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Expression of recombinant *Thermomonospora fusca* xylanase A in *Pichia pastoris* and xylooligosaccharides released from xylans by it

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Abstract

The mature peptide of *Thermomonospora fusca* xylanase A (TfxA) was successfully expressed in *Pichia pastoris* under the control of *AOX1* promoter. The activity of recombinant *T. fusca* xylanase A (reTfxA) in culture supernatant was 117.3 ± 2.4 U/mg, which is 3 times higher than that of the native TfxA. The optimal temperature and pH for reTfxA were 60 °C and 6.0, respectively. When treated at 70 °C and pH 6.0 for 2 min, the residual activity of the reTfxA was 70%. The reTfxA was very stable over a wide pH range (5.0–9.0). After incubation over pH 5.0–9.0 at 25 °C for 1 h, all the residual activity of reTfxA was over 80%. The K_m and k_{cat} values for reTfxA were 2.45 mg/ml and 139 s^{-1}, respectively. HPLC analysis revealed that xylobiose (X2) was the main hydrolysis product released from birchwood xylan and wheat bran insoluble xylan by reTfxA. Hydrolysis results of xylooligosaccharides showed that reTfxA was an *endo*-acting xylanase and xylobiose, xylotriose (X3), xylotetraose (X4), xylopentaose (X5), and xylohexaose (X6) could be hydrolysed. This is the first report on the expression of reTfxA in yeast and on the determining and quantifying of the hydrolysis products released from xylans and xylooligosaccharides by reTfxA.

Keywords: *Thermomonospora fusca* xylanase A (TfxA); Expression; Hydrolysis; Xylooligosaccharides; HPLC

1. Introduction

Xylan is the major component of plant cell wall and the most abundant renewable hemicellulose. The action of the main β-endoxylanase (EC 3.2.1.8) is to convert xylan to xylooligosaccharides (Biely, 1993). According to sequence similarities and hydrophobic cluster analysis, xylanases have been classified into families 10 (or F) and 11 (or G) of glycosyl hydrolases (Henrissat & Bairoch, 1993). Many xylanases used in industry are from mesophiles, yet xylanases from thermophilic sources may be of tremendous utility in some biotechnological processes. Particularly, thermophilic xylanase could be useful in application where a cooling step would be uneconomical or where high temperature is required to increase the availability and/or solubility of substrates, to reduce viscosity and/or to reduce the risk of contamination. A number of thermostable xylanases-producing microorganisms have been isolated from a variety of sources, such as terrestrial and marine solfataric fields, thermal springs, and self-heating decaying organic debris (Vieille & Zeikus, 2001).

Xylanase A from the thermophilic soil actinomycete *Thermomonospora fusca* is thermophilic and thermostable. The amino acid sequence of its catalytic domain (CD)

Abbreviations: *TfxA, Thermomonospora fusca* xylanase A; reTfxA, recombinant TfxA expressed in *P. pastoris*; tfx, the gene encoding mature TfxA; LB, Luria–Bertani; HPLC, high-performance liquid chromatography; X, xylose; X2, xylobiose; X3, xylotriose; X4, xylotetraose; X5, xylopentaose; X6, xylohexaose; AFM, atomic force microscope; DP, degree of polymerization; XOs, xylooligosaccharides.

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showed that TfxA belongs to family 11 and it has high homology with other family 11 xylanases (Irwin, Jung, & Wilson, 1994). The crystal structure of TfxA has been determined. It consists of a single domain composed predominantly of β-strands and has a very characteristic fold, which has been compared to the shape of a right hand (Kulkarni, Shendye, & Rao, 1999). The activity of native TfxA is low for industrial purposes and it is difficult to purify. *Pichia pastoris* has been proven to be an efficient system for the expression of many heterologous proteins. At present, more than 300 genes have been expressed in it (Cereghin & Cregg, 2000). These results motivated us to express the mature peptide of TfxA in *P. pastoris*. Xylan degradation by reTfxA was detected by atomic force microscopy (AFM) and HPLC. Xylooligosaccharides are used as a functional food in many countries (Moure, Gullon, Dominguez, & Parajo, 2006). This is the first report on the expression of reTfxA in yeast and on determining and quantifying the xylooligosaccharides released from xylan by reTfxA.

2. Materials and methods

2.1. Materials

The *Pichia pastoris* expression kit was from Invitrogen (Carlsbad, CA). Medium components were from Difco. Restriction endonucleases were from TaKaRa (Shiga, Japan). T_4 DNA ligase and PCR kit were from Promega (Madison, WI). Primers were synthesised by Sangon (Shanghai, China). Birchwood xylan was from Sigma Chemical Company (St. Louis, MO). The standard xylooligosaccharides (X2–X6) were from Megazyme. Xylose (X) was from Merck (Darmstadt, Germany). Wheat bran-insoluble xylan was provided by Dr. Chen (Southern Yangtze University). The recombinant pBS-T tfx plasmids containing TfxA gene were stored at −20 °C in our laboratory. All other chemicals used were of reagent grade obtained from standard sources.

2.2. Construction of expression plasmid

The tfx gene encoding the mature peptide of TfxA was amplified from recombinant pBS-T tfx plasmid, by using the primers of P1 (5′-CĜAATTCGCTGT TACATCCAACGAGACCG-3′) with the *Eco*RI recognition site (underlined) and P2 (5′-GĈGGCCGCGTTGGCGCTGCAGGACACCGT-3′) with the *Not*I recognition site (underlined). The resulting PCR product, tfx, was digested with *Eco*RI and *Not*I and ligated into pPIC9K vector previously digested with the same enzymes. The recombinant pPIC9K-tfx plasmid was transformed into *Escherichia coli* TOP10F′.

2.3. Transformation of Pichia pastoris and expression of reTfxA

The pPIC9K-tfx plasmid was linearized with *Bgl*II and transformed into competent *pastoris* strain GS115 cell with an Electroporator (Eppendorf-2510, Hamburg, Germany). Seventy-five His^+ Mut^s phenotype transformants were tested for expression of xylanase in 5-ml YPG medium (1% yeast extract, 2% peptone, 1% glycerol) at 30 °C with constant shaking at 250 rpm. To maintain induction, methanol was added to the culture to a final concentration of 0.25% (v/v) every 24 h. After 96 h induction, the xylanase activity was measured according to standard methods. The transformant with the best expression performance was used for further studies.

Scale-up expression was achieved in a 2-l baffled shaking flask. The highest-expressing *P. pastoris* clone, named pPTfxA6, was grown in 0.5 l BMGY medium (2% tryptone, 1.34% yeast nitrogen base (YNB), 0.4 μg/ml biotin, 1% glycerol, 0.1 M potassium phosphate, pH 6.0) with constant shaking (250 rpm) at 30 °C for 24 h. Cells grown in BMGY were harvested and resuspended in 0.5 l BMMY medium (the same as BMGY but with 0.5% methanol instead of glycerol), then incubated for 96 h. To maintain induction, methanol was added to the culture to a final concentration of 0.25% (v/v) every 24 h.

2.4. Purification and SDS–PAGE of reTfxA

Recombinant *P. pastoris* cells were removed from the culture media by centrifugation at 5000*g* for 5 min. Cell-free supernatant (450 ml) was treated with ammonium sulfate to 60% saturation to precipitate the enzyme. The precipitated enzyme was then resuspended in 3.0 ml McIlvaine's buffer (0.2 M sodium phosphate, 0.1 M citric acid, pH 6.0) and loaded onto a Sephadex G-100 column (Pharmacia, 50 cm × 2 cm). The enzyme was eluted with the same buffer system at a flow rate of 12 ml/h. The active fractions were pooled and analysed for xylanase activity.

Aliquots of culture supernatant (10 μl) obtained at different induction times were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). In the Laemmli system (Laemmli, 1976), the stacking and separating gels consisted of 5% and 15% polyacrylamide, respectively. Proteins were visualized with Coomassie brilliant blue R-250 straining.

2.5. Enzyme assays

The xylanase activity was assayed with 1% birchwood xylan (w/v) as substrate and the liberation of reducing sugars was estimated by the dinitrosalicylic acid (DNS) procedure (Miller, Blum, Glennom, & Burton, 1959). Protein concentration was measured by the dye-binding method of Bradford, and bovine serum albumin (BSA) was used as the standard (Bradford, 1976). The kinetic parameters for reTfxA and TfxA activity were calculated from initial velocities, using concentrations ranging from 0.1 to 10 mg/ml birchwood xylan.

One unit of xylanase activity was defined as the amount of the enzyme that catalysed the formation of 1.0 μmol of reducing sugar from xylan in 1 min under its optimal conditions (at 60 °C and pH 6.0). For each assay in this study,

triplicate measurements were conducted, to obtain a mean value of activity.

2.6. Effect of temperature on the activity and thermal tolerance of reTfxA

The temperature optimum was measured at pH 6.0 (McIlvaine's buffer system) from 40 to 90 °C. Thermal stability of reTfxA was determined by assaying residual activity after incubation at 40–90 °C in McIlvaine's buffer (pH 6.0) for 2 min.

2.7. Effect of pH on the activity and stability of reTfxA

The effect of pH on xylanase activity was measured over a range of pH 3.0–7.0 (McIlvaine's buffer system) and 8.0–10.0 (0.2 M glycine, 0.2 M NaOH buffer system) at 60 °C. To determine the pH stability, reTfxA was incubated in various pH buffers at 25 °C for 1 h, and the residual activities were measured at 60 °C and pH 6.0.

2.8. Analysis of hydrolytic products of xylans

The 1.0% (w/v) birchwood xylan and bran-insoluble xylan solutions in McIlvaine's buffer (pH 6.0) were hydrolysed by reTfxA at 40 °C with constant shaking. In the two reaction mixtures, the substrates were excessive and the amount of reTfxA was same. The aliquots at different time intervals (6, 12, 24, and 36 h) and standard xylooligosaccharides were analysed by HPLC with Sugar-Pak™1 column (300 mm × 6.5 mm; Waters, Milford, MA), pure water as mobile phase (0.5 ml/min) and injection volumes of 20 μl. Sugar peaks were screened using a Waters 2401 refractive index detector. Xylose (X) and standard xylooligosaccharides (X2–X6) were resolved in pure water. Samples of sugar (X–X6) were analysed by HPLC separately. The areas of sugar peaks from HPLC results were combined with the concentrations of sugars and then the standard concentration curves of xylooligosaccharides (X–X6) were obtained. The hydrolytic products of xylan were quantified on standard curves.

2.9. Investigation of the hydrolysis of birchwood xylan by AFM

Birchwood xylan (1%) was incubated with reTfxA at 40 °C for 12 h. The mixture was heated at 100 °C for 5 min, and then it was centrifuged. The 1% birchwood xylan mixed with inactive reTfxA was boiled and centrifuged too, and was used as control. Before being scanned by AFM (SPM-9500J3, Shimadzu, Kyoto, Japan), the supernatants were diluted 50 times with water.

2.10. The mode of action of xylooligosaccharides by reTfxA

The mode of action of reTfxA was determined using different xylooligosaccharides (X2–X6) as substrates. The standard xylooligosaccharides solutions (pure water, pH 7.0) were incubated with purified reTfxA at 40 °C. The samples at different time intervals were determined and quantified by HPLC. The injection volume was 20 μl.

3. Results

3.1. Expression and purification of reTfxA in P. pastoris

The His^+ Mut^s transformant, pPTfxA6, with the highest activity in small-culture analysis was selected for scale-up expression of reTfxA. After 96-h induction, the maximal specific activity of reTfxA in a 2-l baffled shaking flask reached 117 ± 2.4 U/mg. Kinetic constants for reTfxA and *Thermomonospora fusca* xylanase A (TfxA) are shown in Table 1.

Partial purification by ammonium sulfate precipitation and gel filtration resulted in a 3.9-fold increase in specific activity of reTfxA (117 ± 2.4 U/mg). Analysis of SDS–PAGE (Fig. 1) showed that the pPTfxA6 produced a specific clear protein band and its molecular mass was about 31.0 kDa.

3.2. Effect of temperature on the activity and stability of reTfxA

Fig. 2 showed that reTfxA activity increased with the rise of temperature, reached a maximum at 60 °C; and then

Table 1
Kinetic constants for TfxA and reTfxA

Xylanase	K_m (mg/ml)	k_{cat} (s^{-1})	k_{cat}/K_m (ml/mg s)
TfxA	2.51	120	47.8
reTfxA	2.45	139	56.6

Note. Xylanase activities were determined at their optimum conditions described in Section 2. The concentration of birchwood xylan ranged from 0.1 to 10 mg/ml. TfxA, *Thermomonospora fusca* xylanase A; reTfxA, recombinant TfxA expressed in *P. pastoris*.

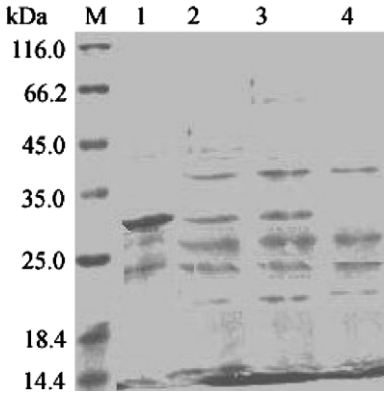

Fig. 1. SDS–PAGE analysis of different reTfxA samples from pPTfxA6. *Note.* The molecular weights of the markers are given to the left. Lane 1 was purified reTfxA. Lanes 2 and 3 were the samples from pPTfxA6 induced by methanol for 96 h. Lane 4 was the control, in which *P. pastoris* was transformed with pPIC9K.

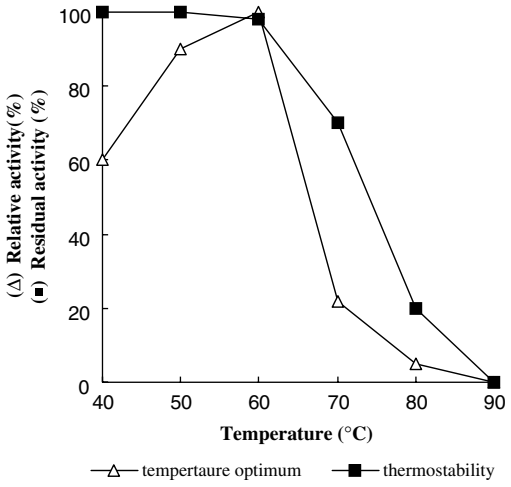

Fig. 2. The temperature optimum (△) and thermostability (■) of reTfxA. *Note*. 1% birchwood xylan was used as substrate. The highest xylanase activity was taken as 100% in assay of temperature optimum (△). The xylanase activity under optimum conditions (60 °C, pH 6.0) was taken as 100% in assay of thermostability (■).

decreased rapidly. The reTfxA was stable below 70 °C, but it lost enzyme activity rapidly above 70 °C. The TfxA was more stable than reTfxA at 70 °C.

3.3. Effect of pH on the activity and stability of reTfxA

The reTfxA showed high activity in a pH range of 5.0–8.0, with the optimal pH at 6.0 (Fig. 3). The reTfxA was very stable from pH 5.0 to 9.0. Over 80% of reTfxA activity was retained after treatment of the enzyme by preincubation over a pH range of 5.0–9.0 for 1 h at 25 °C.

3.4. Hydrolysis products of birchwood xylan by reTfxA

The hydrolysis products of birchwood xylan by reTfxA were analysed by HPLC (Fig. 4, panels a–d). Xylobiose was the major hydrolysis product of birchwood xylan. As the reaction time increased, the xylose and xylobiose concentrations increased with a simultaneous decrease in the concentrations of xylotriose, xylotetraose, xylopentaose, and xylohexaose. After 36 h of incubation, about 44.7% of the total hydrolysis products of birchwood xylan were xylobiose and its concentration was 2.57 mg/ml (Table 2).

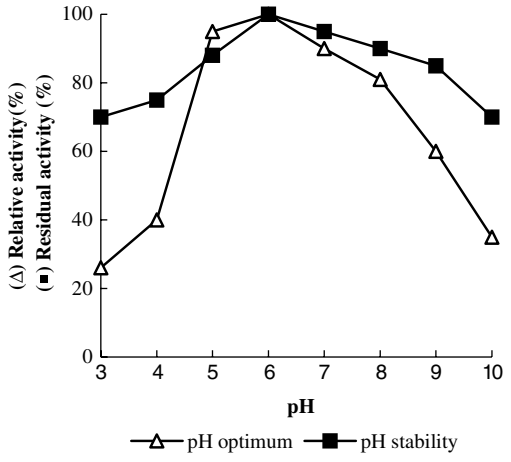

Fig. 3. The pH optimum (△) and pH stability (■) of reTfxA. One percent birchwood xylan was used as substrate. The highest xylanase activity was taken as 100% in assay of pH optimum (△). The xylanase activity under optimum conditions (60 °C, pH 6.0) was taken as 100% in assay of pH stability (■).

3.5. Hydrolysis products of bran insoluble xylan

Xylobiose was the major hydrolysis product of wheat bran-insoluble xylan by reTfxA (Fig. 5). During the period of hydrolysis, concentrations of the six xylooligosaccharides increased. As the reaction went on, the relative amounts of xylose, xylobiose, and xylotriose increased; while those of xylotetraose, xylopentaose, and xylohexaose decreased. After 36 h of incubation, about 34.3% of the total reaction products were xylobiose and its concentration was 1.43 mg/ml (Table 3).

3.6. Changes in structure of birchwood xylan hydrolysed by reTfxA

The operational mode of the atomic force microscope (AFM) is to control the distance between tip and sample with a feedback mechanism, to maintain a constant interaction force during scanning. A topographical image can then be displayed from point-by-point adjustments (Baker, Halber, Sugiyama, & Miles, 1997). The AFM analysis showed that the untreated birchwood xylan has a characteristic and well-arranged surface (Fig. 6, panel 0). After 12 h hydrolysis, the surface of birchwood xylan was altered significantly. The degradation led to a thinner and layer of birchwood xylan (Fig. 6, panel 1).

3.7. Hydrolysis of standard xylooligosaccharides by reTfxA

The hydrolysis mode of reTfxA was determined using different standard xylooligosaccharides (X2–X6) as substrate. X2–X6 could be hydrolysed by reTfxA. X2 and X3 were hydrolysed slowly (Fig. 7, panels a and b). X2 and X3 were major products released separately from X4 and X5 (Fig. 7, panels c and d). A small amount of X4 was produced and no X was detected during the hydrolysis of X5, which suggested that the formation of X4 might be the result of transglycosylation action. The overall reaction might be: $X5 + E = X5(E)$; $X5(E) = X3 + X2(E)$; $X2 + X2(E) = X4 + E$; where E represents free reTfxA and X2(E) represents reTfxA xylobiose complex. X6 was rapidly hydrolysed by reTfxA and generated a xylooligosaccharides mixture (X2–X5). X3 was the major product released from X6 (Fig. 7, panel e). X5 existed in the hydrolysis products of X6 and no X was detected, suggesting the formation of X5 was the result of transglycosylation reaction.

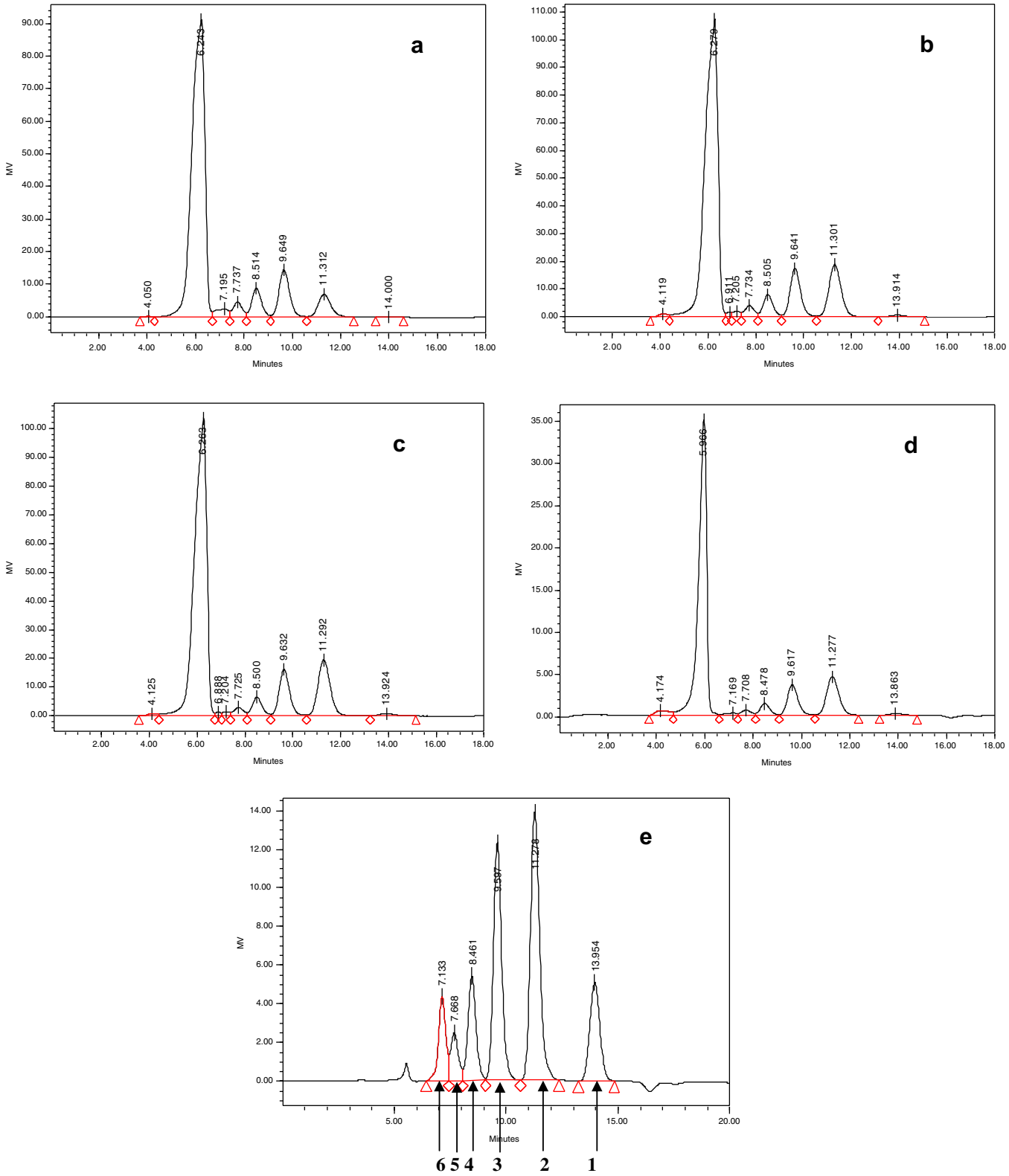

Fig. 4. HPLC profiles of birchwood xylan degradation by reTfxA and the standard xylooligosaccharides. *Note.* The samples (panels a–d) analysed by HPLC after 6, 12, 24, and 36 h degradation from birchwood xylan by reTfxA, respectively. Panel E is the standard xylooligosaccharides mixture analysed by HPLC. The positions of xylose (1), xylobiose (2), xylotriose (3), xylotetraose (4), xylopentaose (5), and xylohexaose (6) are shown.

Table 2
Birchwood xylan hydrolysis products concentration and their percentage content

Reaction time (h)	Hydrolytic products concentration (mg/ml) and percentage content					
	Xylose	Xylobiose	Xylotriose	Xylotetraose	Xylopentaose	Xylohexaose
6	0.004 (*0.09%*)	0.930 (*21.43%*)	1.64 (*37.8%*)	0.900 (*20.74%*)	0.435 (*10.03%*)	0.430 (*9.91%*)
12	0.102 (*1.69%*)	2.44 (*40.58%*)	1.97 (*32.77%*)	0.840 (*13.97%*)	0.450 (*7.49%*)	0.210 (*3.50%*)
24	0.130 (*2.22%*)	2.50 (*42.66%*)	2.05 (*34.98%*)	0.690 (*11.78%*)	0.340 (*5.80%*)	0.150 (*2.56%*)
36	0.135 (*2.34%*)	2.57 (*44.70%*)	2.09 (*36.35%*)	0.550 (*9.57%*)	0.260 (*4.529%*)	0.145 (*2.52%*)

4. Discussion

The practical application of thermophilic enzymes depends on the availability of enzyme in sufficient quantity. This immediately raises problems if the enzyme is isolated directly from the source organism. The conditions required for the growth of thermophiles are imcompatible with standard industrial fermentation and the downstream

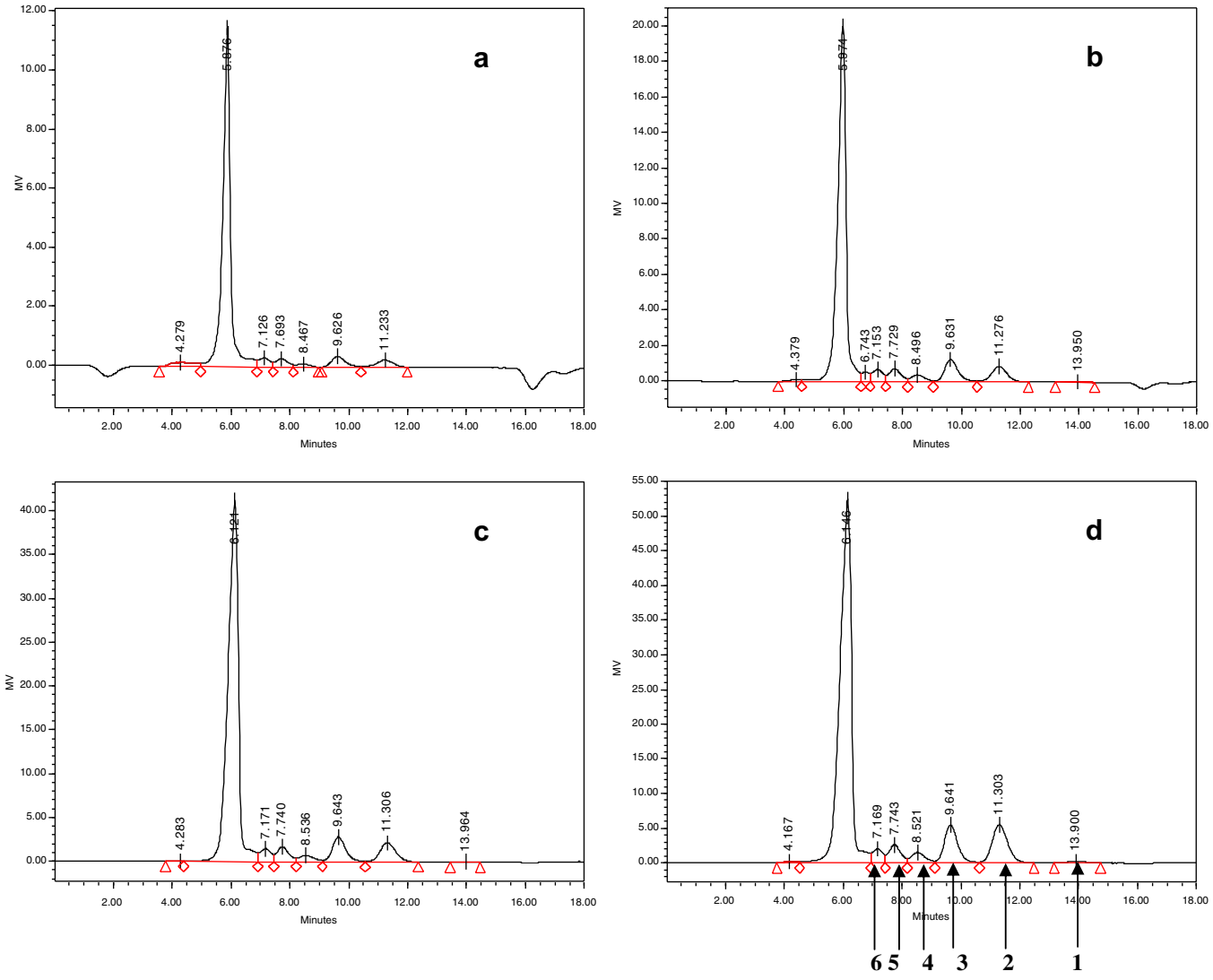

Fig. 5. HPLC profiles of bran-insoluble xylan degradation by reTfxA. *Note*. Panels a–d were the samples analysed by HPLC after 6, 12, 24, and 36 h degradation of bran insoluble xylan by reTfxA, respectively. The positions of xylose (1), xylobiose (2), xylotriose (3), xylotetraose (4), xylopentaose (5), and xylohexaose (6) are shown.

Table 3
Bran-insoluble xylan hydrolysis product concentration and their percentage content

Reaction time (h)	Hydrolytic products concentration (mg/ml) and percentage content (italic)					
	Xylose	Xylobiose	Xylotriose	Xylotetraose	Xylopentaose	Xylohexaose
6	0.003 (*0.16%*)	0.536 (*28.08%*)	0.51 (*26.73%*)	0.182 (*9.53%*)	0.343 (*18.0%*)	0.334 (*17.50%*)
12	0.006 (*0.26%*)	0.674 (*28.96%*)	0.653 (*28.06%*)	0.195 (*8.38%*)	0.408 (*17.54%*)	0.391 (*16.8%*)
24	0.014 (*0.46%*)	0.891 (*29.35%*)	0.861 (*28.18%*)	0.245 (*8.07%*)	0.53 (*17.26%*)	0.506 (*16.48%*)
36	0.054 (*1.29%*)	1.43 (*34.30%*)	1.25 (*29. 90%*)	0.318 (*7.60%*)	0.581 (*13.90%*)	0.544 (*13.01%*)

processing plant (Hough & Danson, 1999). Therefore, most applications of thermophilic enzymes rely on their expressions in mesophilic hosts. As a eukaryote, methylotrophic yeast *P. pastoris* has been developed as an excellent host for the commercial production of heterologous proteins. At present, xylanases from *Aspergillus niger*, *Streptomyces olivaceoviridis*, *Bacillus pumilus*, *Thermomyces lanuginosus*, *Aspergillus terreus*, *Rhodothermus marinus*, *Thermobifida fusca*, and *Aureobasidum pullulans* have been expressed in *P. pastoris* (Berrin et al., 2000; Chantasingh, Pootanakit, Champreda, Kanokratana, & Eurwilaichitr, 2006; Cheng, Yang, & Liu, 2005; Damaso, Almeida, Kurtenbach, Andrade, & Albano, 2003; Jiang, Song, & Ma, 2003; Ramchurn, Mateus, Holst, & Karlsson, 2005; Tanaka, Okuno, Moriyama, Muguruma, & Ohta, 2004; Zhang, Yao, Wang, & Zhang, 2003).

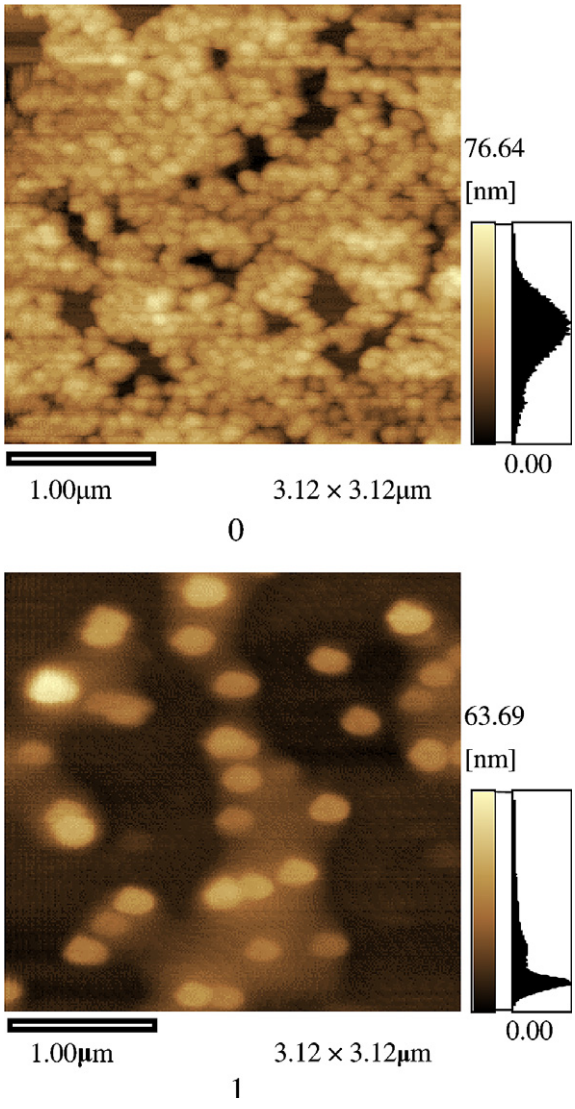

Fig. 6. AFM photographs of birchwood xylan hydrolysed by reTfxA. Panel 0 is the control (1% birchwood xylan was mixed with inactive reTfxA). Panel 1 is the birchwood xylan hydrolysed by reTfxA for 12 h.

The first characterisation and gene cloning of TfxA were reported by Ghangas et al., who expressed the enzyme in *Streptomyces lividans* and *E. coli* with 7.0 and 0.52 U/ml xylanase activities, respectively (Ghangas, Hu, & Wilson, 1989). In the work, the activity of reTfxA in culture supernatant was 117.3 ± 2.4 U/mg, which is much higher than that of the native TfxA (39.0 U/mg). The reTfxA was purified more easily from *P. pastoris* culture supernatant by two steps rather than from the hard-to-grow actinomycete *T. fusca*.

The optimum temperature of reTfxA was 60 °C. After incubation at 70 °C, and pH 6.0 for 2 min, the residual activity of reTfxA was 70%. It was supposed that the *N*-terminus of TfxA contributed to its significant thermostability (Sapag et al., 2002). In our previous study, the obtained hybrid xylanases, in which the *N*-terminuses of *A. niger* xylanase A and *Bacillus subtilis* xylanase A were substituted with the corresponding region of *T. fusca* xylanase A, exhibited significant thermostability at 70 °C (Liu, Weng, & Sun, 2006; Sun et al., 2005; Weng & Sun, 2005).

Although the substrate-binding domain often appeared in cellulase and F/10 family xylanase, it is unwonted in G/11 family xylanase (Din et al., 1994). The biggest difference between TfxA and other family 11 xylanases is that the former contains the xylan-binding domain (XBD). The natural substrates for hemicellulases are complex, comprising both cellulose and hemicellulose. Previous study showed that XBD and linker sequences play an important role in the activity of hemicellulases against

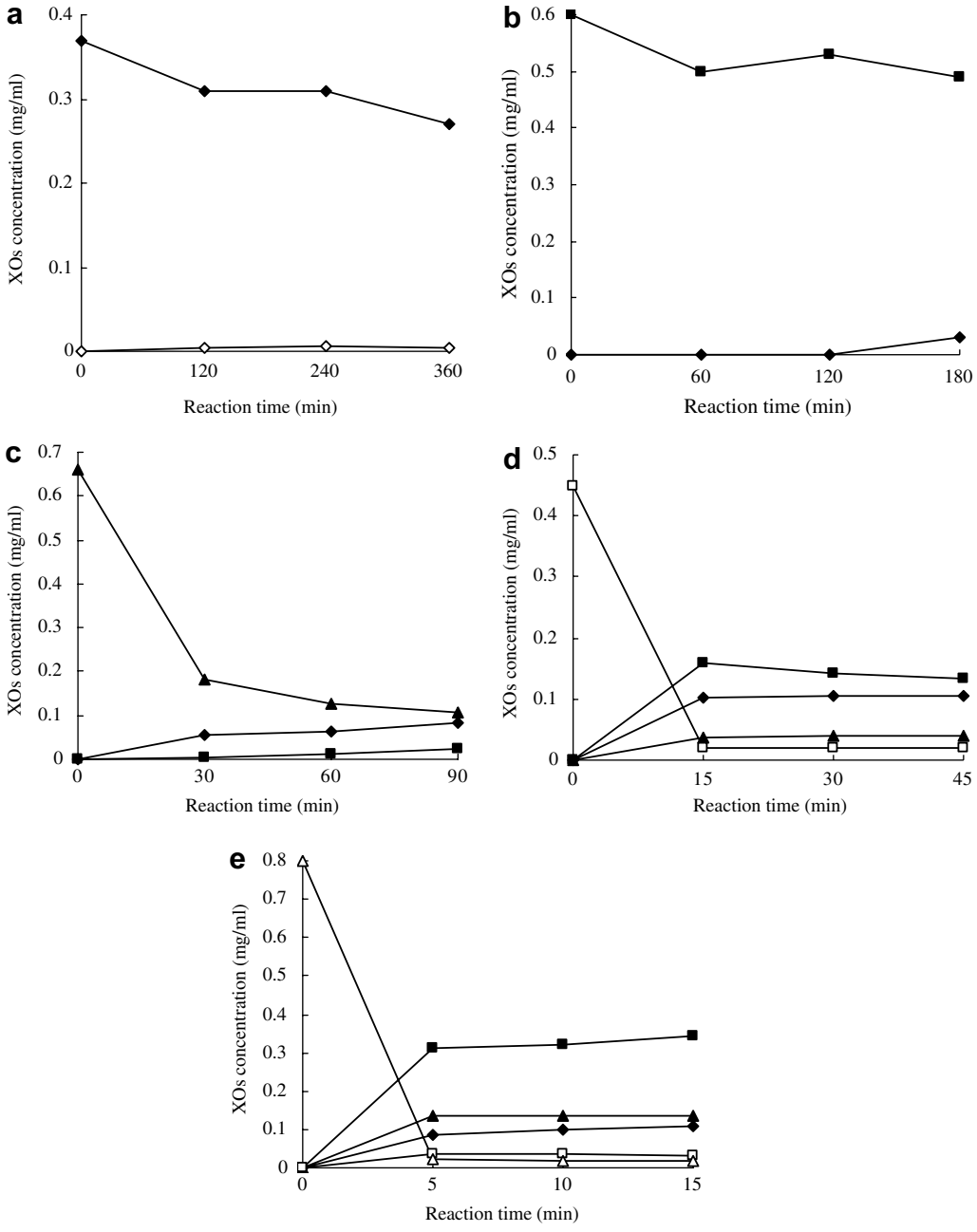

Fig. 7. HPLC analysis of xylooligosaccharides hydrolysed by reTfxA. *Note*. The reTfxA was incubated with 0.37 mg/ml xylobiose (a), 0.6 mg/ml xylotriose (b), 0.66 mg/ml xylotetraose (c), 0.45 mg/ml xylopentaose (d), and 0.8 mg/ml xylohexaose (e) at 40 °C for different times. At regular time intervals, aliquots of the reactions were analysed by HPLC for xylose (□), xylobiose (□), xylotriose (■), xylotetraose (▲), xylopentaose (□), and xylohexaose (△).

plant cell walls and other cellulose/hemicellulose complexes (Sunna, Gibbs, & Bergquist, 2000). The steric hindrance by insoluble cellulose from natural substrates would reduce the activity of xylanase against soluble xylan and linker sequences may provide the catalytic domain (CD) with the necessary flexibility to facilitate full access to the soluble xylan.

AFM analysis showed that the compact surface of birchwood xylan became relaxed after being hydrolysed by reTfxA for 12 h. Xylobiose was the major product from birchwood xylan and wheat bran-insoluble xylan by reTfxA. This made reTfxA potentially suitable for production of xylooligosaccharides. Xylooligosaccharides, used as a functional food in many countries (Moure et al., 2006), can selectively be used by the beneficial gastrointestinal microflora, *Bifidobacterium* spp. (Suwa et al., 1999). Reported beneficial effects of *Bifidobacterium* spp. on human health include suppressing the activity of enteroputrefactive bacteria, preventing the proliferation of pathogenic intestinal bacteria, and facilitating the digestion and

absorption of nutrients (Vazquez, Alonso, Dominguez, & Parajo, 2000).

X2–X6 could be further hydrolysed by reTfxA. X2 was the minimum oligomer hydrolysed by reTfxA. No X was detected in the hydrolysis products of X3–X6 by reTfxA. The main products of X4, X5, and X6 by reTfxA were X2, X3, and X3, respectively. These results revealed that reTfxA preferentially cleaved the internal glycosidic bonds of xylooligosaccharides and it was an *endo*-acting xylanase. Transglycosylation reaction occurred during the hydrolysis of X4, X5, and X6 by reTfxA. Endo-mode enzyme shows low susceptibility of substrate of DP = 2, such as xylobiose, chitobiose, and maltose (Biely, Krátky, & Vrasanská, 1981). Jiang et al. reported that the xylobiose degradation might processed by a transglycosylation reaction (Jiang et al., 2004). No XOs with DP >2 were detected in the hydrolysis product mixture of X2 by reTfxA. The results suggest that reTfxA might directly hydrolyse the X2, in a similar way to *Aspergillus sojae* xylanase (Kimura & Tajima, 1998).

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References

Baker, A. A., Halber, W., Sugiyama, J., & Miles, M. J. (1997). High-resolution atomic force microscopy of native *Valonia* cellulose I microcrystals. *Journal of Structural Biology, 119*, 129–138.

Berrin, J. G., Williamson, G., Puigserver, A., Chaix, J. C., Mclauchlan, W. R., & Juge, N. (2000). High-level production of recombinant fungal endo-b-1,4-xylanase in the methylotrophic yeast *Pichia pastoris*. *Protein Expression and Purification, 19*, 179–187.

Biely, P. (1993). Enzymological aspects of the production of microbiol hemicellulose. In M. P. Coughlan & G. P. Hazlewood (Eds.), *Hemicellulose and Hemicellulases* (pp. 29–51). London: Portland Press.

Biely, P., Krátky, Z., & Vrasanská, M. (1981). Substrate-binding site of endo-1,4-b-xylanase of the yeast *Cryptococcus albidus*. *European Journal of Biochemistry, 119*, 559–564.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry, 72*, 248–254.

Cereghin, J. L., & Cregg, J. M. (2000). Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS Microbiology Review, 24*, 45–66.

Chantasingh, D., Pootanakit, K., Champreda, V., Kanokratana, P., & Eurwilaichitr, L. (2006). Cloning, expression, and characterization of a xylanase 10 from *Aspergillus terreus* (BCC129) in *Pichia pastoris*. *Protein Expression and Purification, 46*, 143–149.

Cheng, Y. F., Yang, C. H., & Liu, W. H. (2005). Cloning and expression of *Thermobifida* xylanase gene in the methylotrophic yeast *Pichia pastoris*. *Enzyme and Microbial Technology, 37*, 541–546.

Damaso, M. C. T., Almeida, M. S., Kurtenbach, E., Martins Jr., O. B., N. P., Andrade, C. M. M. C., & Albano, R. M. (2003). Optimized expression of a thermostable xylanase from *Thermomyces lanuginosus* in *Pichia pastoris*. *Applied Environmental Microbiology 69,* 6064–6072.

Din, N., Damude, H. G., Gilkes, N. R., Miller, R. C., Warren, R. A. J., & Kilburn, D. G. (1994). C_1–C_x Revisited: Intramolecular synergism in a cellulase. *Proceedings of the National Academy of Science of the United States of America, 91*, 11383–11387.

Ghangas, G. S., Hu, Y. J., & Wilson, D. B. (1989). Cloning of a *Thermomonospora fusca* xylanase gene and its expression in *Escherichia coli* and *Streptomyces lividans*. *Journal of Bacteriology, 171*, 2963–2969.

Henrissat, B., & Bairoch, A. (1993). New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemistry Journal, 293*, 781–788.

Hough, D. W., & Danson, M. J. (1999). Extremozymes. *Current Opinions in Chemistry and Biology, 3*, 39–46.

Irwin, D., Jung, E. D., & Wilson, D. B. (1994). Characterization and sequence of a *Thermomonospora fusca* xylanse. *Applied Environmental Microbiology, 60*, 763–770.

Jiang, Z. B., Song, H. T., & Ma, L. X. (2003). Secreted expression of *Bacillus pumilus* xylanase gene in *Pichia pastoris* and study on enzymatic properties. *Chinese Journal of Biotechnology, 19*, 50–55.

Jiang, Z. Q., Deng, W., Zhu, Y. P., Li, L. T., Sheng, Y. T., & Hayashi, K. (2004). The recombinant xylanase B of *Thermotoga maritima* is highly xylan specific and produces exclusively xylobiose from xylans, a unque character for industrial applications. *Journal of Molecular Catalysis B-Enzymatic, 27*, 207–213.

Kimura, I., & Tajima, S. (1998). The modes of action of two endo-1,4-b-D-xylanases from *Aspergillus sojae* on various xylooligosaccharides. *Journal of Fermentation and Bioengineering, 85*, 283–288.

Kulkarni, N., Shendye, A., & Rao, M. (1999). Molecular and biotechnological aspects of xylanases. *FEMS Microbiology Reviews, 23*, 411–456.

Laemmli, U. K. (1976). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*, 680–685.

Liu, M. Q., Weng, X. Y., & Sun, J. Y. (2006). Expression of recombinant *Aspergillus niger* xylanase A in *Pichia pastoris* and its action on xylan. *Protein Expression and Purification, 48*, 292–299.

Miller, G. L., Blum, R., Glennom, W. E., & Burton, A. L. (1959). Measurement of methods for assay of xylanase activity. *Analytical Biochemistry, 2*, 127–132.

Moure, A., Gullon, P., Dominguez, H., & Parajo, J. C. (2006). Advances in the manufacture, purification and applications of xylo-oligosaccharides as food additives and nutraceuticals. *Process Biochemistry, 41*(9), 1913–1923.

Ramchurn, S. O., Mateus, B., Holst, O., & Karlsson, E. N. (2005). The methylotrophic yeast *Pichia pastoris* as a host for the expression and production of thermostable xylanase. *FEMS Yeast Research, 5*, 839–850.

Sapag, A., Wouters, J., Lambert, L., Ioannes, P. D., Eyzaguirre, J., & Depiereux, E. (2002). The endoxylanases from family 11: Computer analysis of protein sequences reveal important structural and phylogenetic relationships. *Journal of Biotechnology, 95*, 109–131.

Sun, J. Y., Liu, M. Q., Xu, Y. L., Xu, Z. R., Pan, L., & Gao, H. (2005). Improvement of the thermostability and catalytic activity of a mesophilic family 11 xylanase by N-terminus replacement. *Protein Expression and Purification, 42*, 122–130.

Sunna, A., Gibbs, M. D., & Bergquist, P. L. (2000). A novel thermostable multidomain 1,4-ß-xylanase from *Caldibacillus cellulovorans* and effect of its xylan-binding domain on enzyme activity. *Microbiology, 146*, 2947–2955.

Suwa, Y., Koga, K., Fujikawa, S., Okazaki, M., Irie, T., & Nakada, T. (1999) *Bifidobacterium bifidum* proliferation promoting composition containing xylooligosaccharide. USA Patent, US 5939309.

Tanaka, H., Okuno, T., Moriyama, S., Muguruma, M., & Ohta, K. (2004). Acidophilic xylanase from *Aureobasidum pullulans*: Efficient expression and secretion in *Pichia pastoris* and mutational analysis. *Journal of Bioscience and Bioengineering, 98*, 338–343.

Vazquez, M. J., Alonso, J. L., Dominguez, H., & Parajo, J. C. (2000). Xylooligosaccharides: Manufacture and applications. *Trends in Food Science and Technology, 11*, 387–393.

Vieille, C., & Zeikus, G. (2001). Hyperthemophilic enzymes: sources, uses, and molecular mechanism for thermostability. *Microbiology and Molecular Biology Reviews, 65*, 1–43.

Weng, X. Y., & Sun, J. Y. (2005). Construction, expression, and characterization of a thermostable xylanase. *Current Microbiology, 51*(3), 188–192.

Zhang, H. L., Yao, B., Wang, Y. R., & Zhang, W. Z. (2003). Expression of xylanase gene xynA from *Streptomyces olivaceoviridis* A1 in *Escherichia coli* and *Pichia pastoris*. *Chinese Journal of Biotechnology, 19*, 41–45.