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Purification and characterization of an acid-stable and organic solvent-tolerant xylanase from *Aspergillus awamori* VTCC-F312

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ABSTRACT: An acid-stable and organic solvent-tolerant extracellular xylanase was isolated and purified from *Aspergillus awamori* VTCC-F312 and its properties were investigated. The xylanase was purified by Sephadex G-100 gel filtration chromatography and DEAE-Sephadex A-50 ion exchange chromatography to homogeneity. The enzyme had a molecular mass of 32 kDa and a specific activity of 217 U/mg protein, with optimum temperature of 50–55 °C and optimum pH of 5. This enzyme was stable at up to 45 °C and no loss of enzyme activity was observed after incubation for 6 h at 40 °C. The xylanase was stable within the pH range of 4–8 with no loss of enzyme activity after incubation at 30 °C for 1–4 h, even the enzyme activity was found to increase by 60% after incubation at pH 4 for 4 h. The K_{m} and $V_{\max}$ values were 12.6 mg oat spelt xylan per ml and 1000 U/mg protein, respectively. Dithiothreitol, β-mercaptoethanol, and EDTA were found to increase the xylanase activity by 19%, 46%, and 138%, respectively. Except for Fe^{3+}, the presence of 5 mM tested metal ions increased the enzyme activity by 18–52%. The enzyme showed high resistance to organic solvents and retained 63–86% and 44–61% of its activity in the presence of tested organic solvents at 30% and 80% (v/v), respectively. Triton X-100 and Tween 80 activated the xylanase to 128% and 116% of its activity, whereas SDS at the concentration of 5% completely inhibited the enzyme. These results suggested that the xylanase from *A. awamori* VTCC-F312 could potentially be used as a feed additive for monogastric animals.

KEYWORDS: detergent-resistant, no disulphide bond, non-metal enzyme, free of cellulase and mannanase activity, corn cob, natural substrate

INTRODUCTION

Xylan is a wide variety of highly complex noncellulosic polysaccharides composed of β-1,4-linked xylose chains with branches containing arabinose and 4-O-methylglucuronic acid. Xylans account for 20–35% of the total dry weight of hard wood and annual plants. The complete hydrolysis of xylan requires the synergistic action of several enzymes of different functions. The enzymes involved in the degradation of main polysaccharide chain are endo-1,4-β-D-xylanases (EC 3.2.1.8) which randomly cleave the xylan backbone, and β-D-xylosidases (EC3.2.1.37) which cleave xylose monomers from the non-reducing end of xylooligosaccharides. Further enzymes including α-L-arabinofuranosidases (EC 3.2.1.55), α-D-glucuronidases (EC 3.2.1.139), acetylxylan esterases (EC 3.1.1.72), ferulic acid esterases (EC 3.1.1.73), and *p*-coumaric acid esterases (EC 3.1.1.-) catalyse the removal of side groups of xylans [1].

Endoxylanases are used for pulp industry to support the bleaching process, reducing the toxic chemicals (chlorine) used to bleach lignin contained in the paper [2]. Other important applications of xylanases are to make the bread fine and soft and extend the storage time [3]; to purify fruit juice, wine and beer [4]; and to form xylitol glucose used in confectionery industry. In the breeding, xylanase expedites the digestion process of food containing xylan and at the same time helps to reduce the viscosity in the digestive system followed by many positive effects such as improved food absorption, improved microorganism populations of the intestine in the advantageous direction, reduced digestion disorder, and drier excrement [5,6].

Among filamentous fungi, *Trichoderma* and *Aspergillus* have been widely studied for the xylanase production, and the genus *Aspergillus* is the most efficient producers of xylanolytic enzymes [7–11]. Thus for different applications, purification and biochemical characterization of xylanases from various *Aspergillus* strains have been carried out, including xylanases from *A. awamori* [12], *A. ficuum* [10], *A. giganteus* [8], *A. nidulans* [7], *A. niger* [3,9,13–15], and *A. sydowii* [11].

In the previous study, cultural conditions for xy-

lanase production by an *A. awamori* strain VTCC-F312 were optimized in shake flask cultures[16] and we developed a nutrient medium containing 6% (w/v) fish powder and 7% (w/v) of corn cobs for optimal xylanase production. The present study described purification and characterization of the xylanase from *A. awamori* VTCC-F312 for application in feed processing industry.

MATERIALS AND METHODS

Chemicals and reagents

Birch wood xylan and oat spelt xylan were purchased from Biochemika (Sigma Aldrich Co.); 3,5-dinitrosalicylic acid (DNS) from Fluka (Sigma Aldrich Co.). Carboxymethyl cellulose (CMC) was from BDH Prolabo Chemicals (a brand from VWR International S.A.S.). Sephadex G-100 and DEAE-Sephadex A-50 for gel filtration and ion exchange chromatography were supplied by Pharmacia Co. (GE Healthcare). Locust bean gum (LBG), DTT, β-mercaptoethanol, DMSO, and SDS were from Sigma Aldrich Co. Tween 20 and Tween 80 were from BioBasic Inc., and Triton X-100 and EDTA were from Merck. All other chemicals were of analytical grade unless otherwise stated.

Strain and culture conditions

The filamentous fungus *Aspergillus awamori* VTCC-F312 purchased from Vietnam Type Culture Collection (http://imbt.vnu.edu.vn/vtcc/), Institute of Micro-Biotechnology (Vietnam National University, Hanoi), was grown in 250-ml Erlenmeyer flasks containing 50 ml of nutrient medium with the following components (g/l): $NaNO_3$, 2; K_2HPO_4, 1; $MgSO_4$, 0.5; KCl, 0.5; fish powder, 60; corn cobs, 70; pH 6 which were optimized conditions for xylanase production by *A. awamori* VTCC-F312 in the previous study[16]. The inoculated flasks were incubated for 4 days at 37 °C on a rotary shaker at 200 rpm. The culture broth was used immediately for purification.

Xylanase purification

The culture was centrifuged for 10 min at 8000g. Ten ml of the crude enzyme extract (975 units) were applied to a Sephadex G-100 column (2.6 cm × 6 cm) pre-equilibrated with 50 mM potassium phosphate buffer pH 7.5 at a flow rate of 25 ml/h. The column was then washed with the same buffer. The eluate was collected in 0.5 ml per fraction. A highly active xylanase pool of 3 ml through Sephadex G-100 column was further applied to a DEAE-Sephadex A-50 column (2.6 cm × 6 cm) pre-equilibrated with 50 mM Tris-HCl buffer pH 8 containing 50 mM NaCl (buffer A), then washed with the same buffer. The protein was eluted with 50 mM Tris-HCl buffer pH 8 containing 1 M NaCl (buffer B) at a flow rate of 20 ml/h until $OD_{280nm} < 0.01$. The eluate was collected in 1 ml per fraction. The fractions showing high xylanase activity were pooled and used as purified enzyme for characterization. All purification steps were carried out at 4 °C, unless otherwise specified.

Xylanase activity estimation

Xylanase activity was determined by measuring the increase in concentration of reducing sugars formed by enzymatic hydrolysis of birch wood xylan. A reaction mixture of 100 µl of the crude or purified xylanase (containing 2 µg of total protein for purified enzyme) was incubated with 400 µl of 0.5% (w/v) birch wood xylan in 20 mM potassium phosphate buffer pH 6.5 at 50 °C for 5 min with an agitation at 150 rpm on a thermostat (Esco Micro Pte Ltd, Singapore). To arrest the reducing sugar released in the reaction mixture, 1.25 ml DNS was added. The reducing sugars were determined by measuring the absorbance at 540 nm[17] using D-xylose as a standard. One unit (U) of the xylanase activity was defined as the amount of enzyme that released 1 µmol of xylose per min under the standard assay conditions. All measurements were carried out in triplicate with the resulting values being the mean of the cumulative data obtained.

SDS-PAGE and protein concentration

The homogeneity and molecular mass of the xylanase was determined by 12.5% SDS polyacrylamide gel electrophoresis[18] with Biometra equipment. Proteins were visualized by staining with Coomassie Brilliant Blue R-250. Protein concentrations were measured by the Bradford method using bovine serum albumin as a standard[19].

Kinetic parameters

The Michaelis-Menten kinetic parameters (K_m and V_{max}) were determined against 1–10 mg/ml of oat spelt xylan as substrate using Lineweaver-Burk plots.

Optimum temperature and pH determination

The pH and temperature optimum of the xylanase were determined by measuring the activity as described above using 20 mM potassium acetate buffer (pH 3–5) and potassium phosphate buffer (pH 6–8) at 50 °C, and in the temperature range of 40–80 °C using 20 mM potassium phosphate buffer pH 6.5, respectively.

Temperature and pH stability

To determine temperature and pH stability, the purified enzyme, 2 µg for each reaction, was preincubated at different temperatures from 37 to 60 °C, pH 6.5 for 1–6 h; and pH range (20 mM potassium acetate pH 3–5 and potassium phosphate pH 6–8) at 30 °C for 1–4 h, respectively. The residual activity was then determined.

Effect of metal ions, detergents, and organic solvents

The purified xylanase, 2 µg for each reaction, was preincubated in the presence of 5–10 mM of various metal ions (Ca^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Mg^{2+}, Ni^{2+}, Zn^{2+}, Ag^{+}, and K^{+}) and EDTA, in the presence of 2% (w/v) of different detergents (SDS, Tween 20, and Triton X-100), and in the presence of 30–80% (v/v) of various solvents (methanol, ethanol, isopropanol, n-butanol, and acetone) at 30 °C for 1 h. The residual activity was then determined.

Effect of inhibitors

The enzyme activity of the purified xylanase, 2 µg for each reaction, was determined in the presence of 10 mM of DTT (dithiothreitol), 5 mM of EDTA, and 5% (w/v) of DMSO (dimethyl sulphoxide), NaN_3 (sodium azide), SDS, β-mercaptoethanol, and Tween 80.

Substrate specificity

To determine the substrate specificity of the xylanase from *A. awamori* VTCC-F312, activities towards 2% (w/v) of natural substrates including coconut fibre, coffee shells, corn cob, peanut shells, rice bran, sawdust, and sugarcane bagasse, or 0.5% (w/v) of polysaccharides including carboxyl methyl cellulose (CMC), locust bean gum (LBG), and oat spelt xylan were determined using DNS method as above mentioned.

RESULTS AND DISCUSSION

Purification of *A. awamori* VTCC-F312 xylanase

The production of xylanase by *A. awamori* VTCC-F312 in the nutrient medium was 97.5 U/ml (specific activity of 40.6 U/mg protein) after growth for 96 h. The culture supernatant was applied to Sephadex G-100 gel filtration chromatography and DEAE-Sephadex A-50 ion exchange chromatography. Through Sephadex G-100, the xylanase gained a specific activity of 157.5 U/mg protein with a purification factor of 3.9 and a yield of 67% (Table 1, Fig. 1), however the eluate through Sephadex G-100 showed several protein bands on SDS-PAGE (Fig. 1). The pooled Sephadex G-100 fractions showing high xylanase activity (fraction 6–11) were applied further to DEAE-Sephadex A-50 ion exchange chromatography (Fig. 1a). The xylanase was purified with a factor of 5.3 and a yield of 22%. The purified enzyme gained a specific activity of 217 U/mg (Table 1) and showed a single protein band on SDS-PAGE (Fig. 1c, lanes 5–7). The molecular mass of this protein was estimated to be 32 kDa.

Table 1 Purification steps of the xylanase from crude enzyme by *A. awamori* VTCC-F-312.

Steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification factor	Yield (%)
Crude enzyme	24.06	975.4	40.55	1	100
Sephadex G-100	4.01	630.4	157.46	3.88	67
DEAE-sephadex	1.01	217.4	216.51	5.33	22

Xylanases from other *Aspergillus* strains were purified to homogeneity by using a similar purification scheme involving ammonium sulphate precipitation, gel filtration chromatography (Sephadex G-200, G-100, G-75), ion exchange chromatography (DEAE-Sephadex A-50, DEAE Sepharose, Q-Sepharose), and affinity chromatography Phenyl Sepharose 6 Fast Flows, Sephacryl S-200 [8–12, 15].

Xylanases have been purified to homogeneity with a molecular mass of 32–35 kDa from *A. awamori* 2B.361 U2/1 [12], *A. niger* DB 106 [13], *A. nidulans* [7], *A. ficuum* AF-98 [10], *A. niger* B03 [14]. The xylanase in this study (32 kDa) belongs to the above mentioned group. Other purified xylanases that showed lower molecular mass of 21–25 kDa were xylanases from *A. sydowii* SBS 45 (xylanase I) [11], *A. giganteus* [8], *A.* cf. *niger* BCC14405 [9], *Aspergillus* sp. FP-470 [20], and *A. niger* C3486 [15] or higher molecular mass from *A. sydowii* SBS 45 (xylanase II, 43 kDa) [11].

The purification factor for xylanases from *Aspergillus* strains was relatively low as 1.3–4 [8, 14] and also in this study, middle high: 30–33 [10, 15, 20] or very high: 77–93 [11]. The purification yield of the xylanase from *A. awamori* VTCC-F312 was the highest (22%) among purification yield reported; low as 3.4–4.5% (xylanase II [8], xylanase I [11, 14]), middle high as 10.5–14.8% (xylanase II [11], xylanase I [8, 15]), and high as 20.7% [9]. The specific activity of the xylanase in this study (217 U/mg) was higher than that (124 U/mg) of the *A. niger* C3486 xylanase [15], relatively high as that (289 U/mg) of the xylanase from *A. ficuum* AF-98 [10] but lower than the xylanases from *A. awamori* 2B.361

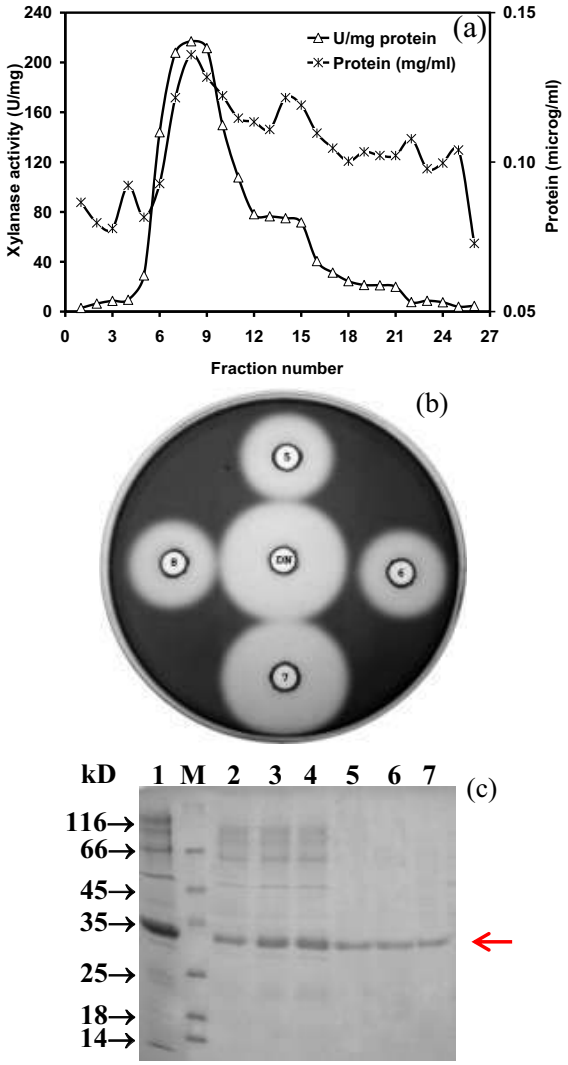

Fig. 1 (a) DEAE-Sephadex A-50 ion exchange chromatography of the xylanase from *A. awamori* VTCC-F312; *triangles*: xylanase activity U/mg protein, *stars*: protein µg/ml. (b) Xylanase activity of fractions 5–8 from DEAE-Sephadex A-50 chromatography from *A. awamori* VTCC-F312; DN: the crude enzyme. (c) SDS-PAGE of the purified xylanase from *A. awamori* VTCC-F312 through Sephadex G-100 and DEAE-Sephadex A-50; lane 1: crude enzyme; lane M: molecular mass markers; lanes 2–4: fractions from G100; lanes 5–7: fractions 5–7 from DEAE-Sephadex A-50 chromatography.

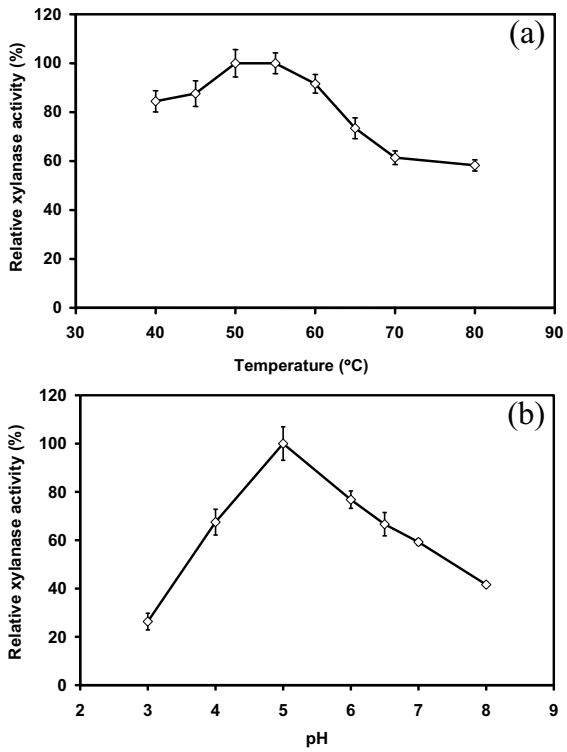

Fig. 2 (a) Temperature and (b) pH optimum of the purified xylanase from *A. awamori* VTCC-F312. Relative xylanase activity was expressed as a percentage of the optimal activity at (a) 60 °C and pH 6.5 (100% xylanase activity was 346 U/mg) and at (b) pH 5 and 50 °C (100% xylanase activity was 566 U/mg).

U2/1 (490 U/mg)[12] and *A.* cf. *niger* BCC14405 (5870 U/mg)[9].

Temperature and pH optimum

The xylanase from *A. awamori* VTCC-F312 was active in a large temperature range from 40–80 °C. The xylanase activity increased gradually from 292.5 U/mg (85%) at 40 °C to the maximum of 346.4 U/mg at 50–55 °C (Fig. 2a) and then decreased gradually to 201.7 U/mg (58%) at 80 °C. This xylanase worked also in a large pH range from 3 to 8. The xylanase activity increased gradually from 149 U/mg (26%) at pH 3 to the maximum of 556.2 U/mg at pH 5 (Fig. 2b) and then decreased gradually to 235.6 U/mg (42%) at pH 8. pH and temperature optimum for xylanases from other *Aspergillus* sp. strains were very similar: pH 5–6.5 and 45–60 °C: *A. nidulans*[7], *A.* cf. *niger* BCC14405[9], *A. niger* DSM 1957[21], *Aspergillus* sp. FP-470[20], *A. awamori*[12], *A. niger* B03[14]; *A. ficuum* AF-98[10], *A. giganteus*[8], and *A. niger* C3486[15], but two xylanases from *A. sydowii* SBS 45[11] showed an extreme pH optimum of 10.

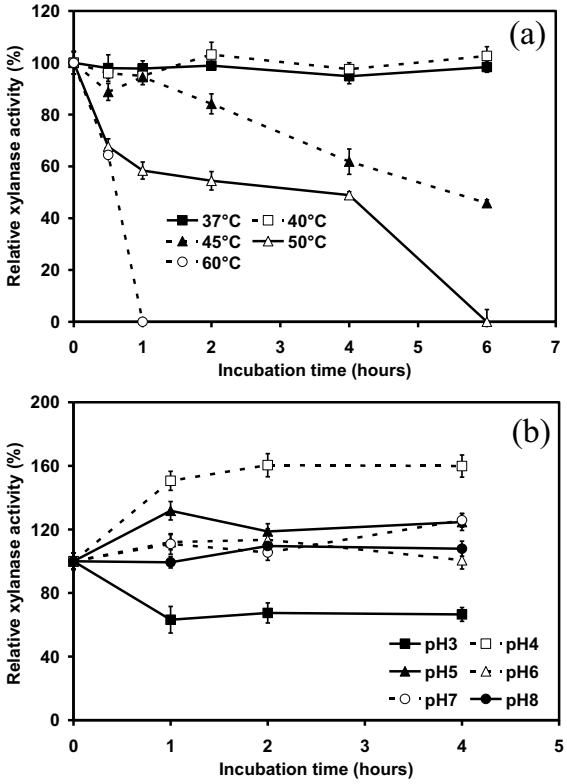

Fig. 3 (a) Temperature and (b) pH stability of the purified xylanase from *A. awamori* VTCC-F312. Relative xylanase activity was expressed as a percentage of the control reaction at time zero (100% xylanase activity was 325 U/mg).

Temperature and pH stability

The xylanase from *A. awamori* VTCC-F312 was stable at temperature up to 50 °C. No loss of the enzyme activity was observed at up to 40 °C after incubation for 6 h and the enzyme maintained one half of the original activity at up to 50 °C after incubation for 4 h (Fig. 3a). The enzyme was also very stable at pH range of 4–8 after incubation for 4 h without loss of the enzyme activity (Fig. 3b). It was surprising that the enzyme activity even increased by up to 60% at pH 4 after incubation for 4 h.

This result agrees with other reports that xylanases from most *Aspergillus* strains were stable at a temperature range of 40–60 °C and pH range of 4–8. The xylanase from *A. niger* DSM 1957 was also stable up to 50 °C and in pH range of 4–8[21]. The xylanase from *A. awamori* 2B.361 U2/1 was very stable at 50 °C and pH 5 and maintained 85% of its original activity after 25 days of treatment[12]. The xylanase from *A. niger* C3486[15] showed a pH stability of 4–5 and thermostability of 55–60 °C, and retained more than 80% and 85% of the activity after incubation for 90 min and 30 min, respectively. The xylanase from *A. niger* B03 was stable at pH range of 5–7 and temperature range of 40–50 °C[14] and retained over 80% of the original activity after 90 min of treatment. The xylanase from *A. giganteus*[8] was stable at 50 °C with a half-life of 22.5 min. The xylanase from *A.* cf. *niger* BCC14405 showed a broad pH stability from 5–10[9] for 4 h at room temperature without loss of the activity at pH 8–10 and residual activity of over 80% at pH 5–8. The xylanase from *Aspergillus* sp. FP-470 showed a high stability at pH 5–10 and retained over 80% of its original activity. Half-lives of the enzyme was 150 min and 6.5 min at 50 °C and 60 °C, respectively[20].

The xylanase from *A. awamori* VTCC-F312 showed a pH stability at pH 4–8 after incubation for 4 h without loss of the enzyme activity, interestingly it showed an increase in enzyme activity after incubation for 1–4 h by up to 60%, which never reported by other studies for *Aspergillus* xylanases. This enzyme was acid-stable so the incubation at pH 4 for 1–4 h could change the catalytic behaviour of the enzyme towards enhancing xylan hydrolysis. A good explanation for this finding requires further experiments.

Effect of metal ions on xylanase activity

The addition of metal ions at the concentration of 5 mM, except for Fe^{3+}, increased the xylanase activity by 18–52%, especially EDTA activated the enzyme with an increase of 138% in xylanase activity (Table 2). The addition of metal ions and EDTA at the high concentration of 10 mM showed a little change in the xylanase activity (±13%) except for Fe^{3+}, Fe^{2+}, and Ag^{+} which reduced the enzyme activity to a half of the original activity (Table 2).

The addition of metal ions (Fe^{3+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, Ca^{2+}, Ni^{+}, K^{+}, and Zn^{2+}) and EDTA at the concentration of 5 mM enhanced the activity of *A. niger* DSM 1957 xylanase by 27–50%, but decreased by 15–47% at the concentration of 2 mM[21]. The xylanase from *A. ficuum* AF-98 was activated by Cu^{2+} up to 116% of activity, but strongly inhibited by Hg^{2+} and Pb^{2+} up to 53% and 89%, respectively[10]. Nair et al[11] reported that metal ions like Al^{3+}, Ba^{2+}, Ca^{2+}, Na^{+}, and Zn^{2+} enhanced the activity of xylanases from *A. sydowii* SBS 45 at the concentration of 10 mM. Fialho and Carmona[8] reported that the addition of 2 and 10 mM of Mg^{2+}, Ca^{2+}, Zn^{2+}, and Ba^{2+} did not show any effect or slight inhibition on the activity of the xylanases from *A. giganteus* whereas ions Hg^{2+} and Cu^{2+} strongly inhibited the xylanases with an activity lost of 50–100%. Most of the metal ions

Table 2 Effect of metal ions on xylanase activity from *A. awamori* VTCC-F312.

Additive	Residual xylanase activity (%)[a]	
	5 mM	10 mM
Ca^{2+}	139.8 ± 9.5	92.4 ± 1.5
Cu^{2+}	149.3 ± 7.5	98.8 ± 1.3
Fe^{2+}	122.2 ± 4.3	54.4 ± 4.1
Fe^{3+}	48.3 ± 0.0	55.4 ± 4.2
Mg^{2+}	117.6 ± 3.5	112.6 ± 4.0
Ni^{2+}	152.4 ± 4.0	104.7 ± 4.1
Zn^{2+}	133.2 ± 1.2	107.0 ± 1.3
Ag^{+}	144.6 ± 3.0	42.5 ± 4.0
K^{+}	133.1 ± 4.1	104.5 ± 2.2
EDTA	237.8 ± 12.1	103.2 ± 1.0

[a] Residual xylanase activity was expressed as a percentage of the control reaction without any additive (100% xylanase activity was 325 U/mg)

tested (Co^{2+}, Zn^{2+}, Ca^{2+}, Na^{+}, Mg^{2+}, and Mn^{2+} at the concentration of 10 mM) showed a slight increase in *A.* cf. *niger* BCC14405 xylanase activity up to 30%[9]. However, the addition of 10 mM of Cu^{2+}, EDTA, or Fe^{2+} led to a 40% reduction in enzyme activity. *A. niger* B03 xylanase was inhibited completely by Cu^{2+} and partially by Pb^{2+}, Fe^{2+}, Fe^{3+}, and Ag^{+}, but activated by Mn^{2+} up to 64%[14].

From these studies, it has been found that the addition of Ca^{2+} and Zn^{2+} at the concentration of 5–10 mM enhanced the activity of the xylanase from *A. awamori* VTCC-F312 (this study), *A. niger* DSM 1957[21], *A. sydowii* SBS 45[11], and *A.* cf. *niger* BCC14405[9]. However, Cu^{2+} showed a confused effect, an increase in the activity of the xylanase from *A. awamori* VTCC-F312 (this study), *A. niger* DSM 1957[21], and *A. ficuum* AF-98[10], but a decrease in the activity of the xylanase from *A. giganteus*[8], *A.* cf. *niger* BCC14405[9], and *A. niger* B03[14].

Effect of detergents on xylanase activity

The effect of ionic (SDS) and nonionic detergents (Tween 20, Tween 80, and Triton X-100) currently used for denaturing of glycoproteins was tested on the xylanase activity. The addition of 2% (w/v) Triton X-100 and of 5% (w/v) of Tween 80 increased the enzyme activity up to 28% (Fig. 4a) and 16% (Table 3) whereas the addition of 2% (w/v) SDS and Tween 20 decreased the enzyme activity by 22% and 38%, respectively (Fig. 4a). However, SDS at the higher concentration of 5% (w/v) completely inhibited the xylanase (Table 3).

The results agreed with the previous study[21]. The xylanase from *A. niger* DSM 1957 was resistant also to tested detergents including SDS, Triton X-100, and Tween 20 at the concentration of 0.2–2% (w/v) and the enzyme activity was enhanced by the addition of SDS up to 85% and Triton X-100 and Tween 20 up to 27%

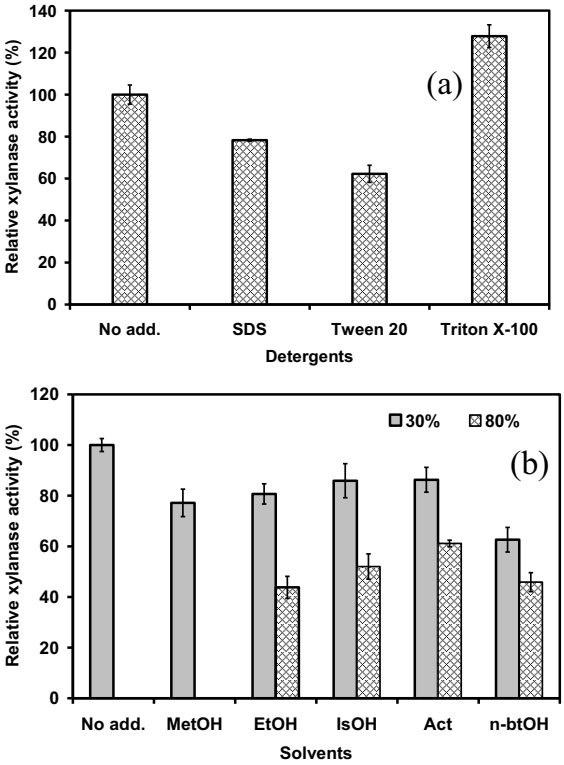

Fig. 4 Effect of (a) organic solvent and (b) detergent on xylanase activity from *A. awamori* VTCC-F312. Relative xylanase activity was expressed as a percentage of the control reaction without any additive (100% xylanase activity was 325 U/mg).

Table 3 Effect of chemical agents on the xylanase activity from *A. awamori* VTCC-F312.

Chemical reagent	Concentration	Residual xylanase activity (%)[a]
Control	–	100.0 ± 0.9
DTT	10 mM	119.0 ± 1.5
EDTA	5 mM	104.4 ± 0.7
DMSO	5%	93.5 ± 2.8
NaN_3	5%	88.0 ± 1.6
SDS	5%	0
β-Mercaptoethanol	5%	146.3 ± 0.5
Tween 80	5%	116.3 ± 1.6

[a] Residual xylanase activity was expressed as a percentage of the control reaction without any additive (100% xylanase activity was 258 U/mg).

after incubation for 2 h[21]. The addition of 1% (v/v) of Tween 80 and Triton X-100 to the xylanases from *Termitomyces* sp.[22] and from the termite *Macrotermes subhyalinus* worker[23] did not change significantly in the xylanase activity, only a slight decrease by 3–4% and 11% in comparison to the control reaction without the additives, respectively. However, SDS strongly inhibited the xylanase from *M. subhyalinus* and *Termitomyces* sp. at 0.1% (w/v) and 1% (w/v) with a residual activity of 11% and 37% of the control, respectively. Fialho and Carmona[8] reported that the xylanase from *A. giganteus* was completely inhibited by SDS[8].

Effects of inhibitors

The disulphide bond reducing agents DTT at the concentration of 10 mM and β-mercaptoethanol at the concentration of 5% showed an increase in the activity of the xylanase from *A. awamori* VTCC-F312 by 19–46% in comparison to the original activity (Table 3). Thus the enzyme did not contain any critical disulphide bond. The chelating agent EDTA enveloping metal ions extensively did not change the xylanase activity (Table 3) that means the enzyme did not require metal ions for its catalysis. Other chemicals including DMSO and NaN_3 showed a slight decrease by 6–12% (Table 3).

The disulphide bond reducing agents DTT and β-mercaptoethanol showed also an activation of other xylanases from *A. giganteus*[8], *A. sydowii* SBS 45[11], *A.* cf. *niger* BCC14405[9], and *A. niger* IBT-90[3]. But EDTA showed a confused effect, no change in the activity of the xylanase from *A. niger* IBT-90[3] and the xylanase II from *A. giganteus*[8], but a decrease in the activity of the xylanase from *A. sydowii* SBS 45[11] and the xylanase I from *A. giganteus*[8].

Effect of organic solvents on xylanase activity

Since organic solvents have been used for solubilizing hydrophobic substrates in enzymatic reactions, their effects on enzyme activity were tested in this study. The addition of the organic solvents at the final concentration of 30% showed an obvious inhibition on the activity of the xylanase from *A. awamori* VTCC-F312 with the residual activity of 63–86%. At the higher concentration of 80%, the enzyme was resistant to the organic solvents and retained 44–61% of the original activity but was completely inhibited by methanol (Fig. 4b).

These results agreed with the previous studies[21–23]. The xylanase from *A. niger* DSM 1957 is resistant to methanol, ethanol, isopropanol, and acetone. No loss of the enzyme activity was observed by the addition of 30% (v/v) of these additives after incubation for 1–2 h, especially acetone enhanced the xylanase activity by 1.5–2 times[21]. At the concentration of up to 5% of all the tested organic solvents (methanol, ethanol, butanol, acetone, acetonitrile, and dioxane), the xylanase from *Termitomyces* sp. retained more than 80% of its initial activity[22]. Activation of the xylanase from *M. subhyalinus* and from *Termitomyces* sp. was observed with the addition of acetone and dioxane at the concentration[23] of up to 15% and with the addition of acetone at the concentration[22] of up to 30%, respectively. However, in contrast to our study, alcohols (methanol, ethanol, propanol, and butanol) completely inhibited the enzyme from *Termitomyces* sp. and *M. subhyalinus* at 30% (v/v) and 60% (v/v), respectively. Primary alcohols including methanol, ethanol, and isopropanol as well as polyhydric alcohol containing glycol and glycerol, all showed inhibitory effects on *A. niger* C3486 xylanase activity which retained around 90% at the concentration of 2% (v/v) and less than 60% of its initial activity at 30%[15].

Kinetic parameters

The K_m and V_{max} values obtained for the purified xylanase from *A. awamori* VTCC-F312 were 12.6 mg oat spelt xylan/ml and 1000 U/mg protein, respectively. The K_m value (12.6 mg oat spelt xylan/ml) was higher than that obtained for the xylanases from *A. sydowii* SBS 45 (6.45 and 7.69 mg/ml)[11] and from *A. niger* B03 (8.19 mg/ml)[14], but lower than that (36.3 and 13.2 mg/ml) obtained for the xylanases from *A. fumigatus*[8]. The V_{max} value (1000 U/mg protein) obtained for the xylanase from *A. awamori* VTCC-F312 was lower than that obtained for the xylanases from *A. sydowii* SBS 45 (2604 and 2381 U/mg protein) but higher than that obtained from *A. niger* B03 (9.5 mg min^{-1} ml^{-1} = 0.38 U/mg protein) when oat spelt xylan was used as substrate.

When birch wood xylan was used as substrate, the K_m value obtained for the xylanases from *A.* cf. *niger* BCC14405, *A. sydowii* SBS 45, *A. ficuum* AF-98, and *A. niger* B03 was 8.9 mg/ml[9], 3.18 and 6.51 mg/ml[11], 3.747 mg/ml[10], and 3.01 mg/ml[14], respectively. The V_{max} value obtained for the xylanases from *A. ficuum* AF-98, *A.* cf. *niger* BCC14405, *A. sydowii* SBS 45, and *A. niger* B03 was 11.1 M min^{-1} mg^{-1} (11.1×10^6 U/mg), 11 100 U/mg, 1191 and 1587 U/mg, and 6.64 mg min^{-1} ml^{-1} (= 0.2626 U/mg protein), respectively.

Substrate specificity

To determine the substrate specificity, the xylanase activity was determined towards oat spelt xylan, CMC,

Table 4 Substrate specificity of the purified xylanase from *A. awamori* VTCC-F-312.

Substrate	Concentration (%, w/v)	Relative xylanase activity (%)[a]
CMC	0.5	0
LBG	0.5	0
Oat spelt xylan	0.5	100 ± 0.3
Coconut fibre	2	35.2 ± 4.7
Coffee shells	2	33.2 ± 2.3
Corn cob	2	47.2 ± 1.6
Peanut shell	2	27.2 ± 3.5
Sawdust	2	0
Rice bran	2	37.2 ± 1.9
Sugarcane bagasse	2	33.3 ± 1.1

[a] Relative xylanase activity was expressed as a percentage of the control reaction with oat spelt xylan as substrate (100% xylanase activity was 522 U/mg).

and LBG with a concentration of 0.5% (w/v) and natural substrates with a concentration of 2% (w/v) (Table 4). Among the tested substrates, the xylanase from *A. awamori* VTCC-F312 displayed the highest activity towards oat spelt xylan, but no activity towards LBG and CMC. This result demonstrated that xylanase from *A. awamori* VTCC-F312 was free of cellulase activity (towards CMC) and mannanase activity (towards LBG). Among natural substrates (Table 4), the enzyme showed the highest activity towards corn cob.

The substrate specificity of this xylanase is similar to that of other xylanases from *A.* cf. *niger* BCC14405[9] and from *A. niger* B03[14] which showed only activity towards xylan and free of amylase or glucanase activity. Xylanase I and II from *A. sydowii* SBS 45[11] showed strong specificity towards birch wood and oat spelt xylan, but no activity was detected against CMC, pectin, and starch. The xylanases from *A. fumigatus* did not hydrolyse avicel or CMC, acting only on xylans[8].

Among natural substrates (Table 4), the enzyme showed the highest activity towards corn cob. This finding coincided with the previous report that the *A. awamori* VTCC-F312 strain produced the highest level of the xylanase in the medium containing 1% (w/v) of corn cob among tested carbon sources (birch wood xylan, corn cob, dried mandarin skin, outer fibrous layer of cassava tube, peanut shells, rice bran, and sugarcane bagasse)[16].

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