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Purification and characterization of low molecular weight extreme alkaline xylanase from the thermophilic fungus *Myceliophthora thermophila* BF1-7

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ABSTRACT

The thermo-alkali-stable xylanase from *Myceliophthora thermophila* BF1-7 was purified and characterized. The enzyme was purified using a procedure including ammonium sulfate precipitation, gel filtration and ion exchange chromatographies. The xylanase was purified to 77.1-fold apparent homogeneity with a recovery yield of 7.48% and maximum specificity was obtained as 2.31 U mg^{-1} protein. The purified xylanase appeared as a single protein band on sodium dodecyl sulfate polyacrylamide gel electrophoresis with a molecular mass of approximately 14 kDa. Xylanase was most active at pH 12.0 and retained 55% of the original activity in the pH range of 9.0—12.0 after incubation at 4 °C for 24 h. The optimal temperature of the xylanase was 50 °C and it retained more than 77% and 56% of its original activity after heating at 50 °C for 30 and 60 min, respectively. Xylanase was inhibited by Hg^{2+} and stimulated by Mg^{2+}, Cu^{2+}, Ag^+, Zn^{2+}, ethylenediaminetetraacetic acid (EDTA) and sodium dodecyl sulfate (SDS) at 1 mM. The K_m and V_{max} values of the purified xylanase were 9.67 mg/mL and 5.38 μmol/min/mg, respectively. The purified xylanase only showed activity on xylan and hydrolyzed beechwood xylan to yield mainly xylotetraose and xylobiose as end products which suggesting that it was an endo-xylanase.

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1. Introduction

Agricultural wastes such as rice straw, corn stover, bagasse, and sawdust contain high amount of lignocelluloses (cellulose, hemicellulose and lignin). Of the three, hemicellulose is the second most abundant polysaccharide in nature after cellulose (Badhan et al. 2007). Xylan is the major component of hemi-celluloses, which has a linear backbone of β-1,4-linked D-xylo-pyranose residues which is further substituted, depending on plant sources to a varying degree with glucuronopyranosyl, 4-O-methyl-D-glucopyranosyl, α-L-arabinofuranosyl, acetyl, as

well as linked to feruloyl and coumaryl components of lignin (Pal and Khanum 2011). Biodegradation of xylan requires the action of endo-xylanase (endo-1,4-β-xylanase, EC 3.2.1.8) and β-xylosidase (xylan-1,4-β-xylosidase, EC 3.2.1.37). Endo-xylanase cleaves the backbone to xylooligosaccharides and β-xylosidase hydrolyzes them to D-xylose (Kolenová et al. 2005). Therefore, those agricultural wastes are the most promising feedstock for xylanase production.

Xylanases can be produced by a number of fungi, bacteria, and yeasts which can use lignocelluloses as a primary carbon source (Wen et al. 2005). However, fungi are potential microorganisms for production of xylanase, because they secrete enzymes into the medium and their enzyme levels are much higher than those of yeasts and bacteria (Singh et al. 2009). Among filamentous fungi, thermophilic fungi produce extracellular enzymes with valuable properties such as thermostability, optimum activity at elevated temperatures, broad tolerance to pH variation, high rates of substrate hydrolysis, and greater resistance to denaturing agents (Kalogeris et al. 2003; Li et al. 2006; Moretti et al. 2012). Thermo-alkaliphilic xylanase shows great activity at high temperatures and at alkaline pH, giving it great potential for application in several industries, especially in the pulp and paper industry, and particularly at the dewatering and refining steps, and for bleaching processes without necessitating changes in pH or temperature (Muthezhilan et al. 2007). In addition, low molecular weight xylanases are of industrial importance because they can better diffuse into the biomass structure or fibrous pulp and can thus efficiently hydrolyze xylan in biomass hydrolysis or pulp bleaching (Walia et al. 2014). Recently, xylanase properties have been observed in many species of thermophilic fungi. These were *Humicola grisea* var. *thermoidea* (Lucena-Neto and Ferreira-Filho 2004), *Thermomyces lanuginosus* CBS 288.54 (Li et al. 2005), *Paecilomyces variotii* IMD RK 032 (Kelly et al. 1989), *Paecilomyces themophila* J18 (Li et al. 2006), *Talaromyces thermophilus* (Maalej et al. 2009), but only a few xylanases are reported to be active and stable at alkaline conditions (Bajaj and Abbass 2011; Chanwicha et al. 2015). Thus, there is a need to identify new sources of alkaline xylanase-producing fungi and record their characterizations.

Myceliophthora species are good producers of xylanase (Badhan et al. 2007). However, there are few reports on the properties of xylanase from this species. Since the xylanase previously purified from *M. thermophila* C1 exhibited maximum activity at acidic conditions (van Gool et al. 2013), until now the properties for alkali-thermostable xylanase from *M. thermophila* have not been reported. This study reports on the purification and some of the properties of low molecular weight extremely alkaline xylanase from the thermophilic fungus *M. thermophila* BF1-7.

2. Materials and methods

2.1. Materials

Agricultural wastes, namely sawdust, rice straw, bagasse, corn stover, rice husk and rice bran were used as substrates. They were collected from areas around Khon Kaen Province, Thailand, and the small pieces (1—2 cm) of each waste product were soaked in 0.5 N NaOH for 2 h. Thereafter, they were washed with tap water until pH reached 7.0, and then dried at 50 °C. The dried materials were ground into smaller particles in a hammer mill and separated by 1.0 mm sieve. Beechwood xylan was purchased from Sigma Chemical Co., St. Louis, MO, USA. Sephadex G-100 gel filtration media was obtained from GE Healthcare (Uppsala, Sweden). Diethylaminoethyl (DEAE)-cellulose was purchased from Sigma—Aldrich (St. Louis, MO, USA). Amersham™ ECL™ gel 4—20% Kit and PlusOne Silver Staining Kit were obtained from GE Healthcare. All other chemicals were of analytical grade.

2.2. Isolation and identification of thermophilic fungi

Myceliophthora thermophila BF1-7 was isolated from compost at Phoenix Pulp & Paper Public Company Limited, Khon Kaen Province, Thailand. The strain was kept on potato dextrose agar (PDA) at 50 °C and was transferred every 3—4 wk. In order to identify the fungal species, morphological characteristics were studied. A molecular analysis was also carried out. Genomic DNA of the strain was extracted directly from pure culture of mycelium with some modification of the method described by Moller et al. (1992). Internal transcribed spacer (ITS) rRNA gene was amplified using a pair of universal primers, ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATCG-3′). Amplification was performed using the GENE Q Thermal Cycler model TC24H/(b) with the following cycling parameters: 94 °C for 5 min, followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C with final extension for 5 min. Then, the PCR product was purified with QIAGEN PCR Purification Kit and sequenced at Biomolecular Analysis Service Unit, Department of Biochemistry, Faculty of Medicine, Khon Kaen University. DNA sequences were submitted to GenBank for homology analysis using BLASTN program. The sequence alignment was performed using the CLUSTALX program (Iwamoto et al. 2002). The neighbor-joining (NJ) method was used for phylogenetic analysis. For the NJ analysis, the data were firstly analyzed using the Kimura 2-parameter distance calculation model, and then used to construct the NJ tree with PHYLIP version 3.69. To determine the support for each clade, a bootstrap analysis was performed with 1000 replications.

2.3. Xylanase production in solid-state fermentation and enzyme extraction

Solid-state fermentation was carried out in Erlenmeyer flasks (250 mL) containing a 5 g mixture of rice bran and rice straw (1:2 w/w), and 16.8 mL (1:3.36 w/v moisture content) of mineral solution [g/L in distilled water: $(NH_4)_2SO_4$ 10.5 g, KH_2PO_4 3.0 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $CaCl_2$ 0.5 g] was added. The pH of the medium was adjusted to 8.87 prior to sterilization (121 °C, 15 min). Five agar mycelial plugs (0.5 cm diam) of a 5-d-old mycelial culture of the strain were inoculated in each flask, and incubated at 44.16 °C under static conditions for 7 d. After the desired incubation period, 50 mL of 0.1 M McIlvaine buffer, pH 8.0 was added to the cultures, and shaken at 180 rpm and at 50 °C for 60 min. Next, the solid materials and fungal biomass were separated by centrifugation at 4 °C (13,551 *g*, 20 min). The supernatant was used for enzyme assays.

2.4. Enzyme assays and protein determination

Xylanase activity was assayed using beechwood xylan as substrate. The reaction mixture, composed of 0.5 mL of enzyme solution and 0.5 mL of 1% beechwood xylan solution in 0.1 M McIlvaine buffer, pH 8.0, was incubated at 50 °C for 15 min, and the reducing sugar released was determined by Somogyi–Nelson method (Nelson 1944). One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of xylose from substrate per minute under the assay conditions. Protein was determined by the Lowry method (Lowry et al. 1951) using bovine serum albumin as standard.

2.5. Purification of xylanase

The xylanase from *M. thermophila* BF1-7 was purified using a standard procedure including ammonium sulfate precipitation, gel filtration and ion exchange chromatographies. The crude enzyme was subjected to 20–80% ammonium sulfate saturation. The precipitated protein collected by centrifugation (13,551 *g*, 20 min) and dissolved in 0.1 M McIlvaine buffer (pH 8.0). Further purification was carried out by gel filtration and ion-exchange chromatographies. A 1.0 mL sample from ammonium sulfate precipitation was loaded on a Sephadex G-100 column (100 cm × 1.0 cm) and equilibrated with 0.1 M McIlvaine buffer (pH 7.0), and the protein was eluted at a flow rate of 0.25 mL/min. All active fractions were combined and applied to a DEAE-Cellulose column (20 cm × 1.5 cm) which was previously equilibrated with 0.1 M McIlvaine buffer (pH 7.0) at a flow rate of 0.5 mL/min. The bound proteins were eluted with a NaCl gradient (2 M) in the same buffer at a flow rate of 0.5 mL/min. The highly active fractions were pooled and dialyzed against 0.1 M McIlvaine buffer (pH 7.0). The homogeneity of dialysate was checked by SDS (sodium dodecyl sulfate)-PAGE (polyacrylamide gel electrophoresis).

2.6. SDS-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed according to a modified method of Laemmli (1970) using Amersham™ ECL™ gel 4–20% Kit. Protein bands were visualized by silver staining using the PlusOne Silver Staining Kit as described in instructions from the manufacturer. The molecular weight standard used was a chromatein prestained protein ladder (10–175 kDa).

2.7. Biochemical characterization of purified xylanase

Investigation for the optimum pH of purified xylanase activity was carried out in four different buffers at pH 3.0–12.0. The optimum pH for xylanase activity was determined to be pH 3.0–12.0 at 50 °C. The following buffers were used: 0.1 M McIlvaine (pH 3.0–8.0), 0.05 M Tris–HCl (pH 8.0–9.0), 0.05 M Glycine–NaOH (pH 9.0–10.0) and 0.05 M Na$_2$HPO$_4$–NaOH (pH 10.0–12.0). The optimum temperature for xylanase activity was determined by assaying the enzyme activity at various temperatures (30–80 °C) in an optimum buffer.

The pH stability of purified xylanase was determined after incubating in the buffers described above at 4 °C for 24 h, then measuring residual activity by standard assay procedure. For thermal stability determination, the purified xylanase in an optimum buffer was incubated at different temperatures (30–80 °C) for 60 min. After cooling the treated enzymes on ice for 30 min, the residual xylanase activity was measured according to the standard assay method.

2.8. Effect of metal ions and reagents on xylanase activity

The purified xylanase was preincubated with 1 and 5 mM solution of different mineral salts (MgSO$_4$, MnSO$_4$, ZnSO$_4$, CuSO$_4$, CoCl$_2$, FeSO$_4$, AgNO$_3$, HgCl$_2$, ZnCl$_2$) and nonmetal reagents (ethylenediaminetetraacetic acid (EDTA), SDS) for 1 h at room temperature (25 ± 2 °C) and compared to the control without the adding of metal ions or reagents. The residual activity was then checked under the standard assay conditions.

2.9. Specificity of purified xylanase and kinetic parameters

The substrate specificity of the enzyme was determined on the following substrates: 1% beechwood xylan (w/v), 1% oat spelt xylan (w/v), 1% CMC (w/v), 1% Avicel (w/v) and 1% cellulose powder (w/v). The activities were assayed at 50 °C for 15 min and determined as relative activity of enzyme for each substrate. For the kinetic experiments, five different concentrations of xylan (2.5, 5, 10, 15 and 20 mg/mL) were prepared in an optimum buffer and incubated with the purified xylanase at 50 °C for 15 min. The kinetic parameters (Michaelis–Menten constant, K_m, and maximal reaction velocity, V_{max}) were estimated by linear regression from double-reciprocal plots according to Lineweaver and Burk (1934).

2.10. Hydrolysis products of beechwood xylan by xylanase

For enzymatic hydrolysis of xylan, reaction mixture consisted of 1% beechwood xylan in 0.05 M Na$_2$HPO$_4$–NaOH buffer pH 12.0 with purified xylanase. The reaction mixture was incubated at 50 °C for 48 h. Aliquots were collected at different intervals (3, 6, 12 and 24 h) and products were analyzed by thin-layer chromatography (TLC). Thin-layer chromatography (Silica gel 60 F254, Merck, Japan) was developed using chloroform: glacial acetic acid: H$_2$O (6:7:2, v/v/v) as the mobile phase according to a slightly modified method described previously (Li et al. 1993). Xylose and xylooligosaccharide (Wako Pure Chemical Industries Ltd., Osaka, Japan) were used as standards. The hydrolysis products were detected by spraying with methanol/sulfuric acid mixture (95:5, v/v) followed by heating at 100 °C for 15 min.

3. Results

3.1. Identification of xylanase-producing thermophilic fungi

The thermophilic fungus, isolate BF1-7 was identified using morphological characteristics and molecular approach. Morphological observation showed that colonies on PDA

were initially white and cottony, and later pale brown and powdery. The microscopic morphology showed that one to three blastoconidia were borne on one hyphal cell or ampulliform swelling. Conidia were obovoid to pyriform, 3.0–4.5 × 4.5–11.0 μm in size, hyaline, smooth and thick-walled (Bourbeau et al. 1992; Morio et al. 2011). In addition, this strain was further identified by ITS sequence of mycelial pure culture. The partial sequence was deposited in the DNA Data Bank of Japan (DDBJ; Accession number LC010279). It was demonstrated that the strain belonged to the genus of *Myceliophthora*, where it showed maximum similarity of 97% with the *Myceliophthora thermophila* WYN8-2. Based on these data, the isolate BF1-7 was identified as *Myceliophthora thermophila* BF1-7 (data not shown).

3.2. Purification and molecular weight of xylanase

The summary of the xylanase purification is presented in Table 1. The crude enzyme extract, having total activity of 143.53 U and specific activity of 0.03 U mg^{-1} protein was subjected to $(NH_4)_2SO_4$ precipitation (20%) at 4 °C. During this step, the specific activity of the enzyme preparation did not increase dramatically. The precipitates recovered were then dissolved in 0.1 M McIlvaine buffer, pH 7.0. The sample from ammonium sulfate precipitation was loaded on to a Sephadex G-100 column and eluted with 0.1 M McIlvaine buffer (pH 7.0) at a flow rate of 0.25 mL/min. The specific activity, fold-purification and the level of recovery in this step were 1.13 U mg^{-1} protein, 37.82% and 11.04%, respectively. The active fractions were finally loaded on to the DEAE-cellulose ion-exchange column and eluted with a linear gradient of 2 M NaCl after the removal of unbound proteins. Overall, the xylanase was purified to 77.1-fold apparent homogeneity with a recovery yield of 7.48%. Specific activity increased during the purification steps from 0.03 to 2.31 U mg^{-1} protein. The purified xylanase appeared as a single protein band on SDS-PAGE gel with a molecular mass of approximately 14 kDa (Fig. 1).

3.3. Effect of pH and temperature on the activity and stability of xylanase

The purified xylanase was most active at pH 12.0 and exhibited a broad optimum between pH 9.0–12.0 (Fig. 2A). It retained 55% of the original activity in the pH range of 9.0–12.0 after incubation at 4 °C for 24 h (Fig. 2B). The optimal temperature of the xylanase was 50–60 °C (Fig. 3A) and it retained more than 77% and 56% of its activity after heating at 50 °C for 30 and 60 min, respectively (Fig. 3B).
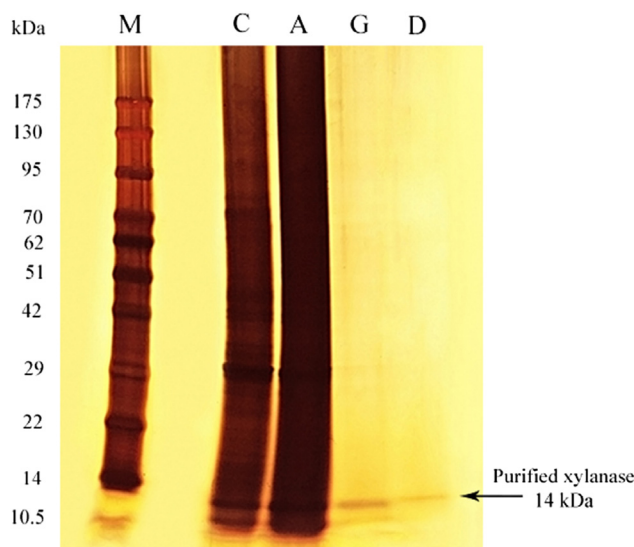

Fig. 1 – SDS-PAGE profiles with silver stain of different stages of xylanase purification. Lane M: low molecular weight calibration kit; C: crude extract; A: ammonium sulfate precipitation; G: after gel filtration; D: after DEAE-cellulose.

3.4. Effect of metal ions and nonmetal reagents on xylanase activity

The effect of different metal ions and nonmetal reagents on the activity of xylanase is shown in Table 2. The presence of Mg^{2+}, Cu^{2+}, Ag^+, Zn^{2+} ($ZnCl_2$), EDTA and SDS at the concentration of 1 mM increased xylanase activity, whilst Zn^{2+} ($ZnSO_4$) and Co^{2+} did not affect to the activity of enzyme. However, the addition of metal ions and nonmetal reagents at a concentration of 5 mM, except for Fe^{2+}, showed a slight inhibition of xylanase activity. Fe^{2+} caused a slight inhibition of xylanase activity at a concentration of 1 mM, but it strongly inhibited xylanase activity at a concentration of 5 mM, while Hg^{2+} strongly inhibited the activity of xylanase for both concentrations of 1 and 5 mM.

3.5. Specificity of purified xylanase and kinetic parameters

The hydrolytic property of the purified xylanase on various substrates was examined (Table 3). The purified xylanase was specific to beechwood xylan and oat spelt xylan. The highest activity was observed with beechwood xylan. The purified xylanase did not act towards carboxymethyl cellulose, Avicel

Table 1 – Summary of the purification of xylanase from *Myceliophthora thermophila* BF1-7.					
Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	4791.43	143.53	0.030	1.00	100.00
20% $(NH_4)_2SO_4$	1147.14	38.52	0.034	1.13	26.84
Sephadex G-100	13.98	15.84	1.133	37.77	11.04
DEAE-cellulose	4.64	10.73	2.313	77.10	7.48

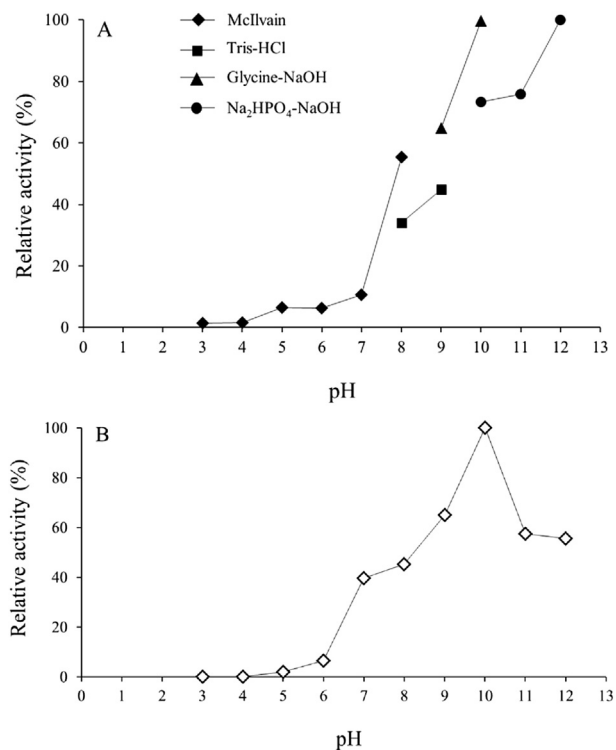**Fig. 2 – Optimal pH (A) and pH stability (B) of the purified xylanase from *Myceliophthora thermophila* BF1-7.**
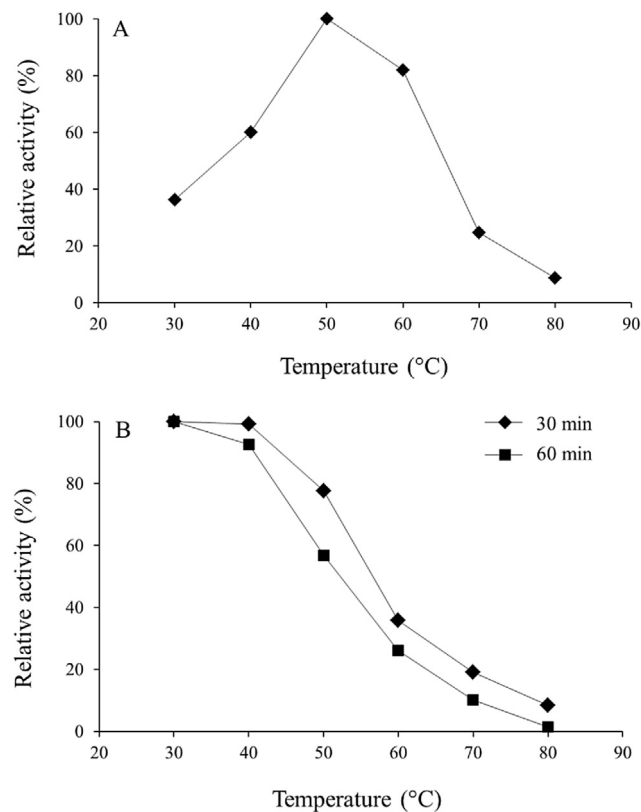
Table 2 – Effect of metal ions and nonmetal reagents on xylanase activity from *Myceliophthora thermophila* BF1-7.

Additive	Relative xylanase activity (%)[a]	
	1 mM	5 mM
Control	100.00 ± 0.00	100.00 ± 0.00
MgSO$_4$·7H$_2$O	134.23 ± 0.82	95.07 ± 0.08
MnSO$_4$·H$_2$O	99.36 ± 0.91	98.92 ± 0.07
CuSO$_4$·5H$_2$O	124.11 ± 2.37	111.18 ± 0.96
ZnSO$_4$·7H$_2$O	102.56 ± 2.45	72.41 ± 1.20
CoCl$_2$·H$_2$O	103.83 ± 2.48	69.01 ± 0.96
FeSO$_4$·H$_2$O	92.62 ± 0.72	21.64 ± 1.21
AgNO$_3$	185.95 ± 3.55	96.51 ± 0.71
HgCl$_2$	0.00 ± 0.00	0.00 ± 0.00
ZnCl$_2$	112.06 ± 1.78	87.67 ± 0.59
EDTA	131.32 ± 4.93	94.59 ± 0.76
SDS	151.72 ± 2.73	64.00 ± 0.45

[a] Relative xylanase activity was expressed as a percentage of the control reaction without any additive (100% xylanase activity was 1757 mU/mg). Mean ± one standard deviation derived from two replicates ($N = 2$).

and cellulose powder. The K_m and V_{max} values of the purified xylanase from BF1-7 were 9.67 mg/mL and 5.38 μmol/min/mg, respectively.

3.6. Hydrolysis properties of the purified xylanase

The hydrolysis pattern of beechwood xylan by purified xylanase was analyzed (Fig. 4). The enzyme liberated mainly xylotetraose and a small amount of xylobiose from beechwood xylan. Thus, the results indicate that the purified xylanase from *M. thermophila* BF1-7 was suggested to be an endoxylanase that randomly cleaved xylan as a substrate.

4. Discussion

For the application of xylanases in the pulp and paper industry, the enzyme should be active and stable at alkaline conditions and high temperatures. The above properties can be introduced at different stages of the bleaching process without requiring changes in pH or temperature

Fig. 3 – Optimal temperature (A) and thermal stability (B) of the purified xylanase from *Myceliophthora thermophila* BF1-7.

Table 3 – Substrate specificity of the purified xylanase from *Myceliophthora thermophila* BF1-7.

Substrate (1% w/v)	Relative xylanase activity (%)[a]
Beechwood xylan	100.00 ± 0.00
Oat spelt xylan	45.87 ± 3.45
Carboxymethyl cellulose (CMC)	0.00 ± 0.00
Avicel	0.00 ± 0.00
Cellulose powder	0.00 ± 0.00

[a] Relative xylanase activity was expressed as a percentage of the control reaction with beechwood xylan as substrate (100% xylanase activity was 2654 mU/mg). Mean ± one standard deviation derived from two replicates ($N = 2$).
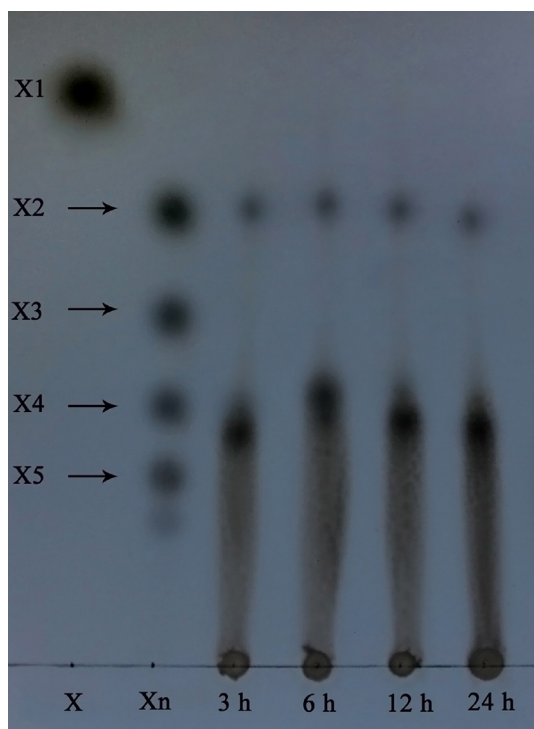
Fig. 4 — TLC analysis of hydrolysis of beechwood xylan by the purified xylanase from *Myceliophthora thermophila* BF1-7. Incubation times (h) are indicated. Xylose (X) and xylooligosaccharide (Xn) were used as standards. Lanes X1: xylose; X2: xylobiose; X3: xylotriose; X4: xylotetraose; X5: xylopentose.

(Muthezhilan et al. 2007). Recently, only a few xylanases from thermophilic fungi have been reported to be active and stable in alkaline conditions (Bajaj and Abbass 2011; Chanwicha et al. 2015). Furthermore, there are no previous studies available on the thermo-alkali-stable xylanase from a thermophilic fungus *M. thermophila*. In this research, an alkali-stable xylanase from *M. thermophila* BF1-7 was purified and characterized. The purification fold of xylanase purification in this study was higher than some of the previous published data from other xylanase-producing fungi. For instance, Pal and Khanum (2011) reported that the purified xylanase from *Aspergillus niger* DFR-5 was purified 36.97-fold using ion exchange and gel filtration chromatography. The purified xylanase from *P. themophila* J18 was purified 2.4-fold to homogeneity with a recovery yield of 38.3% (Li et al. 2006). The observations of Li et al. (2005) showed that the xylanase from *T. lanuginosus* CBS 288.54 was purified to 33.6-fold apparent homogeneity with a recovery yield of 21.5%. Elsewhere, Fang et al. (2008) reported purification of xylanase to 14.88-fold with 25.8% recovery from *A. carneus* M34. The purified xylanase from *M. thermophila* BF1-7 appeared as a single protein band on SDS-PAGE gel with a molecular mass of approximately 14 kDa (Fig. 1), which was less than a low molecular weight xylanase reported in previous literature (Kelly et al. 1989; Cesar and Mrsa 1996; Lucena-Neto and Ferreira-Filho 2004; Li et

2005; Li et al. 2006; Fang et al. 2008; Maalej et al. 2009; Shi et al. 2015).

The purified xylanase was most active at pH 12.0 and exhibited a broad optimum at pH 9.0–12.0, which was a higher pH than those reported in other fungi. The optimum pH of crude xylanase from *M. thermophila* M.7.7 was 5.0 (Moretti et al. 2012), whilst the purified xylanase from *P. themophila* J18 was active at pH 7.0 and exhibited a broad optimum at pH 6.5–7.5 (Li et al. 2006). Also, the optimum pH of xylanase from *Talaromyces thermophilus* was pH 7.0–8.0 (Maalej et al. 2009). Lucena-Neto and Ferreira-Filho (2004) reported that the purified xylanase from *Humicola grisea* var. *thermoidea* was most active in a pH ranging of 4.5–6.5. According to Shi et al. (2015), the optimum pH of the purified recombinant Xyn11B from *Humicola insolens* Y1 was pH 6.0. In addition, the purified xylanase from *T. lanuginosus* CBS 288.54 was most active in the pH ranging from 7.0 to 7.5 (Li et al. 2005). The purified xylanase from strain BF1-7 retained over 55% of original activity in pH range 9.0–12.0 after incubation at 4 °C for 24 h. These data were in contrast to that reported by Moretti et al. (2012), who found that crude xylanase from *M. thermophila* M.7.7 was stable in a pH range of 5.0–10.0. The purified xylanase from *Thermomyces lanuginosus* DSM 5826 was stable at pH 5.0–9.0 (Cesar and Mrsa 1996). Maalej et al. (2009) reported that the purified xylanase of *T. thermophilus* retained more than 80% of its activity after incubation for 24 h at different pH values ranging between 4.8 and 9.2. Also, the purified recombinant Xyn11B of *H. insolens* Y1 was stable at pH 5.0–9.0 (Shi et al. 2015). In addition, the purified xylanase from *T. lanuginosus* CBS 288.54 was stable in pH range of 6.5–10.0 (Li et al. 2005).

The xylanase was optimally active at 50–60 °C which was similar to xylanase (55–65 °C) from the thermophilic fungus *H. grisea* var. *thermoidea* (Lucena-Neto and Ferreira-Filho 2004). As with xylanases isolated from *Paecilomyces variotii* IMD RK 032, the xylanase was optimally active at 50 °C (Kelly et al. 1989). Also, the optimum temperature of the purified xylanase from *A. carneus* M34 was 50 °C (Fang et al. 2008). In addition, the optimum temperature of recombinant Xyn11B was 50 °C (Shi et al. 2015). Xylanases produced by thermophilic fungi were usually more thermostable than those from mesophilic fungi (Li et al. 2006). However, the thermal stability of the purified xylanase from various strains differed depending on the experimental conditions (Li et al. 2005).

The inhibition of the enzyme activity by Hg^{2+} ions may be due to its interaction with sulfhydryl groups, suggesting that there is an important cysteine residue in or close to the active site of the enzyme (Lv et al. 2008; Wipusaree et al. 2011). From our present study, Fe^{2+} and Hg^{2+} inhibited the activity of the xylanase enzyme, which were similar to those reported in other literature (Collins et al. 2002; Lv et al. 2008; Yang et al. 2010; Wipusaree et al. 2011). The xylanase from *M. thermophila* BF1-7 was activated by Cu^{2+} at 5 mM, which was identical to that of xylanase from *Aspergillus awamori* VTCC-F312 (Do et al. 2012). In contrast, Wipusaree et al. (2011) reported that xylanase from *Alternaria alternata* was strongly inhibited by Cu^{2+} at the concentration of 5 mM. Endoxylanase from *Aspergillus niger* B03 was also inhibited by Cu^{2+} (Dobrev and Zhekova 2012). However, the effect of metal ions on xylanase activity depends on the types of metal ion and its concentration and the nature of the enzyme. If its concentration

is too high or unbalanced, these ions or reagents showed a dose-dependent inhibitory effect against the enzyme by destroy its active protein structure (Bankeeree et al. 2014).

The purified xylanase in this study was specific to beechwood xylan and oat spelt xylan (xylan-containing substrates). The highest activity was observed in beechwood xylan. These results indicated that substrate specificity of xylanase depends on type of xylan substrates. Xylan from beechwood has a backbone consisting of more than 90% β-1,4-linked xylose residues, whereas oat spelt xylan is a type of arabinoxylan, the main chain of which is largely branched with arabinose residues (Gruppen et al. 1992; Kormelink et al. 1993). In addition, the purified xylanase did not act towards carboxymethyl cellulose, Avicel and cellulose powder. These results indicate that the purified xylanase from *M. thermophila* BF1-7 was a true xylanase. Both K_m (9.67 mg/mL) and V_{max} (5.38 μmol/min/mg) in the present study were in agreement with the values reported from other fungal xylanases, which ranged from 0.09 to 40.9 mg/mL for K_m and from 0.106 to 6300 μmol/min/mg for V_{max} (Beg et al. 2001). In this study, the K_m value was similar to that the K_m value of xylanase from *Trichoderma longibrachiatum* CS-185 was 10.14 mg/mL (Chen et al. 1997). In addition, the present study provided better value of K_m in comparison to the value previously reported from *Aspergillus terreus* (22 mg/mL) (Sorgatto et al. 2012), *T. thermophilus* (22.5 mg/mL) (Maalej et al. 2009) and *Penicillium sclerotiorum* (23.4 mg/mL) (Knob and Carmona 2010). Whilst the V_{max} value (5.38 μmol/min/mg) estimated here was quite high compared to the V_{max} of xylanase from *A. alternata* (2.14 μmol/min/mg) (Wipusaree et al. 2011) and *T. thermophilus* (1.235 μmol/min/mg) (Maalej et al. 2009).

The purified xylanase from *M. thermophila* BF1-7 hydrolyzed beechwood xylan to yield mainly xylotetraose and a small amount of xylobiose as end products. This indicated that the purified xylanase of this fungus was endo-xylanase. These results agreed with the observations of Chen et al. (1997), who found xylanase from *T. longibrachiatum* CS-185 hydrolyzed oat spelt xylan to mixtures of xylooligosaccharides with xylobiose, xylotetraose, and xylopentaose to be the major ones. After 0.5 h of xylooligosaccharides hydrolysis by the purified xylanase from *T. lanuginosus* CBS 288.54, xylobiose and xylotetraose were observed in the reaction mixture, which could be due to glycosyl transfer reaction of endoxylanase (Li et al. 2005). Li et al. (2006) reported that the xylanase from *P. themophila* J18 hydrolyzed beechwood xylan randomly, yielding xylotriose and xylobiose as the main end products. Moreover, it hardly hydrolyzed xylotriose and xylobiose. According to Fang et al. (2008), xylanase from *A. carneus* M34 liberated mainly xylotriose and xylotetraose from beechwood xylan.

In the present study, low-molecular weight and alkaline-tolerant endo-xylanase from *M. thermophila* BF1-7 was purified. The molecular mass of the purified xylanase was approximately 14 kDa. The purified xylanase was most active at very high pH, with optimum conditions at pH 12.0, and temperature at 50 °C. Also, it was stable at alkaline conditions at pH 9.0–12.0 and a temperature of 50 °C. Thus, these enzyme characteristics suggest great potential for application in commercial paper bleaching processes without changing pH. In addition, the low-molecular weight of this enzyme can better diffuse into the biomass structure or fibrous pulp and can thus efficiently hydrolyzed xylan in pulp bleaching. To verify the potential use of purified xylanase from isolate BF1-7 in bleaching process, further experiments under actual conditions need to be performed. Moreover, the present findings provide the most comprehensive current knowledge, with no literature previously available on low molecular weight and extreme alkaline xylanase among thermophilic fungi.

Acknowledgments

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