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ORIGINAL ARTICLE

Purification of β-xylosidase from *Aspergillus tamarii* using ground oats and a possible application on the fermented hydrolysate by *Pichia stipitis*

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Abstract In this study we determined that *Aspergillus tamarii* Kita is able to utilize *Avena sativa* L. (oats) for the production of β-xylosidase under static or shaking conditions in submerged liquid-state (LSF), solid-state (SSF) and slurry-state (SlSF) cultures. The produced enzyme was purified and characterized. Maximum yield occurred under shaking conditions in SSF cultures (33.7 U/ml), with 24.9 and 5.5 U/ml produced in SISF and LSF cultures, respectively. Peptone was found to be the best nitrogen additive and enhanced enzyme production (41.5 U/ml). The produced enzyme was precipitated by ammonium sulfate (60 %) and further purified by gel filtration through a Sephadex G-100 and ion exchange column of diethylaminoethyl cellulose, with a yield of 40.57 % and 35.73-fold purification. Enzyme activity was optimal at pH 5.5 and 55 °C. The purified enzyme retained full activity even at the end of a 1-h incubation at this optimal condition. Midpoint of thermal inactivation (*T*m) was recorded at 60 °C after 90 min of exposure. The Michaelis–Menten constant, maximal reaction velocity, turnover number and specificity constant of the purified enzyme were calculated to be 0.075 mg/ml, 71.42 U/mg of protein, 7.14/S and 95.2 mg/ml/s, respectively. The inability of the purified enzyme to hydrolyze celluloses indicated that the enzyme was a free cellulase. The most efficient enzyme activators were Mg^{2+}, followed by Mn^{2+} and Zn^{2+} in that order. The molecular mass of the purified enzyme was 91 kDa as determined by SDS-PAGE. The possibility of using the fermentation of ground oat hydrolysate for the production of ethanol and xylitol in the presence of *Pichia stipitis* Pignal was assessed. The maximum production of ethanol and xylitol were obtained after 72 h of fermentation, resulting in 11.06 and 21.51 g/l respectively.

Keywords *Aspergillus tamarii* · β-xylosidase · Oat · *Pichia stipitis* · Ethanol · Xylitol

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Introduction

Hemicellulose is the second most abundant plant biomass fraction in nature (Kim 2005; Comlekcioglu et al. 2011; Zhou et al. 2012; Shi et al. 2013; Zimbardi et al. 2013). Xylan, which is the principal hemicellulose component, consists of xylose units and is a major constituent of plant cell walls. Xylanolytic enzymes have become the focus of increased interest due to their applications in the paper, food processing and textile industries, leading to reduced primary costs (Haltrich et al. 1996; Kulkarni et al. 1999; Saha 2003; Semenova et al. 2009; Kanna et al. 2011; Terrasan et al. 2013). The potential applications of xylanases with or without cellulase include the bioconversion of lignocelluloses to sugar, ethanol and other useful substances, clarification of juices and wines, extraction of plant oils, coffee and starch and improvement to the nutritional value of silage and green feed (Wong and Saddler 1992; Knob et al. 2010). For these reasons, xylan degradation has been carried out by xylanolytic enzymes produced by a variety of microorganisms, including bacteria, yeasts and fungi (Carmona et al. 1997; Coughlan and Hazlewood 1993; Fawzi 2010; Kanna et al. 2011; Zimbardi et al. 2013).

β-Xylosidase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.37) hydrolyzes xylobiose and short chain xylooligosaccharides from the nonreducing end to xylose. This enzyme is essential for the complete breakdown of xylan to xylose (Biswas et al. 1988).

Filamentous fungi have received much attention for their potential applications as xylanolytic enzyme producers, primarily because fungi produce more xylanolytic enzymes than yeasts or bacteria. In this context, Haltrich et al. (1996) and Bhat (2000) attempted to obtain xylanolytic enzymes from members of the genera *Trichoderma* and *Aspergillus* for commercial use. *Aspergillus tamarii* has been found to grow well and to produce a high cellulase-free xylanase activity in both submerged and solid state fermentation systems using corn cob, wheat bran and sugar cane bagasse as the main substrates (Kadowaki et al. 1997; Ferreira et al. 1999).

Xylose from hemicellulose constitutes a potential low-cost material for the biotechnological production of xylitol and fuel ethanol and, in terms of abundance, only glucose constitutes a higher fraction of the total carbohydrates of lignocellulosic hydrolysates (Hector et al. 2008; Girio et al. 2010). As a result, the ability of the fermenting microorganisms to utilize the xylose available from the hydrolysate is a vital determining factor for improving the commercial production of cellulosic ethanol and potentially of bio-based chemicals through economically competitive processes (Sakai et al. 2007). The environmental impacts associated with the use of fossil fuels and the rapid depletion of the world‘s reserves of such fuels are the two important reasons for promoting the production of biofuels from available biomass (Wang et al. 2007). This has led to increasing attention being directed towards ethanol production from all types of grains (Latif and Rojoka 2001). Usvalampi (2013) reviewed ethanol production from xylose by yeasts and pointed out that xylose fermentation using yeasts has many advantages over fermentation using bacteria, including the higher ethanol tolerance of yeasts, the relative ease in harvesting and recycling yeast cell compared to bacterial cells from the fermentation broth and the resistance of yeast fermentation to contamination from bacteria and viruses. Among the yeasts used for xylose fermentation, only six species have been found to produce significant amounts of ethanol, and only three of these, namely *Candida shehatae*, *Pachysolen tannophilus* and *Pichia stipitis*, have been studied extensively (Sanchez et al. 2002). *Pichia stipitis* is one of the natural xylose-fermenting yeast strains, and it has been shown to be the most useful species for the direct fermentation of xylose to a high ethanol yield (Agbogbo and Coward-Kelly 2008; Ghindea et al. 2010).

The majority of the industrial enzymes on the market are produced by submerged liquid fermentation (SLF). However solid-substrate fermentation (SSF) has been shown to improve both enzyme yield and the cost of enzyme production (Pandey 1994; Saad and Fawzi 2012). SSF refers to the growth of microorganisms on solid materials in the absence of free liquid (Fadel 2001; Fawzi 2009; Zimbardi et al. 2013). Saake et al. (2003) and Puls et al. (2006) reported that aat grains contain a remarkably high amount of soluble arabinoxylan (a xylan backbone with L-arabinofuranose). Therefore, we selected oat grains as the substrate in our present study.

The aims of our study were twofold. The first was to produce and purify β-xylosidase utilizing oat grains (*Avena sativa* L.) and LSF, SSF and slurry-state (SlSF) fermentation protocols that involve inoculation with *A. tamarii* Kita. The second goal was to investigate the probability of using the fermented hydrolysate (crude enzyme broth of *A. tamarii*) for the production of ethanol and xylitol using *Pichia stipitis* Pignal, a haploid, homothallic, hemiascomycetous yeast.

Materials and methods

Preparation of materials Grains of the common oat (*Avena sativa* L.) were obtained from the Vegetables Research Center, El-Dokkey, Giza, Egypt, ground to pass through a 50-μm sieve and then oven-dried at 55 °C for 24 h until they reached a constant weight. The powder was stored in dry flasks at room temperature until use.

Microorganisms The *Aspergillus* strain used in this study, *A. tamarii* Kita IMI 380870, was previously isolated by Prof. Dr. Ahmed A. El-Gindy (Ain Shams University, Cairo) from an Egyptian soil sample and identified by Dr. Z. Lawrence (CABI, UK Centre, UK). The fungus was maintained on malt extract agar at 4 °C and routinely cultured. Stock cultures of *A. tamarii* were stored in the form of spore suspension of up to 1×10^6 spores/ml in a 25 % (v/v) glycerol solution at 30 °C.

Pichia stipitis Pignal NRRL Y-7124 was obtained from the Agricultural Research Service Culture Collection, United States Department of Agriculture, New Orleans, LA). Cultures of this yeast were maintained on malt extract agar slants at 4 °C (Ghindea et al. 2010). For inoculum preparation, yeast cells in the maintenance medium were transferred to a previously sterilized Erlenmeyer flask containing 100 ml of medium A (composition in (g/l: xylose, 30.0; glucose, 5.0; arabinose, 5.0; urea, 2.3; $MgSO_4 7H_2O$, 1.0; yeast extract, 3.0). The inoculated flask was incubated at 37 °C and 200 rpm for 48 h, following which, when the end of the exponential growth phase had been reached, the cells were recovered by centrifugation (2,000 *g*, 20 min), washed twice with sterile distilled water and resuspended in the fermentation medium. Stock cultures of *P. stipitis* were stored in a mixture of 25 % (v/v) glycerol and liquid medium A.

Enzyme production and fermentation media Flasks containing the different fermentation media were prepared in triplicate, and the initial pH value was adjusted to 5.5. After sterilization at 121 °C for 20 min, each flask was inoculated with 1 ml of the *A. tamarii* spore suspension (10^6 spore/ml) and incubated for 3–7 days at 30 °C under static and shaking

(150 rpm) conditions [GFL Shaking Incubator; Gesellschaft für Labortechnik mbH (GFL), Burgwedel, Germany].

The media used were:

LSF medium, composed of (in g/l): freshly prepared ground oats, 30; NH_4Cl, 2.0; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 1.0, in a final volume of 50 ml per flask.

SSF medium, in which 5 ml of LSF medium—without the ground oats—was added to 10 g of oat spelts.

SISF medium, in which 20 ml of distilled water was added to SSF medium to achieve the slurry state condition.

Enzyme extraction At the end of the incubation period, the cell-free filtrate in the LSF system was obtained by filtering through Whatman filter paper No. 1 in a Buchner funnel. In the SSF and SlSF systems, the fermented matter was thoroughly mixed with 10 ml of cold distilled water by keeping the flasks on a rotary shaker for 1 h at 150 rpm. The mixture was then filtered through muslin cloth. The volume of all the filtrates (enzyme extracts) obtained from the different protocols was restored to 50 ml through the addition of cold distilled water and served as the crude enzyme preparation.

β-Xylosidase and protein assays β-Xylosidase activity was determined by incubating 1.0 ml of 1 % (w/v) *p*-nitrophenyl-β-D-xyloside (*p*NPβX) with 1.0 ml of diluted enzyme in 0.05 M citrate buffer at pH 5.5. (Panbangred et al. 1983). After incubation at 50 °C for 30 min, the reaction was stopped by adding ice-cold 0.5 M Na_2CO_3 (1.0 ml), and the color that developed as a result of *p*-nitrophenol (*p*NP) liberation was measured at 405 nm. One unit (U) of β-xylosidase activity was defined as the amount of enzyme that liberated 1 μmol *p*NP per minute in the reaction mixture under these assay conditions.

Protein concentration was determined using bovine serum albumin dissolved in 0.17 M NaCl as a standard (Bradford 1976).

Effect of different nitrogen sources on β-xylosidase The effect of different organic and inorganic nitrogen sources was explored. Equimolecular amounts of three organic nitrogen sources [casein, 5.0 g; peptone, 5.0 g; yeast extract, 3.0 g] and three inorganic nitrogen sources {ammonium nitrate [NH_4NO_3], 3.0 g; ammonium sulfate [$(NH_4)_2SO_4$], 2.0 g; sodium nitrate ($NaNO_3$), 2.0 g} were replaced by the original nitrogen sources in the optimal media.

β-Xylosidase purification The first step in β-xylosidase purification was dialysis of the cell-free filtrate (CFF) at 4 °C. This was followed by precipitation of the cell-free dialysate (CFD) with $(NH_4)_2SO_4$ (60 % concentration). The solution was left overnight at 4 °C and then centrifuged, dissolved in a minimum volume of 50 mM Tris–HCl buffer and dialyzed again by the same buffer. The dialyzed enzyme solution (concentrated to 5 ml) was applied onto a Sephadex G-100 column (2.5 × 82 cm; Sigma-Aldrich, St. Louis, MO) pre-equilibrated with Tris–HCl buffer (pH 8). The column was eluted with the same buffer at 10 ml/h. Active fractions (5.0 ml each) were pooled, lyophilized and further purified on a DEAE-cellulose column (fast flow, fibrous form; Sigma-Aldrich). The DEAE-cellulose column was eluted with a gradient of 0–0.8 M NaCl prepared in the corresponding buffer, at a flow rate of 20 ml/h, and 5-ml fractions were collected and dialyzed once again to remove Na^+ and Cl^-. The enzyme was then lyophilized and stored at 0 °C for further analysis (Peterson and Sober 1962; Palmer 1991).

Characterization of the purified β-xylosidase

Effect of pH and pH stability The effect of pH on purified β-xylosidase enzyme activity was assessed by adding 1.0 ml of enzyme solution to 1.0 ml of 1 % *p*NPβX at different pH values (3.5–9) obtained by using 0.05 M citrate-phosphate and 0.05 M Tris–HCl buffer. After incubation at 50 °C for 30 min, the reaction was stopped and the enzyme activity deduced from the amount of sugar liberated. To determine pH stability, the enzyme was incubated at varying pH values (3.5–9) for 60 min. The residual enzymatic activity was assayed.

Effect of temperature and thermal stability The effect of temperature on enzyme activity was assessed by incubating the enzyme with its substrate at various temperatures ranging from 30 to 85 °C. Enzyme activity was measured to determine the optimum temperature for activity. However, for the determination of thermal stability, the enzyme was incubated for different lengths of time (15–90 min) at fixed temperatures (55–65 °C).

Substrate specificity tests The enzyme was incubated with different substrates; cellulose, cellobiose, carboxymethylcellulose, pectin, birch wood xylan and oat spelt xylan (1 % w/v) at 50 °C for 30 min. The reaction was then stopped and the residual activity for enzyme assayed. Enzyme activity with its substrate, *p*NPβX, was defined as 100 % relative activity.

Kinetic parameters The effect of *p*NPβX concentration (tested range 6–20 mg/ml) on enzyme activity was evaluated under optimal assay conditions. The kinetic parameters [Michaelis–Menten constant (K_m), maximal reaction velocity (V_{max}), turnover number (K_{cat})] were estimated by linear regression from double-reciprocal plots according to Lineweaver and Burk (1934).

Effect of different chemical additives In this experiment we examined the effect of different chemical additives by incubating 1.0 ml of enzyme solution with 10 mM of each of the metal ions and chemical reagents ($CuSO_4$, $CuCl_2$, $CoCl_2$, EDTA, $FeSO_4$, $MgSO_4$, $MgCl_2$, $MnSO_4$, NaCl, $ZnSO_4$) in 0.05 M citrate buffer, pH 7.0, with 1 % *p*NPβX as a substrate at 50 °C for 30 min. The remaining activity of the enzyme was measured under the standard conditions described above. Enzyme activity in the absence of additives was defined as 100 % relative activity.

Molecular mass determination Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was carried out in 10 % polyacrylamide gel slabs at pH 8.3 by using 25 mM Tris–HCl buffer containing 0.1 % (w/v) SDS as described by Laemmli (1970). The electrophoresis was performed overnight (18 h) at room temperature (20 °C). The gels were stained with Coomassie Brilliant Blue (0.25 % w/v) in methanol/acetic acid/water (5:1:4, v/v/v) for 2 h and afterwards bleached at 40 °C in a solution containing 25 % (v/v) methanol and 7 % (v/v) acetic acid in water. The molecular mass of the purified enzyme was estimated using standard protein markers (phosphorylase b, 97 kDa; bovine serum albumin, 67 kDa; egg albumin, 45 kDa; glyceraldehyde-3-phosphate, 36 kDa; carbonic anhydrase, 29 kDa; Sigma-Aldrich)

Xylitol production To screen for xylitol production, sugar tubes containing 20 ml of crude enzyme broth from the SSF (33.7 U/ml) as the ground oats hydrolysate were prepared; the xylose content in the hydrolysate was 5.03 g/l. Tween-80 (0.1 ml) was then added, followed by the addition of concentrated solutions of yeast extract and peptone (5 ml each) to the enzyme broth to a final concentration of 2 and 3 % (v/v), respectively (Latif and Rojoka 2001). Finally, the cells of yeast (*P. stipitis*) were added (2.5 ml yeast suspension of 1-day-old culture), and the sugar tubes were incubated at 37 °C and pH 5.0 for 48 h. After 48 h of anaerobic fermentation, the culture broth was separated and analyzed for xylitol. Xylitol production was repeatedly estimated at intervals of 72- and 96-h.

High-performance liquid chromatography analysis of xylose, ethanol and xylitol Samples were first filtered through a 0.45-μm membrane, and the carbohydrates in the filtrate were analyzed using a high-performance liquid chromatography (HPLC) system (model VPV 5.03; Shimadzu Corp., Kyoto, Japan) equipped with the refractive index RID-10A Shimadzu detector, LC-16ADVP binary pump, DCou-14 A degasser, Shodex PL Hi-Plex Pb column (Sc 1011 No. H706081), guard column Sc-Lc Shodex and a heater set at 80 °C. The mobile phase was double distilled water, and the flow rate was 1 ml/min. Standard solutions were prepared by diluting working standards. The injection volume of each standard was 20 μl. For xylitol determination, the column was set at 60 °C. The carrier was acetonitrile/H_2O (75:25), the flow rate was 1.0 ml/min and the injection volume was 20 uL (Nojiri et al. 2000; Martínez-Montero et al. 2004). Since the concentration of standard was known, the concentration of the sample was estimated according to the concentration of standard injected to the HPLC system.

Statistical validation of treatment effects

The mean, standard deviation, *t* score and probability "*P*" values of three replicates of the investigated factors and the

Table 1 Production of β-xylosidase by *Aspergillus tamarii* at different time points during the incubation period under liquid-state fermentation, solid-state fermentation and slurry-state fermentation conditions

Time-course (h)	β-xylosidase production (U/ml)					
	LSF		SlSF		SSF	
	Static	Shaking	Static	Shaking	Static	Shaking
72	1.2±0.02	2.6±0.01	4.8±0.05	6.6±0.05	9.7±0.09	11.4±0.12
96	5.4* ±0.03	8.1 ±0.02	12.5*±0.22	18.3*±0.23	21.1* ±0.02	26.2*±0.24
120	6.1*±0.11	5.5*±0.22	15.1*±0.26	24.9* ± 0.8	22.4* ±0.05	33.7*±0.32
144	7.8*±0.09	11.9*±0.14	21.9*±0.18	23.5*±0.12	23.6* ±0.09	32.5*±0.52
168	6.5*±0.07	15.7*±0.08	19.2*±0.23	21.7*±0.31	22.8*±0.12	32.3*±0.82
Least significant difference (LSD)						
1 %	0.88	4.65	5.88	5.95	6.23	8.64
5 %	0.54	2.87	2.97	3.11	4.89	5.73

*Difference from the control value at 72 h of incubation is highly significant at $P<0.01$ according to the LSD test (a set of individual *t* tests)

LSF, Liquid state fermentation; SSF, solid state fermentation; SlSF, slurry state fermentation

Measurement data are presented as the mean of 3 readings±standard deviation (SD).

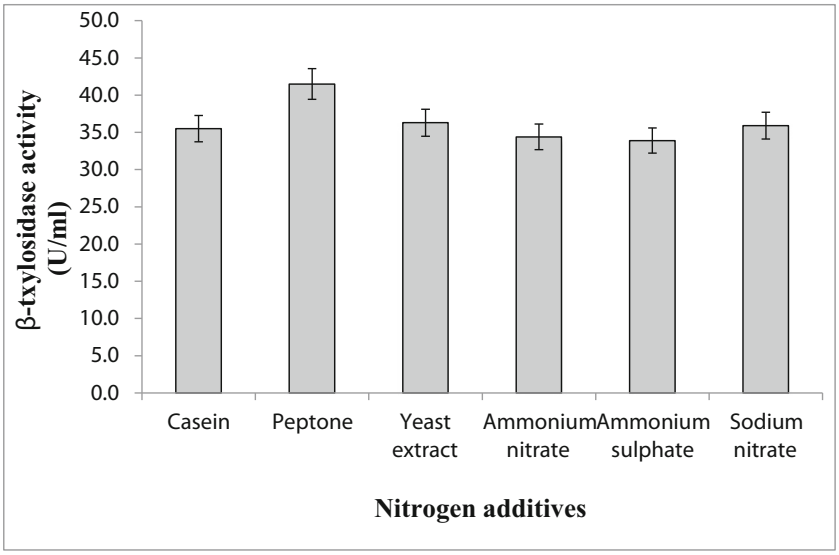

Fig 1 The effect of different organic and inorganic nitrogen sources on the production of β-xylosidase from *A. tamarii*

control were computed according to the mathematical principles described by Glantz (1992). The results were considered to be highly significant, significant or non-significant at $P<0.01$, <0.05 or >0.05, respectively.

Results and discussion

β-Xylosidase production by *A. tamarii* under LSF, SSF or SlSF conditions and with static or shaking incubation was monitored at different time points during the incubation periods (72–168 h). The results (Table 1) of the time-course measurements of β-xylosidase production by *A. tamarii* under LSF, SSF or SlSF conditions at 30 °C showed that the maximum yield was achieved after 120 h of shaking incubation and after 144 h of static incubation. β-Xylosidase production under shaking conditions was significantly higher than that under static conditions within the same fermentation protocol. Analysis of enzyme production in relation to the fermentation protocol revealed that the maximum amount of enzyme was produced under SSF conditions (33.7 U/ml), followed by SISF (24.9 U/ml) and LSF (15.5 U/ml); these data were calculated to 3.86, 2.74 and 1.5 U/mg protein, respectively (not shown in Table 1). Biswas et al. (1988) reported that β-xylosidase production by *Aspergillus ochraceus* on solid substrates is higher than in LSF. In LSF, β-xylosidase is partially intracellular and is subsequently secreted into the medium during fermentation, while the enzyme in SSF is completely secreted out of the cells (Kim 2005; Zimbardi et al. 2013). However, caution is advised when the incubation periods are compared, even for the same fungal species, due to variations in the fermentation state and the substrate utilized. SSF has several advantages over a liquid culture, including low capital costs for equipment, high volumetric productivity and decreased operational costs. On the other hand, the problems associated with SSF include a lower overall productivity, the critical importance of the moisture content of the medium, the heat build-up in fermenting solids and the requirement of substrate pre-treatment to facilitate microbial attachment (Wang 1999). To bridge this gap, alternative techniques, such as solid/slurry state fermentations, and/or less expensive substrates may be used. However, very little information is currently available on SlSF, and the data which are available are mostly restricted to anaerobic fermentations (De-Gregorio et al. 2002).

Table 2 A Summary of treatments used for the purification of β-xylosidase from *A. tamarii*

Treatment	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Recovery (%)	Purification (folds)
Cell-free filtrate (200 ml)	2,034	8,200	4.03	100	1.0
Cell-free dialysate	2,033	8,112	3.99	98.92	0.99
Cell-free precipitation $(NH_4)_2SO_4$ (60 %)	481	5,927	12.32	72.28	3.05
Gel filtration (Sephadex G-100)	98.6	4,435	44.97	54.08	11.15
Ion-exchange chromatography DEAE-cellulose	23.1	3,327	144.02	40.57	35.73

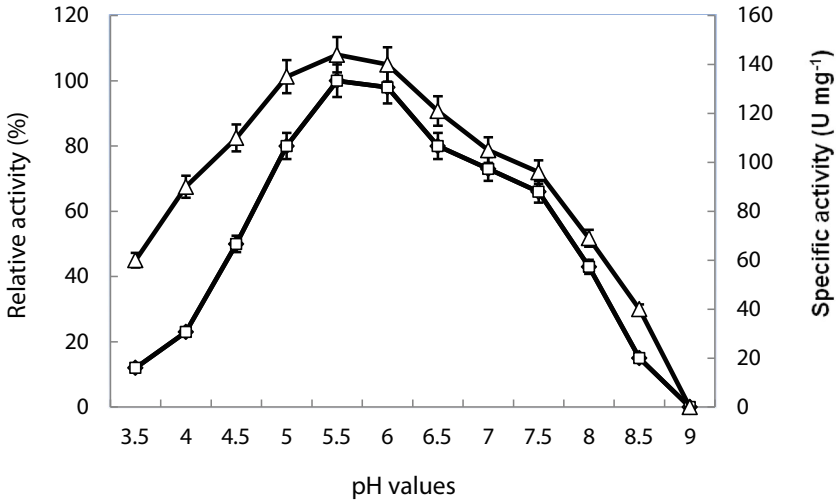

Fig 2 pH profile (*open triangle*) and pH stability (*open box*) of purified β-xylosidase from *A. tamarii*

Analysis of the effect of organic and inorganic nitrogen sources on β-xylosidase production by *A. tamarii* revealed that peptone was the best nitrogen additive that enhanced enzyme production (41.5 U/ml), followed by yeast extract and casein (Fig. 1). In previous studies, organic nitrogen was found to be superior to inorganic nitrogen sources in terms of enhancing the production of enzymes (Reid 1983; Fawzi 2011).

In this study, we purified to homogeneity an extracellular ß-xylosidase isolated from the cell-free culture supernatant of *A. tamarii* grown on oatmeal substrate. A summary of the purification steps is provided in Table 2. The enzyme was precipitated by 60 % $(NH_4)_2SO_4$, with a 72.28 % yield and fold-purification of 3.05, followed by gel filtration using Sephadex G-100. In this step, ß-xylosidase showed a fold-purification of 11.15, with a yield of about 54.08 % and specific activity of 44.97 U/mg protein. The purification procedure was completed by anion exchange chromatography on a DEAE-cellulose column using a linear sodium chloride gradient. The final enzyme preparation displayed only a fold-purification increase in specific activity of 35.73 (144.02 U/mg protein) compared with the culture filtrate, with a level of recovery relative to the original activity of 40.57 % (Table 2). The specific activity of the purified ß-xylosidase was approximately similar to that of ß-xylosidase from *Fusarium proliferatum* (Saha 2003). In comparison, previous studies have shown that purified ß-xylosidases from a number of fungi have very low specific activities, including those from *Trichoderma lignorum* (2.4 U/mg protein), *Neocallimastrix frontalis* (0.9 U/mg protein), *Talaromyces emersonii* (3.42 U/mg protein) and *Aspergillus carbonarius* (3.29 U/mg protein) (Garcia-Campayo and Wood 1993; Tuohy et al. 1993; Kiss and Kiss 2000).

A number of enzymatic properties of ß-xylosidase purified from *A. tamarii* was studied. The optimal pH was around 5.5–6.0 (Fig. 2). Interestingly, according to the pH stability data, the purified enzyme was completely stable at pH 5.5 for 60 min, and it retained >95 % of its activity after being incubated for 60 min at pH 6.0. The residual activity was

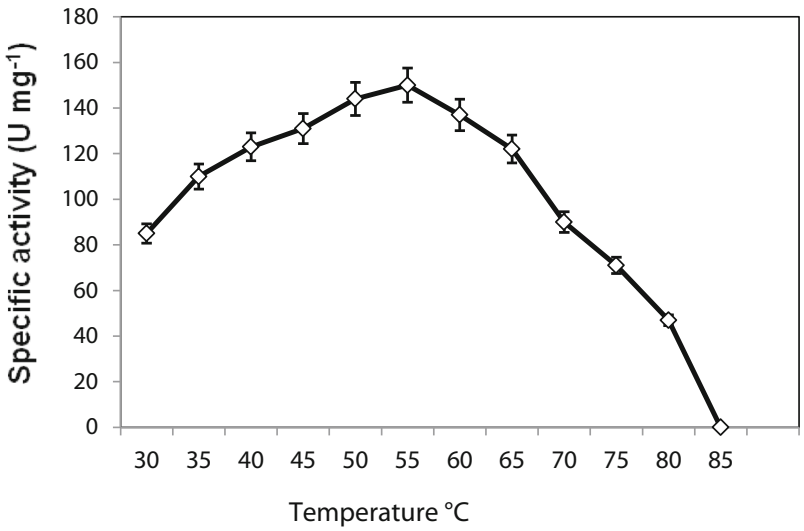

Fig 3 The effect of temperature on the purified β-xylosidase from *A. tamarii*

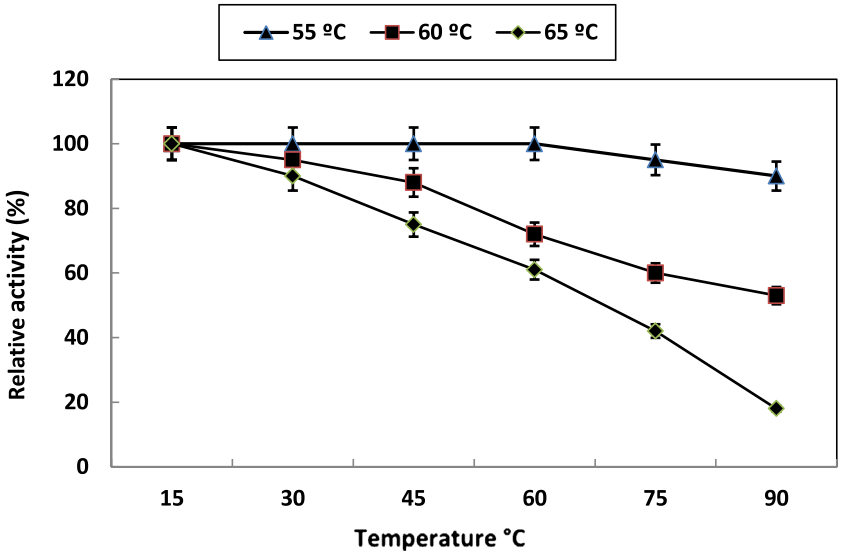

Fig 4 Thermal stability of the purified β-xylosidase from *A. tamarii*

almost 80 % at pH 5.0 and 6.5. These data agree to a certain extent with previously reported data on ß-xylosidase isolated from various fungi (Kiss and Kiss 2000; Saha 2003; Knob et al. 2010). In terms of the effect of temperature on ß-xylosidase activity, the optimum temperature of the purified enzyme was found to be 55 °C (Fig. 3), which is similar to the optimum temperature determined for ß-xylosidase isolated from *F. proliferatum* (Saha 2003). The enzyme retained 100 and 90 % of its activity after incubation at 55 °C for 1 h and 1.5 h, respectively. The midpoint of thermal inactivation (*T*m) was recorded at 60 °C after 90 min of exposure (Fig. 4). The thermal stability of this enzyme was higher than that from *T. emersonii* (Tuohy et al. 1993) and was similar to that from *F. proliferatum* (Saha 2003). The specificity of the enzyme against a number of substrates is shown in Table 3. The enzyme was highly specific against oat spelt xylan (97.9 %) and birch wood xylan (87.7 %), while almost no activity was detected when cellulose, cellobiose, caboxymethyl cellulose (CMC) and pectin were used. This inability of the enzyme to hydrolyze celluloses indicates that the enzyme was a free-form cellulase (Kadowaki et al. 1997; Ferreira et al. 1999).

Table 3 The effect of different substrates on the activity of purified β-xylosidase from *A. tamarii*

Substrate	Relative activity (% of control)
p-Nitrophenyl-β-D-xyloside	100
Cellulose	0.0
Cellobiose	0.0
Carboxymethyl cellulose	0.0
Pectin	0.0
Birch wood xylan	87.72±7.01
Oat spelt xylan	97.92±10.11
LSD	
1 %	2.364
5 %	1. 472

Use of the assay substrate at increasing concentrations resulted in saturation of the enzyme at a concentration of 12 mg/ml (data not shown). This characteristic differs from that of other fungal enzymes, such as that isolated from *A. carbonarius* (Kiss and Kiss 2000). The initial velocity of the reaction was measured as a function of substrate concentration and plotted as a double reciprocal, in accordance with the Lineweaver–Burk analysis (data not shown). Plotting the measurements revealed a K_m value of 0.075 mg/ml and a V_{max} value of 71.42 U/mg of protein. These values were lower than those recorded for β-xylosidase from *F. proliferatum* (Saha 2003). Lower K_m values denote a higher affinity of the enzyme with the substrate (Hamilton et al. 1998).

Calculation of the turnover number (K_{cat},V_{max}/E_t) and specificity constant (K_{cat}/K_m) obtained values of 7.14/S and 95.2 mg/ml/s, respectively. Either a large value of K_{cat} (rapid turnover) or a small value of K_m (high affinity for substrate) results in a large K_{cat}/K_m.

Most of the chemical additives tested had a significant effect on the purified enzyme (Fig. 5). The effect of metal ions on enzyme activity may be due to the change in electrostatic bonding which would change the tertiary structure of enzyme. However, exposure to $CuSO_4$, EDTA, or $FeSO_4$ led to a 35–55 % reduction in enzyme activity, suggesting that disulfide bonds are essential to maintenance of the active conformation of the enzyme (Palmer 1991). The studied enzyme, therefore, exhibits a number of highly appealing and promising features that recommend it as a strong candidate for future industrial applications.

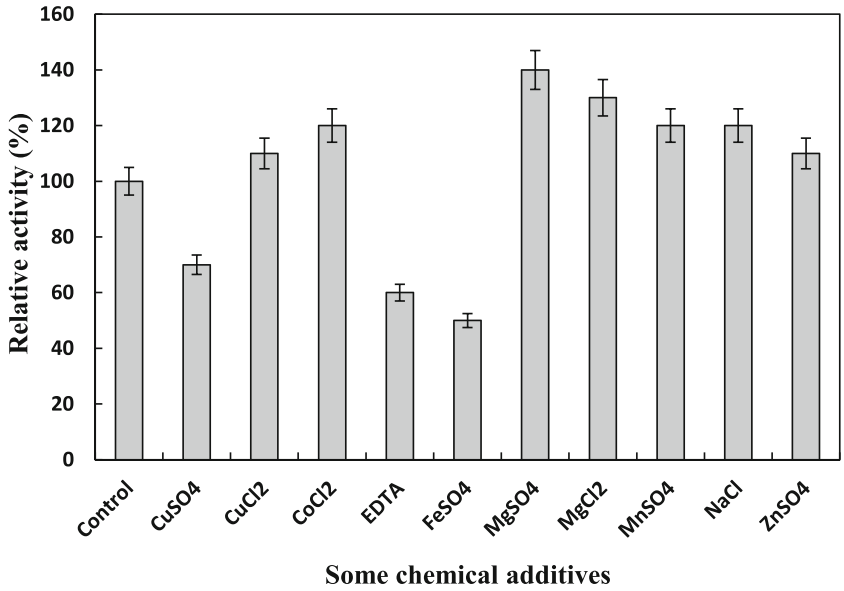

Fig 5 The effect of different chemical additives on the purified β-xylosidase from *A. tamarii*

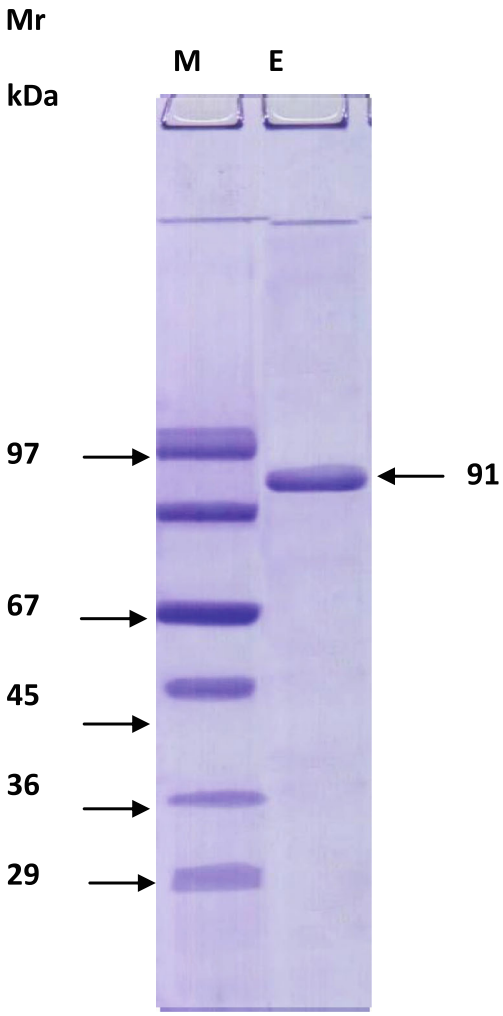

Fig. 6 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified β-xylosidase from *A. tamarii* eluted from a DEAE-cellulose column (91 kDa). *M* Marker, *E* enyzme

The molecular weight of the enzyme was calculated after gel electrophoresis in relation to a number of protein markers to be 91 kDa (Fig. 6). This was in complete agreement with that from *F. proliferatum* (Saha 2003).

With respect to the production of ethanol and xylitol from the β-xylosidase broth of *A. tamarii* using *Pichia stipitis*, our results (Table 4) show that during the consumption of xylose with increasing fermentation time, the maximum production of ethanol and xylitol was obtained after 72 h of fermentation, reaching 11.06 and 21.51 %, respectively, followed by a decrease in ethanol and xylitol production with increasing fermentation time. Kurian et al. (2010) showed that when the maximum production of ethanol after 72 h fermentation was reached, the growth of yeast in the medium was prevented. Oliver and Colicchio (2012) stated that very few yeasts can tolerate ethanol concentrations of >15 % by volume in a fermenting media and that the ability of a yeast stain to continue the fermentation process in the presence of high ethanol concentrations is thus strain-dependant. These authors suggested that high ethanol concentrations affect the porosity of the yeast plasma membrane and stated that most yeast strains cannot tolerate more than 8 % ethanol in the medium. In the present study, the yeast reached its stationary growth

Table 4 Xylose consumption and xylitol productivity from the fermentation of β-xylosidase using *Pichia stipitis*

Fermentation time (h)	Xylose[a] (g/l)	Ethanol (g/l)	Xylitol (g/l)
48	2.3	7.2	16.29
72	0.94	11.06	21.51
96	0.52	8.85	19.31

[a] At the beginning of the fermentation process, the total sugar in the hydrolysate was 51.21 g/l. After the fermentation process, it was 5.03 g/l

phase and maximum ethanol production within 48 and 72 h of inoculation. Different results have been obtained in different studies according to the yeast species utilized and the prevailing fermentation conditions (Ghindea et al. 2010). Xylitol is industrially produced by the chemical reduction of xylose derived mainly from photosynthetic biomass hydrolysates. The biotechnological production of xylitol was extensively studied as an alternative to the industrial one in order to clarify the metabolic pathways involved in microbial growth in the presence of non-conventional compounds (Ghindea et al. 2010; Usvalampi 2013).

Conclusion

We report the maximum production of β- xylosidase from *A. tamarii* under shaking conditions and solid state fermentation using oat grains. Our results indicate that the purified β-xylosidase of *A. tamarii* exhibits a number of highly appealing and promising features that can make it a strong candidate for future industrial applications. The crude SSF ground oat hydrolysate of *A. tamarii* was subjected to anaerobic fermentation by *P. stipitis*, and considerable amounts of xylitol and bioethanol were obtained. To the best of our knowledge, this is the first trial to produce xylitol and ethanol from oats.

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