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Catalytic properties of *Talaromyces thermophilus* α-L-arabinofuranosidase and its synergistic action with immobilized endo-β-1,4-xylanase

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ABSTRACT

When grown on wheat bran, *Talaromyces thermophilus* produces a wide spectrum of hemicellulases, mainly endo-β-1,4-xylanase, α-L-arabinofuranosidase and β-xylosidase. The extracellular α-L-arabinofuranosidase was purified to homogeneity by sequential operation of ammonium sulfate precipitation, Q-sepharose column chromatography, gel filtration on Sephacryl S-200 and MonoQ column. The pure α-L-arabinofuranosidase had a specific activity of 49 U/mg of protein and was purified 26.7-fold. The molecular mass of the enzyme was estimated to be 35 kDa, determined by SDS-PAGE and by gel filtration. The α-L-arabinofuranosidase exhibited maximal activity at pH 6.0–7.0 and an optimal temperature at 55 °C. The half-life of the α-L-arabinofuranosidase at 60 °C was approximately 2 h and it was very stable over a wide pH range for 24 h at 4 °C. The apparent Michaelis constant K_m value of the α-L-arabinofuranosidase was 0.77 mM for *p*-nitropenyL-α-L-arabinofuranoside. The turnover number (K_{cat}) and catalytic efficiency (K_{cat}/K_m) were found to be 14.3 s^{-1} and 1.8 104 M^{-1} s^{-1}, respectively. Metal ions such as Hg^{2+} and Cu^{2+} inhibited enzyme activity, whereas it was strongly activated by Mn^{2+}. The α-L-arabinofuranosidase was specific for the α-linked arabinoside in the furanoside configuration and can also retain 52% of its activity in the presence of *p*-nitrophenyl-β-D-xylopyranoside as substrate. α-L-arabinofuranosidase acted synergistically with the immobilized endo-β-1,4-xylanase for the breakdown of alkali-extracted arabinoxylan and in the improvement of xylobiose and monosaccharide production.

1. Introduction

Plant cell wall arabinoxylans have complex structures that require the sequential and synergistic action of a number of different enzymes for their complete hydrolysis [1]. Arabinoxylans are composed of a backbone of (1,4)-linked β-D-xylopyranosyl residues where single α-arabinofuranosyl substituents are attached to the C(*O*)-2, C(*O*)-3 or to both C(*O*)-2,3 of the xylose residues [2]. The xylan backbone can also be substituted with α-D-glucopyranosyl uronic acid, or its 4-*O*-methyl derivative and acetyl groups [3]. In addition, ferulic acid and *p*coumaric acid may be covalently linked to arabinoxylan via esterification at the C_5 position of some of the arabinosyl units [4].

Because of this heterogeneous composition of arabinoxylans, their enzymatic degradation requires the action of a battery of debranching and depolymerizing activities. Debranching activities mainly include α-L-arabinofuranosidase (EC 3.2.1.55) and subsequently, feruloyl esterases (EC 3.1.1.73), α-glucuronidases (EC 3.2.1.139) and/or acetyl xylan esterases (EC 3.1.1.72), whereas depolymerization relies on endo-1,4-β-xylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37) activities [5,6].

In fact, α-L-arabinofuranosidases are exo-type enzymes that generally catalyse the cleavage of the terminal α-L-arabinofuranosyl residues of arabinoxylan, arabinan and arabinogalactan [7]. This class of enzymes is important since removal of the arabinose residues from the xylan backbone may have a synergistic effect with endoxylanases, presumably because endo-xylanase hydrolysis of glycosidic bonds is inhibited by arabinose-substituted xylose residues [8]. In general, these enzymes are produced by bacteria [9], actinomycetes [10] and several fungi [11–13] but only a few α-L-arabinofuranosidases from thermophilic fungus had been reported [14]. Depending on their amino acid sequences, α-L-arabinofuranosidases have been classified into four of glycoside hydrolase families (families 43, 51, 54 and 62) (http://afmb.cnrs-mrs.fr/CAZY/). This classification is based on the amino acid sequence of their active sites rather than their substrates specificity [15].

Researchers have recently realized that effective α-L-arabinofuranosidase 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, the efficient delignification of pulp, the proper

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utilization of plant materials for animal feed [14] and the improved hydrolysis of grape monoterpenyl glycosides during wine fermentation [16]. α-L-arabinofuranosidase was used for polysaccharides hydrolysis to liberate L-arabinose, which is used commercially as a low-calorie sweetener. L-Arabinose inhibits sucrase and prevents elevation of blood glucose levels induced by sucrose intake [17]. As a result, it can potentially inhibit obesity and prevent or treat diseases associated with hyperglycemia [18].

In view of this, there is a need to develop suitable α-L-arabinofuranosidases for use in the conversion of hemicelluloses to fermentable sugars which could be used for the subsequent production of bio-ethanol, and other value-added chemicals [6]. In this context, we have isolated a new *Talaromyces thermophilus* strain named AX4 that proved efficiency in the production of large amounts of thermostable xylanolytic enzymes, including an endo-xylanase [19], a β-xylosidase [20], an α-L-arabinofuranosidase and a β-D-mannosidase [21].

We describe here the purification and catalytic properties of the α-L-arabinofuranosidase produced by this fungus. We also studied the mode of action of this enzyme and its synergistic effect with immobilized endo-β-1,4-xylanase for the breakdown of alkali-extracted wheat arabinoxylan.

2. Materials and methods

2.1. Chemicals

Q-Sepharose (Big beads), Sephacryl S-200 (high resolution) and Standard proteins (LMW gel filtration calibration kit) were from Amersham Biosciences (Uppsala, Sweden). Xylan (Oat spelt), *p*-nitropenyl-α-L-arabinofuranoside, *p*-nitrophenyl-α-L-arabinopyranoside, *p*-nitropenyl-β-D-xylopyranoside, *p*-nitrophenyl-β-D-cellobioside, *p*-nitrophenol-β-D-mannopyranoside, methylumbelliferyl-4,7-β-D-arabinofuranoside, and 3,5-dinitrosalicylic acid were purchased from Sigma–Aldrich (Steinhiem, Germany). Polyethylene glycol (PEG) was from Fluka (Steinhiem, Germany). Column for high-pressure liquid chromatography (HPLC, Bio-Sil SEC-125, 7.8 mm × 300 mm) was from Bio-Rad (Montpellier, France).

2.2. Micro-organism

The thermotolerant fungal strain 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 is: Tunisian Collection of Micro-organisms CTM10.103 (Centre of Biotechnology of Sfax, Tunisia).

2.3. Cultivation in bioreactor

The cultivation was carried out in a 3.6 l stirred tank bioreactor (Infors, AG GH-4103 Bottmingen, Switzerland) with 1.6 l working volume. The bioreactor (dished bottom glass jacketed reactor) was equipped with 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 diameter. The cultivation temperature was maintained at 50 °C and dissolved oxygen was kept at above 20% of the saturation for the medium by varying the agitation rate and flow rate of air. The pH was controlled at 6.7 ± 0.1 by automatic addition of ammonium hydroxide (4 M) and phosphoric acid (2 M). The *T. thermophilus* was cultivated in the optimized liquid medium [21]: KH_2PO_4, 1 g; K_2HPO_4, 2.5 g; $MgSO_4$, 1.2 g; $CaCl_2$, 0.3 g; yeast extract, 1 g; Tween 80, 1 ml; water, 1 l and 3.7% wheat bran as sole carbon source. The pH of the medium was 6.7 and was supplemented with 1 ml/l of an oligoelements solution ($MnSO_4$, 1.6 g/l; $ZnSO_4$, 1.4 g/l; $FeSO_4$, 5 g/l and $CoCl_2$, 2 g/l). The medium was sterilized by autoclaving (120 °C, 20 min); appropriate antibiotics (ampicillin 50 μg/ml and tetracycline 20 μg/ml) were added and subsequently inoculated by 2 ml suspension of spores (5.10^8 spores/ml). Ten milliliters broth samples was taken regularly during the course of fermentation and examined for growth, contamination, and hemicellulase activities. At the end of the fermentation (5 days), the mycelium was harvested by centrifugation at 6000 rpm for 10 min, and the supernatant was used to purify the α-L-arabinofuranosidase enzyme.

2.4. Enzyme essays

α-L-Arabinofuranosidase, β-xylosidase and β-mannosidase activities were determined by measuring the release of *p*-nitrophenol from, respectively, *p*-nitrophenyl-α-L-arabinofuranoside (*p*NPA), *p*-nitrophenol-β-D-xylopyranoside (*p*NPX) and *p*-nitrophenol-β-D-mannopyranoside (*p*NPM). The assay mixture contained 200 μl of each substrate (2 mM) in 50 mM potassium phosphate buffer (pH 7.0), and 200 μl of enzyme solution. After incubation for 10 min at 50 °C, the reaction was stopped by the addition of 1.6 ml of Na_2CO_3 (1 M) [22]. The absorbance (nm) due to the release of *p*-nitrophenol was measured at 405 nm. One unit (IU) of enzyme activity was defined as the amount of enzyme that released 1 μmole of *p*-nitrophenol/min in the reaction mixture under the assay conditions.

The xylanase activity was assayed by measuring the reducing groups released from Birchwood xylan [23]. The reaction mixture consisted of 500 μl of 1% 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 releases 1 μmol xylose equivalents/min under the assay conditions.

2.5. α-L-Arabinofuranosidase purification

The production of α-L-arabinofuranosidase by *T. thermophilus* strain in submerged batch fermentation was examined. The strain was grown in optimized nutrient medium containing wheat bran as carbon source. Cultivation was performed at 50 °C for 5 days. The extracellular proteins were recovered by centrifugation and the supernatant (1 l) was treated with ammonium sulfate (60% saturation). The precipitate was collected by centrifugation at 9000 rpm for 15 min, dissolved in 20 mM phosphate buffer, pH 7.0, and then dialyzed overnight against the same buffer. The dialyzed enzyme solution was loaded on a Q-sepharose column (2.5 cm × 22 cm) pre-equilibrated with 20 mM phosphate buffer, pH 8. The column was extensively washed with the same buffer. α-L-Arabinofuranosidase activity was eluted with a gradient of 0–0.5 M NaCl in the same buffer, at a flow rate of 30 ml/h. The active fractions were pooled, concentrated using PEG 6000 and dialyzed overnight against 20 mM phosphate buffer, pH 7.0. The α-L-arabinofuranosidase obtained from the ion exchanger was further purified by gel filtration Sephacryl S-200 (high resolution) (1.5 cm × 96 cm) equilibrated and eluted by 25 mM phosphate buffer, pH 7.0 at a flow rate of 0.5 ml/min. Fractions showing α-L-arabinofuranosidase from the last purification step were then pooled, concentrated by Centricon 10 (Amicon) and analyzed by FPLC using Mono Q column anion exchange chromatography (Q_{12} Bio-Rad, 15 × 68 mm) equilibrated with 20 mM phosphate buffer, pH 8. The column was operated at a flow rate of 5 ml/min and active fractions (3 ml each) were eluted with a gradient of 0-0.5 M NaCl in the same buffer. Purified α-L-arabinofuranosidase was dialyzed overnight in 20 mM phosphate buffer, pH 7.0 and used for further studies.

2.6. Molecular mass determination

The molecular mass of the purified α-L-arabinofuranosidase was estimated using SDS-PAGE electrophoresis and gel filtration (HPLC). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [25] was carried out to determine the purity and the molecular weight of the enzyme. The gel was stained with Coomassie brilliant blue R-250 and destained in 10% acetic acid and 20% of absolute ethanol. A low molecular weight calibration kit for SDS electrophoresis (Fermentas, France): β-galactosidase (116 kDa), Bovine serum albumin (66.0 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35.0 kDa), REase Bsp981 (25.0 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.4 kDa) was used as molecular weight standard.

The purified α-L-arabinofuranosidase was also injected on a high performance liquid chromatography (HPLC; KNAUER advance scientific instruments, Germany) gel filtration column (Bio-Sil SEC-125, 7.8 mm × 300 mm, BioRad, France). The elution was realized with 50 mM phosphate buffer pH 7.0 at a flow rate of a 0.8 ml/min. Molecular weight standard from Amersham used to calibrate the column were ribonuclease A (13.7 kDa), chymotripsinogen A (25 kDa), ovalbumin (43 kDa) and albumin (67 kDa).

2.7. Zymogram analysis

α-L-Arabinofuranosidase activity was also revealed by zymogram analysis. For this purpose, proteins are mixed with the same loading buffer as in SDS-PAGE, but they are not boiled. After electrophoresis, the gel is incubated for 2 h in 50 mM phosphate buffer pH 7.0 to get rid of SDS and allowing the renaturation of proteins. Then, the gel was superposed against an overlay of 1% agar containing 5 mM of MUA or 2 mM of *p*NPA. After a suitable period of incubation (15 min) at 50 °C, activity was observed under UV light (MUA) or directly following the appearance of a yellow spot (*p*NPA).

2.8. Influence of temperature, pH, and chemicals

α-L-Arabinofuranosidase activity was determined under standard conditions except different temperatures were assayed within the range 30–70 °C as indicated in the results. The enzyme activity was then determined at various pH values ranging from 4.0 to 11.0 at the optimal temperature. The thermostability of the enzyme was evaluated by measuring the residual activity after each hours of pre-incubation of the enzyme in the absence of substrate at various temperatures between 50 and 70 °C. The pH stability was studied by pre-incubating the enzyme at pH ranging from 5.0 to 11.0 at 4 °C for 24 h and the residual enzymatic activity was determined under standard conditions. The effects of metal ions and some chemicals were assessed by pre-incubating the enzyme with the test compound at 4 °C for 15 min and then assaying the residual enzymatic activity under standard conditions. Apo-α-L-arabinofuranosidase was made by dialyzing the enzyme against 5 mM EDTA dissolved in 20 mM phosphate buffer pH 7.0 for 20 h. The EDTA was removed by dialyzing apo-enzyme intensively against 15 l of bi-distilled deionized water in 24 h [26]. Activity of apo-α-L-arabinofuranosidase was determined in the presence of different ions (Ca^{2+}, Mn^{2+}, Co^{2+} and Na^{+}). The enzyme was incubated for 2 h at 4 °C in the presence of each metal before measuring the activity.

2.9. Determination of kinetic parameters

The kinetic parameters of the purified enzyme were determined for *p*NPA as substrate and were estimated using a specific program of enzymology (Hyper 32 exe program, version 1.0.0, 2003). The enzyme was incubated with *p*NPA at various concentrations (ranging from 0.5 to 20 mM) at optimal conditions. The enzyme kinetic parameters, K_m (mM) and K_{cat} (s^{-1}) were calculated from Lineweaver–Burk plot of Michaelis–Menten equation.

2.10. Endo-xylanase immobilization

T. thermophilus endo-xylanase retained 100% of its activity after 1 h of incubation at 100 °C. This high thermostability makes difficult the total elimination of the endo-xylanase by just boiling [19] consequently we exploited its immobilization by covalent binding in chitosan support for subsequently subtracted from mixture reaction by centrifugation or filtration. For this purpose (0.5 g) of chitosan was dissolved in 50 ml of 0.1 M HCl containing 1.5% (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 (8.5 U/ml) of the endo-xylanase 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 [19]. We shall recall that the immobilized endo-xylanase presented 81% and 72% of activity and immobilization yield, respectively.

2.11. Chemical arabinoxylan extraction and monosaccharide composition

Arabinoxylan fraction was chemically extracted from wheat bran after two steps. The first step led to the delignification of wheat bran and the second to the solubilization of the arabinoxylan fraction. The delignification step adapted from [27], involves the oxidation of sodium hypochlorite and allows not only the removal of lignin but also the defibrillation of the polysaccharide network due to the swelling of cellulose fibers. Therefore, it facilitates the extraction of hemicellulose [27]. 50 g of destarched wheat bran (particle size less than 0.5 mm diameter) was incubated in 0.3% of H_2SO_4 and 40% of $NaClO_2$ at 70 °C during 2 h [27]. The mixture was centrifuged (15 min at 7000 rpm), the supernatant was eliminated and the pellet was recovered and washed by distilled water and lyophilized. The arabinoxylan extraction step was advocated by the incubation of delignified wheat bran at 60 °C for 2 h in the alkaline solution (KOH 1 M). The heat treatment in alkaline medium, results in the saponification of the ester bonds linking ferulic acid to arabinose, releasing individual arabinoxylan molecules from cell wall structure [28]. After this step, the pH of the mixture was neutralized by adding HCl solution 12 M and centrifuged for 20 min at 7000 rpm. The supernatant was recovered and concentrated. The heteroxylans were finally precipitated by ethanol (2 Vol.; for 18 h; at 4 °C), recovered by filtration (Whatman GF/A glass microfiber filters), washed with ethanol then acetone, and dried in an oven at 40 °C [28]. The yield of arabinoxylan by this extraction process was approximately 45% (Data not shown). To determine the monosaccharide composition and content, 10% of arabinoxylan was hydrolyzed using 0.4 M HCl for 2 h at 100 °C. After hydrolysis, the sample was cooled and subsequently neutralized by addition of NaOH. Each sample was then filtered and the monosaccharide composition and content (g/l) were determined by HPLC.

2.12. High-performance liquid chromatography

The hydrolysis products were monitored by high-performance liquid chromatography (HPLC; Bio-Rad Aminex 87-C, column 7.8 mm × 300 mm). A solution of simple sugar and disaccharide (glucose, xylose, arabinose and xylobiose), at 1 g/l each, were used as a standards.

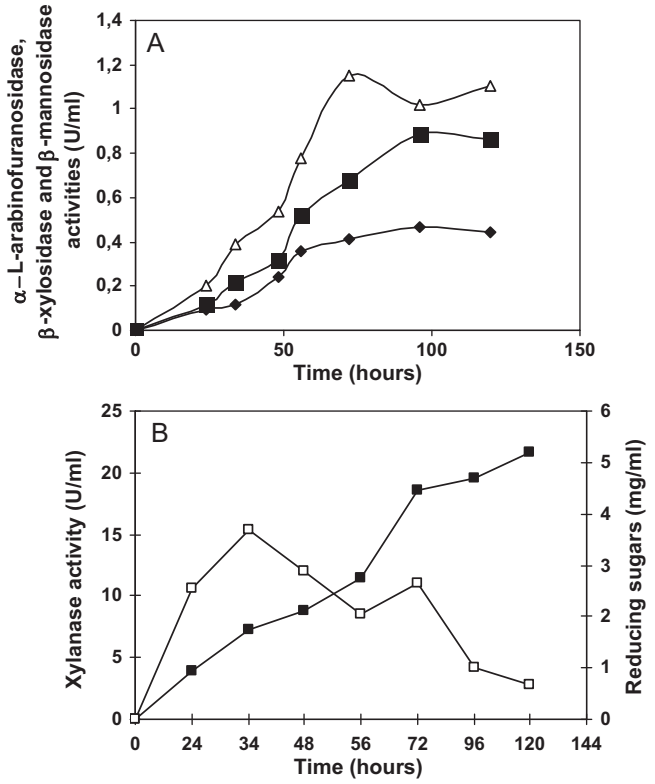

Fig. 1. Time course profiles of hemicellulases production by *T. thermophilus* in batch fermentation using wheat bran as only carbon source. (A) Kinetic production of α-L-arabinofuranosidase (■), β-xylosidase (△) and β-D-mannosidase (♦). (B) Kinetic production of the endo-xylanase (■) and reducing sugars (□). Enzyme and reducing sugars essays were detailed in Section 2.

3. Results

3.1. Hemicellulases production by T. thermophilus in batch fermentation

The production of hemicellulases by *T. thermophilus* strain was studied in a 3.6-l bioreactor by using previously optimized culture medium [21]. When grown on wheat bran as the only carbon source, this filamentous fungus produces a wide spectrum of hemicellulases (Fig. 1). In contrast, no cellulase activity was detected in culture filtrate. Compared with the culture of *T. thermophilus* in Erlenmeyer flask, the batch fermentation led to an improvement in the rate of production of all xylanolytic hydrolases. The xylanase activity is predominant with a production rate of 22 U/ml instead of 5 U/ml after 120 h of culture [19]. Wheat bran induced high titres of β-xylosidase (1.2 U/ml) and α-L-arabinofuranosidase (0.85 U/ml), furthermore, it does not appear to be an effective inducer of β-mannosidase activity (0.4 U/ml). The time course of α-L-arabinofuranosidase production begins after 24 h of culture and reached a maximum after 100 h corresponding to 2.25 U/mg of specific activity. At the end of the culture (120 h) a decrease of reducing sugars (0.6 mg/ml) was observed in the growth medium.

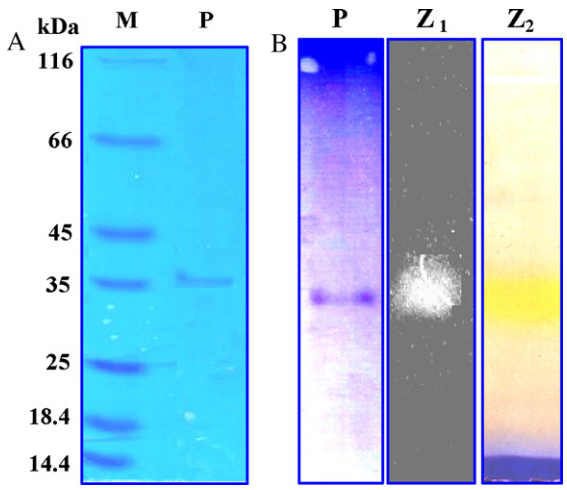

Fig. 2. SDS–PAGE of purified α-L-arabinofuranosidase from *T. thermophilus* (A) and the overlay zymogram gel corresponding (B). The enzyme was electrophoresed at pH 8.6 on a 10% acrylamide gel and stained with Coomassie Brillant Blue R-250. M: Molecular weight standards; P: Purified α-L-arabinofuranosidase (~10 μg); Z_1: α-L-arabinofuranosidase activity was detected with 5 mM 4-methylumbelliferyl-α-L-arabinofuranoside (MUA); Z_2: α-L-arabinofuranosidase activity was detected with 2 mM *p*NPA.

3.2. α-L-Arabinofuranosidase purification

Following growth of *T. thermophilus* for 120 h at 50 °C on a wheat bran-containing medium, the culture supernatant was concentrated with 60% of ammonium sulfate and directly adsorbed to a Q-sepharose column. The α-L-arabinofuranosidase activity was eluted as a sharp peak between 0.2 and 0.25 M NaCl. Following concentration and desalting of the active fractions, the specific activity increased with 58% recovery of the activity (Table 1). This material was applied directly to a Sephacryl S-200 column and the active fractions were collected as a single sharp peak, which was applied on to a Mono-Q column and a pure α-L-arabinofuranosidase was finally eluted in a single peak. A summary of the purification procedures is presented in Table 1. The purified enzyme behaves as a single band on SDS polyacrylamide gel electrophoresis (SDS–PAGE; Fig. 2), coinciding with the activity band, as revealed on zymogram with MUA or *p*NPA (Fig. 2B). The final purification step resulted in a yield of 8% of the activity and a 26.7-fold increase in the specific activity.

3.3. Enzyme properties

The molecular mass of the α-L-arabinofuranosidase was determined to be about 35 kDa, either by gel filtration (data not shown) or following SDS-PAGE analysis, indicating that it exists as monomeric protein.

The α-L-arabinofuranosidase displayed maximal activity at 55 °C (Fig. 3A) and between pH 6.0 and 7.0 (Fig. 4). The activity was stable over 7 h at 50 °C and was slightly affected at 55 °C with a half-life of 4 h. At 70 °C, the enzyme was rapidly inactivated and had a half-life of 30 min (Fig. 3B). After incubation for 24 h

Table 1
Summary of the purification strategy for α-L-arabinofuranosidase from *T. thermophilus*.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Recovery (%)	Purification fold
Culture supernatant	328	742	1.8	100	1
Ammonium Sulfate (60%)	190	521	2.7	70	1.5
Q-Sepharose	99	436	4.5	58	2.4
S-200 gel filtration	22	271	12.3	36	6.7
Mono-Q Sepharose	1.2	59	49	8	26.7

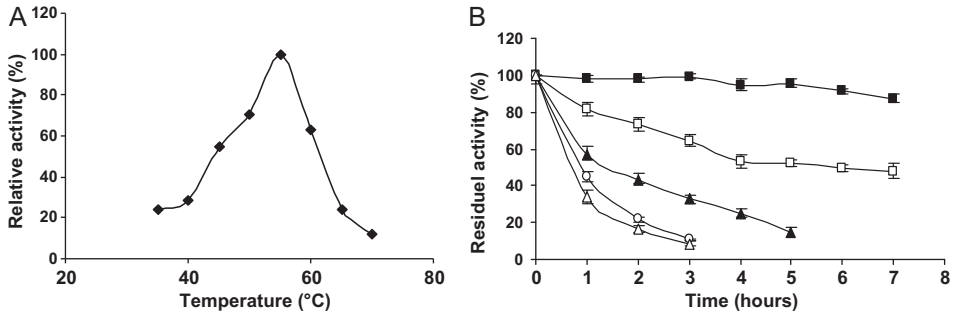

Fig. 3. Effects of temperature on activity (A) and stability (B) of *T. thermophilus* α-L-arabinofuranosidase. Enzyme activity was assayed in (50 mM) phosphate buffer pH 7 at different temperatures. For thermal stability, the pure enzyme was incubated in the same buffer at 50 °C (■), 55 °C (□), 60 °C (▲), 65 °C (○) and 70 °C (△). For different time intervals, residual activity was determined by standard method.

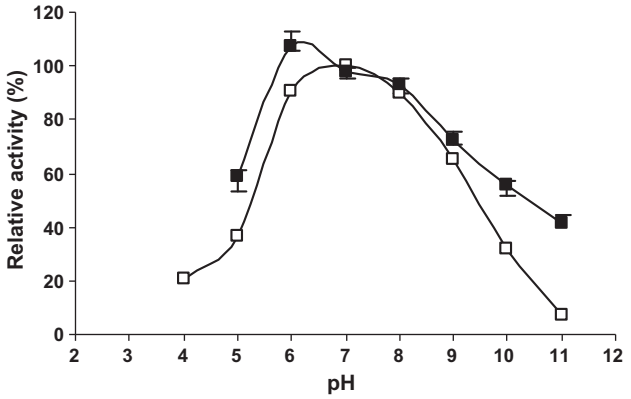

Fig. 4. Effects of pH on activity (□) and stability (■) of *T. thermophilus* α-L-arabinofuranosidase. For pH optimum, enzyme activity was assayed by the standard method after changing the buffer to obtain the desired pH. The buffers used were citrate (pH 4.0–5.0), phosphate (pH 6.0–7.0), AMPSO (pH 8–9), and glycine-hydroxide (pH 10–11). pH stability was determined by pre-incubating the enzyme for 24 h at 4 °C at these same different pH and the residual activity was assayed by the standard method.

at 4 °C at various pH (ranging from 5.0 to 11.0) (Fig. 4), the α-L-arabinofuranosidase was shown to be very stable between pH 6.0 and 8.0. We even found that the enzyme, after incubation at pH 6.0, was slightly activated relatively to its starting activity, measured under standard conditions. At pH 9.0, α-L-arabinofuranosidase retains up to 70% of its initial activity.

The influence of various reagents on the purified α-L-arabinofuranosidase activity was studied (Table 2). The activity

Table 2
Effect of various reagents on the activity of the purified *T. thermophilus* α-L-arabinofuranosidase. The data presented are an averages and standard errors of two independent experiments.

Reagents	Concentration (mM)	Relative activity (%)
Control	–	100
$CaCl_2$	5	133 ± 6
$BaCl_2$	5	99 ± 4
$CoCl_2$	5	123 ± 2
$MnSO_4$	5	135 ± 8
$MgSO_4$	5	114 ± 3
$ZnCl_2$	5	11 ± 4
$FeSO_4$	5	58 ± 4
$CuSO_4$	5	7 ± 2
$HgCl_2$	5	2 ± 0
NaCl	5	119 ± 5
EDTA	10	13 ± 6
Urea	10	118 ± 2
2-Mercaptoethanol	10	120 ± 1
DTT	10	100 ± 2
SDS	10	30 ± 6

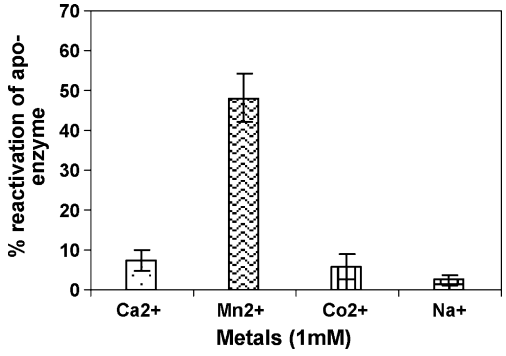

Fig. 5. Effect of metals on the reactivation of apo-α-L-arabinofuranosidase from *T. thermophilus*. Error bars represent the standard deviation.

was significantly inhibited by Hg^{2+}, Cu^{2+}, and Zn^{2+} cations. Inhibition was also observed in the presence of SDS and ethylenediaminetetraacetic acid (EDTA). The enzymatic activity was slightly stimulated in the presence of Mn^{2+}, Ca^{2+}, Co^{2+} and Na^{+} ions. The removal of metals from α-L-arabinofuranosidase resulted into almost complete loss of activity and apoenzyme of α-L-arabinofuranosidase displayed only about 7.6% residual activity. The effect of different metal ions like Mn^{2+}, Ca^{2+}, Co^{2+} and Na^{+} on the reactivation of apo-enzyme was determined (Fig. 5). The activity of apo-α-L-arabinofuranosidase was recovered (48% of reactivation) in the presence of 1 mM Mn^{2+}, other metals exhibited a low reactivation rate.

3.4. Substrate specificity to pNP glycosides

Relative rate of hydrolysis of various *p*-nitrophenyl glycosides by the purified α-L-arabinofuranosidase were examined (Table 3). The enzyme showed the highest activity towards *p*-nitrophenyl-α-L-arabinofuranoside (100%) but was not able to hydrolyse *p*-nitrophenyl-α-L-arabinopyranoside. α-L-Arabinofuranosidase was less active (52%) on *p*-nitrophenyl-β-D-xylopyranoside as substrate. Only 3.4% of that activity was found with *p*-nitrophenyl-β-D-mannopyranoside and no activity

Table 3
Substrate specificity of purified α-L-arabinofuranosidase.

Synthetic substrate	Relative activity (%)
p-Nitrophenyl-α-L-arabinofuranoside	100
p-Nitrophenyl-α-L-arabinopyranoside	0
p-Nitrophenyl-β-D-xylopyranoside	52 ± 4
p-Nitrophenyl-β-D-glucopyranoside	0
p-Nitrophenyl-β-D-mananoside	3.4 ± 1.5
p-Nitrophenyl-β-D-cellobioside	0

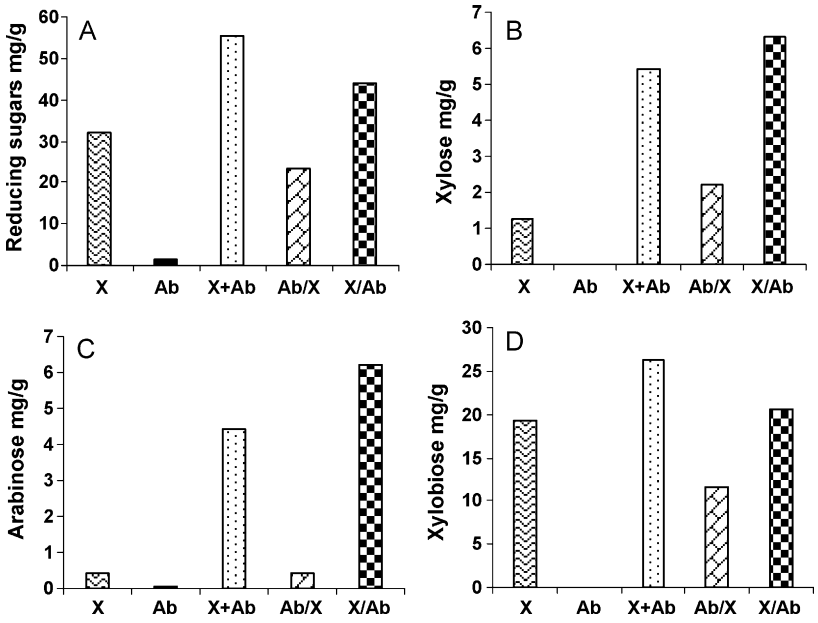

Fig. 6. Synergistic action of α-L-arabinofuranosidase and immobilized endo-β-1,4-xylanase. Plots show the release of reducing sugars (A), xylose (B), arabinose (C), and xylobiose (D) from 5% (w/v) wheat arabinoxylan. This substrate was treated for 4 h with 0.65 U/ml α-L-arabinofuranosidase alone (Ab); with immobilized family 11 endo-β-1,4-xylanase alone (X), with immobilized endo-β-1,4-xylanase for 4 h, with subsequent elimination by centrifugation, followed by α-L-arabinofuranosidase for 4 h (X/Ab); with α-L-arabinofuranosidase for 4 h with subsequent inactivation by boiling, followed by endo-β-1,4-xylanase for 4 h (Ab/X) or with both enzymes simultaneously (X + Ab).

could be detected with *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-cellobioside.

The rate dependence of the enzymatic reaction on the *p*NPA concentration at pH 7.0 and 55 °C followed Michaelis–Menten kinetics. Reciprocal plot analysis showed an apparent K_m value of 0.77 mM. The turnover number (K_{cat}) and catalytic efficiency (K_{cat}/K_m) of the enzyme were found to be 14.3 s^{-1} and 1.8 104 M^{-1} s^{-1}, respectively, for pNPA.

3.5. Synergistic action with immobilized endo-xylanase

Analysis of hydrolysis products of arabinoxylan by HPLC shows that it was composed of 38%, 23% and 6% of xylose (X), arabinose (A) and glucose, respectively. The A/X ratio of the arabinoxylan was then calculated to be 0.6. This extracted arabinoxylan fraction was used to test the combinatory effects of α-L-arabinofuranosidase and the immobilized family 11 endo-β-1,4-xylanase (both purified from culture filtrate of *T. thermophilus*) on the release of arabinose, xylose and xylobiose. Various combination of 0.65 U/ml α-L-arabinofuranosidase and 1 g of immobilized family 11 endo-xylanase (14 U/g of support) were incubated with 5% (w/v) wheat bran arabinoxylan in 50 mM phosphate buffer. Reactions were allowed to proceed at 55 °C for 4 h. The hydrolysis reaction products were analyzed by HPLC (xylose, arabinose and xylobiose were used as standards). Substrates were incubated with each enzyme individually, with both enzymes simultaneously, or sequentially as follow: condition (a), first incubation of the substrate with the immobilized endo-β-1,4-xylanase for 4 h followed by its subsequent elimination by centrifugation (10 min at 7000 rpm), then by incubation with α-L-arabinofuranosidase for a second cycle for 4 h. Condition (b), consist in the incubation of the substrate with the α-L-arabinofuranosidase for 4 h at 50 °C, followed by heat inactivation (15 min at 100 °C) and finally a last cycle of 4 h beginning by the addition of the immobilized endo-xylanase. Fig. 6 illustrates, that α-L-arabinofuranosidase was unable to act alone in the presence of a complex substrate such as arabinoxylan. In this condition, the rate of reducing sugars release was very low, as confirmed by HPLC analysis, showing the absence of liberated xylose and arabinose. On the other hand the endo-xylanase was capable of releasing almost 30 mg/g of reducing sugars (Fig. 6A), containing 1.26 mg/g, 0.4 mg/g and 19 mg/g of xylose, arabinose and xylobiose, respectively (Fig. 6A–C). Family 11 xylanases are known to be able to cleave arabinose from polymeric substrates [29], although no activity against *p*NPA could be detected. When both enzymes acted simultaneously, we noticed a clear improvement in the rate of reducing sugar release (>50 mg/g, superior to each enzyme acting individually). Sequential treatment of the substrate with endo-xylanase followed by α-L-arabinofuranosidase increases the rate of arabinose released from 0.44 mg/g for the endo-xylanase alone to 6.2 mg/g (Fig. 6C). The α-L-arabinofuranosidase was active only when the arabinoxylan was pre-treated initially with an endo-xylanase. α-L-Arabinofuranosidase was unable to release xylose from polymeric xylan but the simultaneous action of both enzymes released more xylose than endo-xylanase acting alone (Fig. 6B). Treatment of the arabinoxylan by the endo-xylanase produced 19 mg/g of xylobiose. Simultaneous action with α-L-arabinofuranosidase (Ab + X) can improve this rate to reach 26 mg/g. However, α-L-arabinofuranosidase was not able to release additional xylobiose from wheat arabinoxylan after pre-treatment with endo-xylanase (Fig. 6D). The treatment with α-L-arabinofuranosidase followed by the endo-xylanase (Ab/X) did not improve hydrolysis efficiency of arabinoxylan, due to the inability of the α-L-arabinofuranosidase to change the texture of the polymer substrate.

4. Discussion

The generation of fermentable sugars from lignocellulosic resources is an important step in the biotransformation of renewable biomass into useful products such fuels, chemicals and polymers [30]. It is clear that a complete xylanolytic system of filamentous fungus including debranching and depolymerizing activities is necessary to achieve a complete hydrolysis of hemicellulose [31]. When cultivated on wheat bran as only carbon source, *T. thermophilus* produces a wide spectrum of hemicellulases (Fig. 1). This hemicellulase complex has been previously reported as a good

alternative for lignocellulosic plant material saccharification and particularly for arabinose and xylose production [21]. No cellulase activity was detected in culture filtrate after growth on wheat bran. In this context, the use of cellulase-free xylanases has a wide range of biotechnological applications, especially in the pulp and paper industries. They are used primarily as bleaching agents to reduce the amount of chlorine required to achieve desirable levels of paper brightness [32].

To better exploit the *T. thermophilus* xylanolytic system, the purification and characterization of the α-L-arabinofuranosidase activity were investigated. Except α-L-arabinofuranosidase belonging to family 62 that cannot hydrolyse effectively the *p*NPA as substrate [33], the results observed after purification suggest that the enzyme preparation from *T. thermophilus* contains only one α-L-arabinofuranosidase enzyme. This was also confirmed by overlay zymography method (data not shown). In contrast, multiple forms of α-L-arabinofuranosidase have been detected in the culture broths of *Aspergillus niger* [11] and *Penicillium capsulatum* [12]. Moreover, the analysis by gel filtration showed that the α-L-arabinofuranosidase from *T. thermophilus* is a monomer with an apparent molecular mass ~35 kDa. Several fungi possess α-L-arabinofuranosidase with a low molecular mass, such as *Aspergillus sojae* (34.4 kDa) [34], *Aspergillus terreus* (39 kDa) [35], and *Aspergillus nidulans* (36 kDa) [36]. The molecular mass of fungal α-L-arabinofuranosidase is ranging from 31 kDa to 105 kDa [6], this indicates considerable diversity in α-L-arabinofuranosidase molecular weight that influences biochemical characteristics. The maximal activity of the purified α-L-arabinofuranosidase from *T. thermophilus* was observed at 55 °C and pH 7.0. This enzyme was very stable over a considerable temperature range, as observed for several α-L-arabinofuranosidase of fungal strains [6]. Stability and activity at high temperatures are desirable properties in this class of enzymes, considering the fact that the most industrial processes using xylanolytic enzymes are carried out at high temperatures [37]. According to their physicochemical properties, some fungal α-L-arabinofuranosidases were active and stable only at acidic pH [6]. However, most bacterial α-L-arabinofuranosidases that are purified until now, have the optimum pH around 7.0, such as *Bacillus stearothermophilus* [9] and *Ruminococcus albus* [38]. The addition of metal ions such as Zn^{2+}, Cu^{2+} and Hg^{2+} inhibited the α-L-arabinofuranosidase activity significantly, suggesting that α-L-arabinofuranosidase is a thiol-sensitive enzyme because these heavy metal ions bind free mercapto groups (–SH) in cysteine residues. Moreover, sensitivity to Hg^{2+} indicates that α-L-arabinofuranosidase has an active-site thiol group [39]. This effect was also observed in the α-L-arabinofuranosidase of *Streptomyces* sp. *PC22* [40], *Aspergillus nidulans* [36] and two *Penicillium chrysogenum* α-L-arabinofuranosidases [13]. Apo-forme of enzyme was reactivated in the presence of 1 mM of Mn^{2+}, probably this α-L-arabinofuranosidase is mostly dependant on this metal ion to maintain an active structure. This property made it novel for this group of enzyme.

The α-L-arabinofuranosidases belonging to family 3 and 43 of glycosyl hydrolases can hydrolyze the *p*NPX following a non-specific reaction [41]. This can be explained by the fact that the D-Xylopyranose and α-L-arabinofuranose are spatially similar such that the glycosidic bonds and hydroxyl groups can be overlaid, leading to bifunctional enzymes [42]. Other notable property of this enzyme was its apparent high affinity for the *p*NPA (K_m: 0.77 mM). Lower K_m values for homogeneous α-L-arabinofuranosidase have been previously reported for a few other enzymes such as α-L-arabinofuranosidase of *A. niger* (0.6 mM) [11] and that of *Streptomyces* sp. *PC22* (0.23 mM) [40]. The *T. thermophilus* α-L-arabinofuranosidase hydrolyses *p*NPA with a catalytic efficiency 3.5 fold higher than α-L-arabinofuranosidase of *Penicillium purpurogenum* [43].

The pentose composition of the extracted arabinoxylan indicated a 0.6 ratio of arabinose/xylose. This result was quite in good agreement with previously reported data [44] and may indicate the quality of the arabinoxylan fraction. The evidence from synergy experiments (Fig. 6) suggests that *T. thermophilus* α-L-arabinofuranosidase is unable to hydrolyze α-L-arabinofuranose bound to internal xylosyl units of alkali-extracted arabinoxylan. This inability was circumvented when the enzyme was acting in concert with family 11 endo-xylanases. Clearly the low-molecular-mass substituted xylo-oligosaccharide products generated by endo-xylanase action are better substrates for the α-L-arabinofuranosidase. Similar result had been reported previously for *Aspergillus awamori* α-L-arabinofuranosidase [31], suggesting that *T. thermophilus* α-L-arabinofuranosidase may belongs to family 51 of glycosyl hydrolases. This group of α-L-arabinofuranosidases degrades only low molecular mass L-arabinofuranose-containing xylooligosaccharides and synthetic substrates [15]. Several factors can affect the binding of α-L-arabinofuranosidase to the arabinoxylan, such as, the steric hindrance caused by the substituents on the xylose backbone of the arabinoxylan molecule and the conformational changes of the polysaccharide chains especially after hydrolysis of ester linkages by alkali treatment [31]. Treatment of arabinoxylan with the simultaneous action of endo-xylanase and α-L-arabinofuranosidase can, therefore, be considered as a potential alternative for the eventual production of xylobiose. This compound is a selective growth stimulant to intestinal *Bifidobacterium*, which is beneficial for the maintenance of healthy intestinal microflora. The selective stimulative effect of xylobiose on *Bifidobacterium* was greater than that of other oligosaccharides [45].

Treatment of the substrate with either only endo-xylanase or sequential reactions of Ab/X should theoretically give the same results but two main causes can affect the result. The first one is the nature of alkali-extracted arabinoxylan (heterogeneity due to its partial purification), the second one concerns experimental errors.

The synergistic interaction of the α-L-arabinofuranosidase and endo-xylanase of *T. thermophilus* is obviously essential for the extensive hydrolysis of complex arabinoxylan polymers.

5. Conclusion

The results presented in this paper indicate that α-L-arabinofuranosidase of *T. thermophilus* play an important role in the assimilation of arabinose moieties from arabinose-containing xylooligosaccharides generated by endo-xylanase. For this reason, α-L-arabinofuranosidase can be suitable for practical applications in food processing, such as efficient degradation of hemicellulosic biomass for production of biofuels or L-arabinose and its derivatives. In the future, accessory enzymes such as α-L-arabinofuranosidases combining with xylanase might be widely applied in animal feed to convert xylan to simple sugars effectively and enhance feed digestibility. Studies on this novel enzyme, such as its gene cloning are now in progress.

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