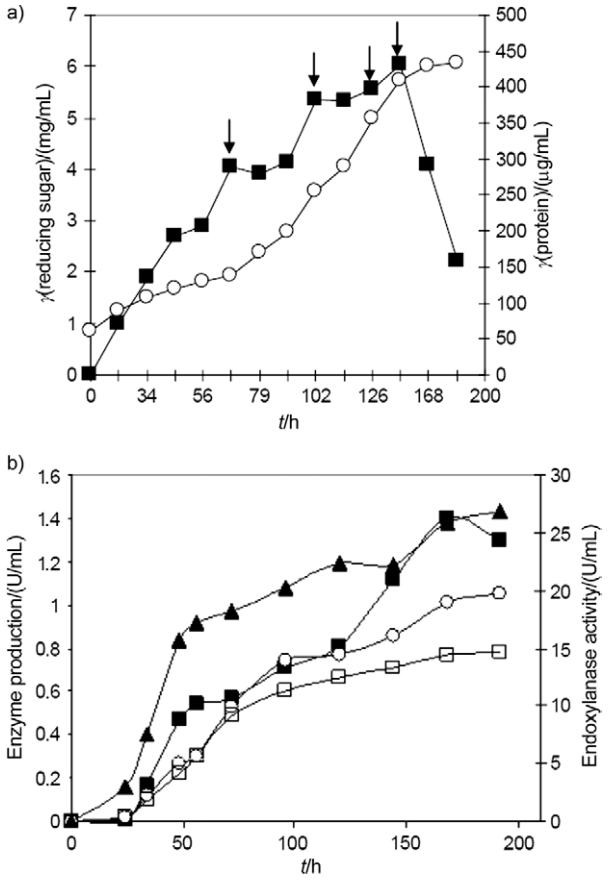

Fig. 1. Time course profiles of hemicellulase production by *T. thermophilus* in fed-batch fermentation using wheat bran as only carbon source: a) production of reducing sugars (■) and proteins (○); the arrows show feeding times; and b) production of β-xylosidase (■), endoxylanase (▲), α-L-arabinofuranosidase (○) and β-D-mannosidase (□)

Sugar tolerance of β-xylosidase

β-Xylosidase is an essential enzyme in the conversion of xylobiose and short xylooligosaccharides to xylose. It is important to observe that during the enzymatic saccharification of xylan, the use of β-xylosidase (which is resistant to xylose inhibition) would be of great advantage for the process. Therefore, we sought to investigate the influence of various concentrations of three simple sugars (xylose, arabinose and glucose) on β-xylosidase activity. Our results show that the β-xylosidase from *T. thermophilus* is resistant to xylose inhibition since it retained 75 % of its residual activity in the presence of 133 mM of xylose (Fig. 2). This enzyme can be considered as the second most xylose-resistant β-xylosidase after that of *Scytalidium thermophilum,* which tolerates up to 200 mM of xylose (*28*). On the other hand, most of β-xylosidases reported to date are inhibited by very low xylose concentrations starting from 10 mM (*10,25,30*) (Table 1). The β-xylosidases from *Paecilomyces thermophila* and *Aspergillus nidulans* have been shown to lose almost half of their activities in the presence of 100 and 25 mM of xylose, respectively (*27,32*). In addition, while all arabinose concentrations tested did not affect β-xylosidase activity, 25 mM of glucose stimulated it up to 120 %. This can be considered as an advantage for *T. thermophilus* β-xylosidase *vs.* that of *Candida utilis*, which is widely used in winemaking and loses 50 % of its activity in the presence of 20 mM of glucose (*18*).

Hydrolytic conversion of xylobiose to xylose

β-Xylosidase is an exoglycosidase degrading xylobiose and short xylooligosaccharides to liberate xylose.

Table 1. Culture conditions and β-xylosidase properties of different fungal species

Microorganism	Culture conditions						Enzyme properties					Reference
	Type of culture	*w*(carbon source) / %	*t*(culture) / h	pH	Temperature/°C	β-xylosidase activity / U/mL	M_r/kDa	Optimum activity pH	Optimum activity Temperature/°C	K_m/mM	*c*(xylose inhibition) / mM	
Fusarium proliferatum	Erlenmeyer flask	xylan 1	69	5.0	30	0.07	91.2	4.5	60	0.77	5	(*25*)
Aspergillus niger	Erlenmeyer flask	xylan 1	168	5.0	30	0.54	78	5.0	45	1.5	–	(*26*)
Fusarium verticillioides	Erlenmeyer flask	xylan 1	48	5.0	30	0.05	94.5	4.5	65	0.85	6	(*10*)
Paecilomyces thermophila	Erlenmeyer flask	corncob 5	120	6.5	45	0.5	53.3	6.5	55	4.3	130	(*27*)
Scytalidium thermophilum	Erlenmeyer flask	xylan 1	24	6.0	40	0.37	45	5.0	60	1.3	>200	(*28*)
Sporotrichum thermophile	bioreactor 20 L	corncob 2.7	72	5.0	50	0.07	45	7.0	50	1.1	–	(*16*)
Trichoderma harzianum	solid state	wheat bran 1	288	5.0	30	0.2	60	4.0–4.5	70	0.053	–	(*29*)
Trichoderma reesei Rut C-30*	bioreactor 3 L	xylan 1	96	5.0	30	6.3	100	4.0	60	0.42	2.3	(*30*)
Talaromyces thermophilus	Erlenmeyer flask bioreactor 3.6 L	xylan 1 wheat bran 2	120 96	7.0	50	0.5 1.4	97	7.0	50	2.37	133	(15) this work

*hypercellulolytic mutant
K_m for *p*NPX as substrate

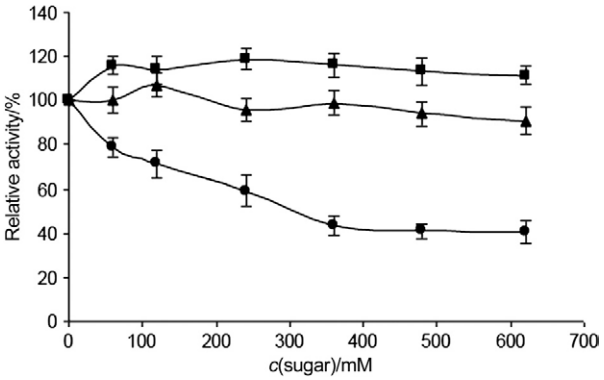

Fig. 2. The effect of sugar concentration on *T. thermophilus* β-xylosidase activity: (■) glucose, (▲) arabinose, (●) xylose

According to Wong *et al.* (*33*), the ability of the hydrolytic conversion of xylobiose to D-xylose is one of the characteristics of true β-xylosidases. In this context, *T. thermophilus* β-xylosidase was incubated in the presence of commercial xylobiose and hydrolysis reaction was monitored by HPLC analysis (Fig. 3). After 1 h of hydrolysis, the majority of xylobiose (92 %) was converted to D-xylose. The comparison of the conversion rate of *T. thermophilus* β-xylosidase with that of other species of filamentous fungi shows the ability of this enzyme to catalyze the cleavage of (1,4)-β-D-xylosidic linkage and liberate xylose (Table 2). Moreover, the β-xylosidase from *T. thermophilus* was also incubated in the presence of different commercial substrates such as oat spelt, beechwood and birchwood xylan but we were not able to detect any

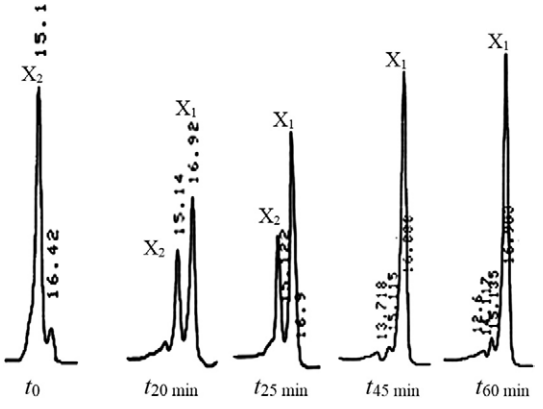

Fig. 3. Chromatographic response analyses of xylobiose hydrolysis by *T. thermophilus* β-xylosidase (0.65 U/mL) at different times. Retention times of xylobiose (X_2) and xylose (X_1) were 16.9 and 15.1 min, respectively; t_0–t_{60}=β-xylosidase hydrolysis time in minutes

hydrolytic activity (data not shown). Most of the purified β-xylosidases reported were not able to degrade oat spelt xylan. Generally, β-xylosidases exhibit little or no action on polymeric xylans (*13,16*). *Trichoderma reesei* β--xylosidase is an exception, because this multifunctional β-D-xylan hydrolase was able to form xylose from xylan (*30*).

Hydrolysis of xylooligosaccharides by the purified β-xylosidase

Having in mind that the β-xylosidase from *T. thermophilus* is inactive on xylan, we sought to study the specificity of this enzyme towards xylooligosaccharides. Birchwood xylan is weakly branched (11 % of substitution degree) and contains a higher percentage of xylose (>90 %) (*34*). Therefore, this substrate was pretreated with acidic solution (HCl, 200 mM) at high temperature (100 °C) for its depolymerisation and the release of xylooligosaccharides (mainly X_2–X_5) (Fig. 4a). This xylooligosaccharide mixture was used to test the ability of the β-xylosidase from *T. thermophilus* to release xylose. Imaging software was used for the quantification of xylooligosaccharides (Fig. 4b). After 6 h of hydrolysis, the spots corresponding to xylobiose (0.09 g/L at t_0) and xylotriose (0.05 g/L at t_0) were totally transformed to xylose with a final concentration of about 0.14 g/L. On the other hand, spots corresponding to xylotetraose and xylopentaose continued to increase during the first three hours of hydrolysis to reach 0.1 and 0.7 g/L, respectively. After 24 h of incubation, all xylooligosaccharides were hydrolysed and converted to xylose (0.24 g/L). This is a typical feature of an exo-type xylanolytic enzyme (*30*). It hydrolyses up to xylopentaose (X_5) and seems to be more applicable in xylan saccharification. β-Xylosidases were grouped according to the substrate polymerisation degree (*35*). The β-xylosidases from *Aspergillus phoenicis* and *Aspergillus versicolor* hydrolysed only up to xylotriose (*36*), while the *Scytalidium thermophilum* β-xylosidase hydrolysed up to xylotetraose (*28*).

Xylose production using immobilised continuous reactor

The generation of fermentable sugars from lignocellulosic resources is an important step in the biotransformation of renewable biomass into useful products such as fuel, chemicals and polymers (*37*). Current developments in this area are mainly focused on a two-step process, where lignocellulosic material is first pretreated using thermochemical methods in order to render the polymer of sugars more accessible to cellulolytic and hemicellulolytic enzyme cocktails that are employed in the hydrolysis step. For this purpose, wheat bran was first treated

Table 2. Hydrolytic conversion of xylobiose by different fungal β-xylosidases

Microorganism	γ(xylobiose)	t(incubation)	Enzyme activity	Conversion rate	Reference
	g/L	h	U/mL	%	
Fusarium proliferatum	10	2	0.30	100	(*25*)
Trichoderma reesei	10	2–6	0.81	90	(*30*)
Aspergillus nidulans	15	9	1.20	90	(*32*)
Talaromyces thermophilus	10	1	0.65	92	this work

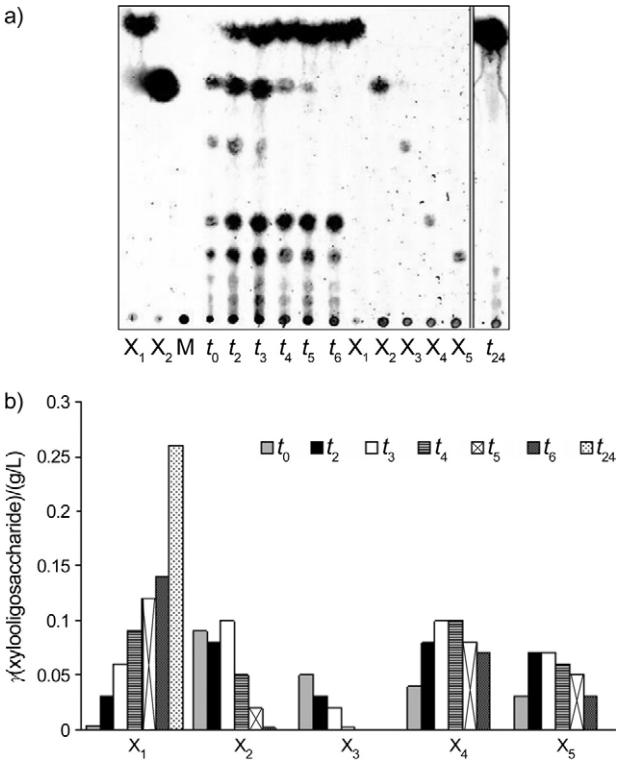

Fig. 4. Hydrolysis of xylooligosaccharides by purified β-xylosidase: a) TLC analysis of the hydrolysed products from xylooligosaccharides (birchwood xylan) by purified *T. thermophilus* β-xylosidase, b) time course of xylose production and xylooligosaccharide hydrolysis. The oligosaccharide standards were: X_1= xylose, X_2=xylobiose, X_3=xylotriose, X_4=xylotetraose, X5=xylopentaose (M=native birchwood xylan, t_0=xylooligosaccharides extracted by thermochemical pretreatment, t_2–t_{24}=β-xylosidase hydrolysis time in hours)

with acidic solution (HCl, 200 mM) at high temperature, and then the liberated soluble oligosaccharides were hydrolysed by continuous chitosan-immobilised β-xylosidase-based process. The released xylose was monitored by TLC and HPLC analyses (Fig. 5). Diluted acid treatment led to a limited hydrolysis called prehydrolysis.

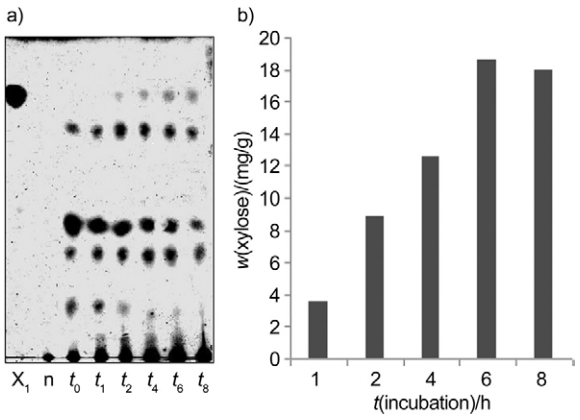

Fig. 5. Continuous production of xylose from wheat bran hydrolysate using continuous chitosan-immobilized β-xylosidase-based process: a) TLC analysis, and b) plots of xylose time course (X_1= xylose, n=native wheat bran, t_0–t_8=β-xylosidase hydrolysis time in hours)

This consists in the hydrolysis of the hemicellulosic fraction, leaving the cellulose and lignin fractions almost unaltered (*23*). Acidic treatment is able to liberate oligosaccharides from wheat bran as proved by thin layer chromatography (Fig. 5a). Purified β-xylosidase was covalently coupled with chitosan through cross-linking mediated by glutaraldehyde. This support provided the highest activity and immobilisation yields (87 and 94 %, respectively) (Table 3). Chitosan is a natural cationic polysaccharide derived from chitin and is known as a good support for enzyme immobilisation because of its hydrophilicity, biocompatibility and biodegradability (*26*). Due to its porous structure, chitosan lowers the diffusion limitation caused by high substrate size and offers a good stability for the immobilised enzyme (*22*).

Table 3. Immobilisation and activity yield of chitosan-immobilised β-xylosidase

Chitosan-immobilised β-xylosidase (covalent coupling)	
Activity yield/%	94±2
Immobilisation yield/%	87±4
w(immobilised protein)/%	65±6

Results are expressed as mean values±SEM (standard error of the mean)

Xylose production was maximal after approx. 6 h of operating reactor corresponding to 18.6 mg per g of wheat bran (Fig. 5b). This amount was twofold higher than that produced by the free enzyme (data not shown). According to Dupont and Selvendran (*38*), the percentage of xylose in wheat bran was determined to be around 29.5 % (by mass). The extraction rate of xylose after combined thermochemical and enzymatic treatment was estimated to be about 6.3 %. It is interesting to note that after 8 h of hydrolysis, the β-xylosidase activity measured in 0.1 g of immobilised enzyme on chitosan retained 53 % of its initial activity. Such stability was often described for enzymes that are covalently immobilised (*26*). Overall, based on these preliminary tests, it appears that a continuous xylosidase-based process for the production of xylose from wheat bran hydrolysate is a feasible concept. Certainly, the performance of our reactor could be improved through careful optimisation of various control parameters (temperature, substrate concentration, flow rate, *etc.*) and through further innovation.

Bioconversion of xylose into xylitol

The main application of xylose is its bioconversion to xylitol, a functional sweetener with important technological properties like anticarcinogenicity, low caloric value and negative heat of dissolution (*7*). The economic interest in xylitol production can be enhanced if the needed xylose solutions can be obtained from the hydrolysis of low-cost lignocellulosic biomass. For this purpose, previously produced xylose was bioconverted into xylitol using the crude intracellular enzyme of *T. thermophilus*. Figs. 6 and 7 show the production of xylitol and the corresponding chromatographic analyses, respectively. After

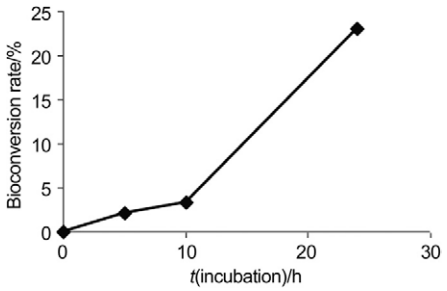

Fig. 6. Bioconversion of xylose to xylitol

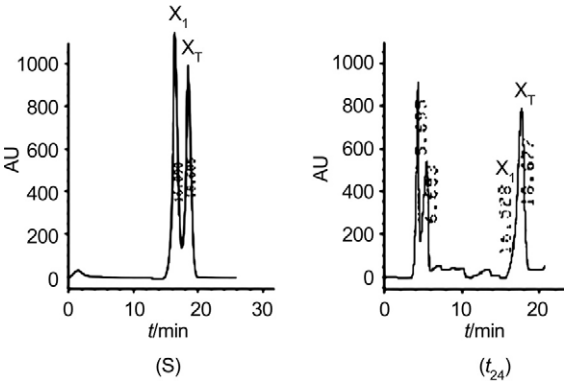

Fig. 7. Chromatographic response analyses. Retention times of xylose (X_1) and xylitol (X_T) were 16 and 18.6 min, respectively. (S)=standard, (t_{24})=reaction time in hours

24 h of incubation, the bioconversion rate of xylose to xylitol reached 23 %. This result could be explained by the presence of an intracellular *T. thermophilus* xylose reductase activity which was able to reduce xylose into xylitol. Intracellular xylose reductase activity had previously been described and purified from very few filamentous fungi (*39*). Xylitol production by biotechnological means appears to be an efficient approach and has economic advantages when compared to chemical processes. The bioconversion concept occurs directly in the hemicellulosic hydrolysate and does not require xylose purification.

Conclusion

The results presented in this paper indicate that β--xylosidase from *T. thermophilus* plays an important role in the assimilation of xylose moieties from xylooligosaccharides. In fact, chitosan-immobilised β-xylosidase was used in a continuous conversion of wheat bran hydrolysate to xylose. The liberated xylose was further converted into xylitol using intracellular enzyme extract of *T. thermophilus*, with xylose reductase activity. The high tolerance of *T. thermophilus* β-xylosidase to sugar inhibition and its ability to release xylose from xylooligosaccharides makes this enzyme a promising candidate for future industrial application, such as efficient degradation of hemicellulosic biomass for production of xylitol or bioethanol.

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