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Characterization and pH-dependent substrate specificity of alkalophilic xylanase from *Bacillus alcalophilus*

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Abstract The gene of endo-beta-1-4 xylanase, *xynT*, was cloned from *Bacillus alcalophilus* AX2000 and expressed in *Escherichia coli*. This XynT, which belongs to glycoside hydrolase (GH) family 10, was found to have a molecular weight of approximately 37 kDa and exhibit optimal activity at pH 7–9 and 50 °C. It exhibits a high activity towards birchwood xylan and has the ability to bind avicel. Under optimal conditions, XynT hydrolyzes all xylooligomers into xylobiose as an end product with a preference for cleavage sites at the second or third glycosidic bond from the reducing end. XynT has a different substrate affinity on xylooligomers at pH 5.0, which contributes to its low activity toward xylotriose and its derived intermediate products. This low activity may be due to an unstable interaction with the amino acids that constitute subsites of the active site. Interestingly, the addition of Co^{2+} and Mn^{2+} led to a significant increase in activity by up to 40 and 50 %, respectively. XynT possesses a high binding affinity and hydrolytic activity toward the insoluble xylan, for which it exhibits high activity at pH 7–9, giving rise to its efficient biobleaching effect on *Pinus densiflora* kraft pulp.

Keywords Alkalophilic xylanase · Biobleaching · pH dependent · Substrate specificity

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Introduction

Hemicellulose is a heterogeneous polysaccharide consisting of β-1,4-linked xylose and side chains substituted with L-arabinofuranosyl, acetyl, diferulate cross-links, glucuronyl, and 4-*O*-methylglucuronyl groups. Complete enzymatic hydrolysis of hemicellulose requires the cooperative action of various enzymes, such as endo-β-1,4-xylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), α-L-arabinofuranosidase (EC 3.2.55), feruloyl esterase (EC 3.1.1.73), α-glucuronidase (EC 3.2.1.139), and acetyl xylan esterase (EC 3.1.1.72). This enzymatic process can lead to the efficient utilization of xylan for bio-fuels and chemicals, delignification of paper pulp, and digestibility enhancement of animal feedstock [32].

Endo-β-1,4-xylanase (XynT) is a critical enzyme in the degradation of hemicellulose, catalyzing its breakdown into xylooligomers or xylobiose. Xylanases are classified into two main families, namely, glycoside hydrolase (GH) families 10 and 11. Family 10 enzymes typically have a high molecular weight (>30 kDa) and low pI and display an $(\alpha/\beta)_8$ barrel fold in which the catalytic cleft is located on the surface of the C-terminal side of the central β-barrel [10, 13]. Family 11 enzymes have a low molecular weight (approx. 20 kDa) and a high pI value and exhibit a β-jelly roll structure. Xylanases produced by fungi (*Aspergillus* sp., *Trichoderma* sp.) and bacteria (*Bacillus* sp., *Streptomyces* sp., *Clostridium* sp.) have been studied intensively

for their industrial applications [8, 36]. In general, xylanases produced by fungi, such as *Trichoderma reesei*, *T. viride*, and *Aspergillus nidulans*, have an optimum pH range between 4 and 5.5 and an optimum temperature in the range 50–60 °C while bacterial xylanases have a slightly higher optimum pH range (pH 6–9 [36, 37]). Despite their high activity at low pH (acidic), most fungal xylanases are unsuitable for application in the paper and pulp industry due to the a requirement for additional steps in subsequent stages [36]. Therefore, many alkalophilic xylanases produced by alkalophilic bacteria (alkalophilic *Bacillus* sp. strain AR-009, *Bacillus* sp. strain 41 M-1, *Bacillus pumilus* 13a) and fungi (*Cephalosporium* sp., *Aspergillus fischeri* FXn1) have been isolated and characterized [2, 7, 12, 14, 27].

In the study reported here, *xynT* gene was isolated from *Bacillus alcalophilus* AX2000, which produces xylanases possessing high activity under strongly alkaline conditions [28]. We describe the molecular properties and substrate specificity of XynT from *B. alcalophilus* AX2000 under different pH conditions and its potential for application in biobleaching.

Materials and methods

Expression and purification of XynT from *Escherichia coli*

The *xynT* gene (GenBank accession no. AY423561) was isolated from genomic DNA of *B. alcalophilus* AX2000 through several subsequent steps, including ligation of genomic DNA fragments cut by the restriction enzyme *Pst*I into expression vector pUC19, selection of *E. coli* colonies on LB solid media containing 0.5 % oat-spelt xylan, and analysis of DNA fragments inserted in pUC19 vector [29]. The gene was sub-cloned into pRset expression vector (Invitrogen, Carlsbad, CA) and transformed into an *E. coli* BL21 host strain. The transformant cells were inoculated and cultured in 500 mL LB liquid media at 37 °C until the OD_{600} was approximately 0.8–1.0, and over-expression of XynT was induced by the addition of lactose to a final concentration of 5 mM lactose. Cells were harvested and suspended with 10 mM lysis buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.8). All subsequent steps were carried out on ice or at 4 °C. The cells were disrupted by sonic treatment (at 30 % amplitude and 0.5 cycles for 20 min) with an ultrasonic processor (UP200S; Hielscher Ultrasonics, Teltow, Germany). After centrifugation, the supernatant was loaded onto Ni–NTA agarose column (Qiagen, Venlo, the Netherlands). The enzyme was washed twice with 100 mL washing buffer (20 mM sodium phosphate, 500 mM NaCl, pH 6.0) and eluted with 1 mL elution buffer (20 mM sodium phosphate, 500 mM NaCl, 250 mM imidazol, pH 6.0). The purified enzyme was exchanged into a final buffer of 20 mM phosphate buffer (pH 7) by dialysis (semipermeable cellulose membrane, Cat. no. D0655-100FT; Sigma, St. Louis, MO). Following incubation with the Bio-Rad Protein Assay Solution (Cat. no. 500-0006; Bio-Rad, Hercules, CA), the enzyme was quantified by measurement at 595 nm with an enzyme-linked immunosorbent assay (MULTISCAN EX; Thermo Fisher Scientific, Waltham, MA) and compared to a standard curve of bovine serum albumin.

Substrates preparation

Oat spelt xylan (Cat. no. X0627), birchwood xylan (Cat. no. X0502), and beechwood xylan (Cat. no. X4252) were purchased from Sigma-Aldrich (St. Louis, MO). Insoluble wheat arabinoxylan (Cat. no. P-WAXYL) and Avicel^{R} PH-101 (Cat. no. 11365) were obtained from Megazyme (Wicklow, Ireland) and Fluka (Sigma-Aldrich), respectively. The xylooligomers xylohexaose, xylopentaose, xylotetraose, and xylotriose were purchased from Megazyme.

Effect of pH and temperature on XynT activity

The optimum pH for XynT enzyme activity was determined by performing the xylanase activity assay on birchwood xylan at various pH (3–11), at 38 °C for 1 h. Citrate–phosphate buffer (pH 3–6), phosphate buffer (pH 7–8), and carbonate–bicarbonate buffer (pH 9–11) were prepared according to the '*Guide to protein purification*' [11]. The optimal temperature for XynT enzyme activity was determined by checking activity in phosphate buffer (pH 7) containing birchwood xylan at 25, 38, 50, 60 and 80 °C for 1 h. Activity measurements were repeated three times. The pH stability was determined by measuring enzymatic activity at pH 7 and 50 °C for 10 min after preincubating the enzyme at pH 5, 7, and 9, respectively, without substrate for 30, 60, 90, and 120 min.

Enzyme assay

To measure the enzyme activity of the recombinant XynT, 5 μg of the enzyme diluted in 0.7 mL phosphate buffer (pH 7.0) was added to 0.3 mL 1 % (w/v) birchwood xylan in the same buffer. The reaction was carried out at 50 °C for 10 min. Reducing sugars liberated from birchwood xylan were measured at 550 nm after boiling in DNS solution [1 % (w/v) dinitrosalicylic acid, 0.05 % (w/v) sodium sulfite, 2 % (w/v) Rochelle salt ($C_4H_4KNaO_6{\cdot}4H_2O$), 0.2 % (w/v) phenol, 2 % (w/v) NaOH] and quantified according to the standard curve of xylose. One unit (1 U) of xylanase activity was defined as the amount of enzyme that

released 1 μmol of reducing sugars (xylose equivalent) per minute [39], and specific activity was defined as units per milligram protein.

Kinetic parameters and substrate specificity

Arabinoxylan, beechwood xylan, birchwood xylan, and oat spelt xylan were prepared in eight different concentrations (0.4, 1, 2, 4, 10, 16, 20, 50 mg mL^{-1}) and incubated with the recombinant XynT at pH 7 and 50 °C for 10 min. The concentrations of reducing sugars released from each substrate were measured as described above. The V_{max} (absolute maximum reaction rate) and K_m (substrate concentration required to achieve ½ V_{max}) values were calculated according to Lineweaver–Burk equation [41].

Substrate specificity of XynT depending on protein amount (1, 3, 5, 10, and 15 μg) was determined by measuring enzyme activity on various substrates. Enzyme solution diluted in 0.7 mL phosphate buffer (pH 7.0) was added to 0.3 mL each of 1 % avicel, beechwood xylan, birchwood xylan, arabinoxylan, and oat spelt xylan in the same buffer. The reactions were carried out at 50 °C for 10 min.

Binding assay

To determine the binding assay of XynT, insoluble substrates were prepared from 2 % avicel, beechwood xylan, birchwood xylan, arabinoxylan, and oat spelt xylan by three sequential washings and centrifugation. The reactions were carried out in 1 mL of phosphate buffer (pH 7.0) containing 50 μg recombinant XynT and one of the insoluble substrates, at 4 °C for 4 h. After centrifugation at 13,000 rpm for 10 min, each pellet was washed three times with phosphate buffer (pH 7.0). The samples collected by centrifugation were suspended in phosphate buffer (pH 7.0), 10 % sodium dodecyl sulfate (SDS), then centrifuged again; each supernatant was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and the protein was transferred onto PVDF membrane (ImmobilonP; Millipore, Billerica, MA) in 39 mM glycine, 48 mM Tris, 10 % SDS, and 20 % methanol. The membrane was blocked with skim milk solution. The immunoblotting analysis was carried out with a penta-histidine antibody (Qiagen Cat. no. 34660) and alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G antibody as the primary and secondary antibody, respectively. The intensity of each stained band was analyzed with UTHSCSA Image Tool software (ver. 2.00; The University of Texas Health Science Center, San Antonio, TX).

Activity patterns of XynT toward xylooligomers

To analyze the mode of action of XynT, we added 120 U of the enzyme to 0.5 mL phosphate buffer (pH 7) or citrate buffer (pH 5) containing either 0.2 mg mL^{-1} of xylotriose, 0.4 mg mL^{-1} of xylotetraose, 1 mg mL^{-1} of xylopentaose, 1 mg mL^{-1} of xylohexaose, or 0.5 % beechwood xylan. The reactions were carried out at 50 °C for 1, 5, 10, or 30 min. The products liberated from substrates were analyzed by high-performance liquid chromatography (HPLC) with refractive index detection (Waters, Milford, MA). After the product solutions had been filtered, they were loaded onto a Rezex RPM column (4.6 × 300 mm; Phenomenex, Torrance, CA) and eluted with deionized water at a flow rate of 0.6 mL min^{-1}.

Scanning electron microscopy

Each sediment of birchwood xylan hydrolyzed by XynT was fixed with a chemical fixative (2 % glutaraldehyde and 2 % paraformaldehyde solution in 0.05 M cacodylate buffer, pH 7.2). After dehydration through an ethanol series, the samples were dried in a freezing dryer, coated with gold, and observed in a scanning electron microscope (SEM; model S2400; Hitachi, Tokyo, Japan) operated at 15 kV.

Biobleaching effect of XynT toward softwood

Unbleached *Pinus densiflora* kraft pulp (0.2 g) was incubated in 10 mL phosphate buffer (pH 7.0) with 200 U XynT at 37 °C for 4 h. The control sample was treated under the same conditions without the enzyme. The bleached control and enzyme-treated pulp samples were filtered through a 0.2-μm micron membrane filter (Cat. no. C020A025A; ADVANTEC MFS, Dublin, Ireland), washed three times with phosphate buffer (pH 7.0), dried in an oven, and its brightness measured by a brightimeter (model S4-M Brightness tester; Technidyne, New Albany, IN).

Effect of metal ions on the xylanase activity

The effect of metal ions and chemical reagents on the activity of recombinant XynT was investigated in the presence of 10 mM $HgCl_2$, $CuSO_4$, $ZnCl_2$, $FeCl_2$, $CaCl_2$, NaCl, $MgCl_2$, KCl, $CoCl_2$, $MnSO_4$, $NiSO_4$, urea, Tween-80, Tween-100, and glycerol. The activities were measured with birchwood xylan at pH 5 and 50 °C for 10 min. Control activity assayed in the absence of the metal ions or reagents was taken as 100 %.

Results

Amino acid sequence of XynT

A comparison of the amino acid sequence of XynT with family 10 alkalophilic xylanases (Xyn10A of *Bacillus*

halodurans and xylanase of *Bacillus* sp.) showed 41 and 48 % identity, respectively. The three inserted amino acid stretches that are unique to alkalophilic xylanases were also present [25], but two inserted stretches of >10 amino acid residues in length (residues 250–268 and 304–314) and one shorter region (residues 99–103) were present in the XynT sequence. Three inserted stretches are less conserved in the sequence of xylanases from *Bacillus* sp. and *B. halodurans*, but the percentage of acidic amino acids (Asp and Glu) in the stretch located between β7b and α7 of XynT is 26 %, which is a relatively higher ratio than those (15 %) of alkalophilic xylanases from *Bacillus* sp. and *B. halodurans* (Fig. 1). Aromatic residues (W88, Y182, W294, and W302) interacting with the substrate and two catalytic residues, one acting as an acid–base catalyst (E137) and the other as a nucleophile (E244) in the active site of XynT, were highly conserved [13, 31]. The catalytic domain of XynT comprised a $(\beta/\alpha)_8$-barrel structure which is typical of GH family 10 [10, 13]. The critical amino acids (inverted open triangles in Fig. 1) between β1 and α1, β2 and α2a, β3 and α3a, and β8 and α8a in the catalytic cleft of XynT formed subsites −1, −2, and −3, and those (inverted open triangles in Fig. 1) between β5 and α5 and β6a and α6 formed subsites +1, +2, +3, +4 [13, 30]. The structure of XynT was analyzed by Pymol™ v0.99 (Delano Scientific LLC, San Carlos, CA) and Scratch Protein Predictor (http://scratch.proteomics.ics.uci.edu/).

Purification and enzyme assay

For the production of recombinant XynT in *E. coli*, the transformed cells were cultured in 500 mL liquid media and enzyme expression was induced by the addition of lactose. SDS-PAGE analysis of the purified recombinant XynT revealed only one protein band with a molecular weight of 37 kDa (Fig. 2). An average yield of protein (calculated by five sequential purifications) was 7.2 mg mL^{-1} (equivalent to 180,000 μmol min^{-1} mL^{-1}), and its specific activity was 25,000 μmol min^{-1} mg^{-1} on birchwood xylan. The enzyme was active at pH 5–10 and 25–80 °C, and optimally active at pH 7 and 50 °C. The enzyme exhibited high stability at pH 7 and retained 69–85 % of its original activity at pH 9 (Fig. 2).

Kinetic parameters and substrate specificity

The hydrolytic activity of the recombinant XynT on various substrates was determined (Table 1). The enzyme was observed to have a higher activity on birchwood xylan and a lower activity on oat spelt xylan and wheat arabinoxylan. The activity of XynT on birchwood xylan increased remarkably at enzyme concentrations ranging between 5 and 15 μg protein when compared to hydrolytic activity on the others substrates (Fig. 3a). The binding affinity of XynT was 1.46- and 2.29-fold higher on insoluble birchwood xylan than on insoluble beechwood xylan and oat spelt xylan (Fig. 3b). These results are equivalent to the kinetic parameters of XynT on birchwood [K_m 4.5 mg mL^{-1}, k_{cat} (turnover number) 2,254.6 s^{-1}] and beechwood xylan (K_m 4.9 mg mL^{-1}, k_{cat} 2,344.7 s^{-1}). Interestingly, the enzyme interacted with crystalline cellulosic substrate (avicel), but no activity was observed (Fig. 3).

SEM imaging and biobleaching

The surface of insoluble birchwood xylan appeared to be greatly modified after treatment with XynT, judging by the highly roughened surface and increased porosity, with a larger number of large holes present. These observations indicated that XynT, although it does not contain carbohydrate-binding modules, has the ability to efficiently degrade insoluble substrates (Fig. 4a).

Compared to the control pulp, the brightness of XynT-bleached pine tree pulp was increased by up to 7.3 % ISO (Fig. 4B).

Mode of action on xylooligomers

The HPLC analysis was carried out to determine the action mode of XynT on various xylooligomers. Xylopentaose (1 mg mL^{-1}) was mainly hydrolyzed into xylobiose and xylotriose, and infrequently xylotetraose was shown as an intermediate product during the reaction. The oligomer xylohexaose (1 mg mL^{-1}) was cleaved into xylotetraose, xylotriose, and xylobiose as intermediate products at the initiation time. According to these results, the enzyme appeared to have a preference for cleavage sites at the second and third glycosidic bonds from the reducing end of xylopentaose and xylohexaose (Fig. 5). Alkalophilic XynT had a different specificity on xylooligomers at pH 5. Xylotetraose, xylopentaose, and xylohexaose were hydrolyzed into xylotriose and -biose as the main products at pH 5 (Fig. 6). However, XynT had even a lower activity on xylotriose, which is a main intermediate product during hydrolysis reaction of the xylanase on xylan substrates, as well as a lower affinity to the xylooligomer (8.621 min peak) released from beechwood xylan at pH 5. The different specificities toward the different xylooligomers may explain, at least partially, why XynT showed a lower activity under acidic conditions compared to its activity under optimal conditions.

Effect of metal ions and detergents on XynT activity

The activities of recombinant XynT in the presence of different metal ions and weak detergents are shown in

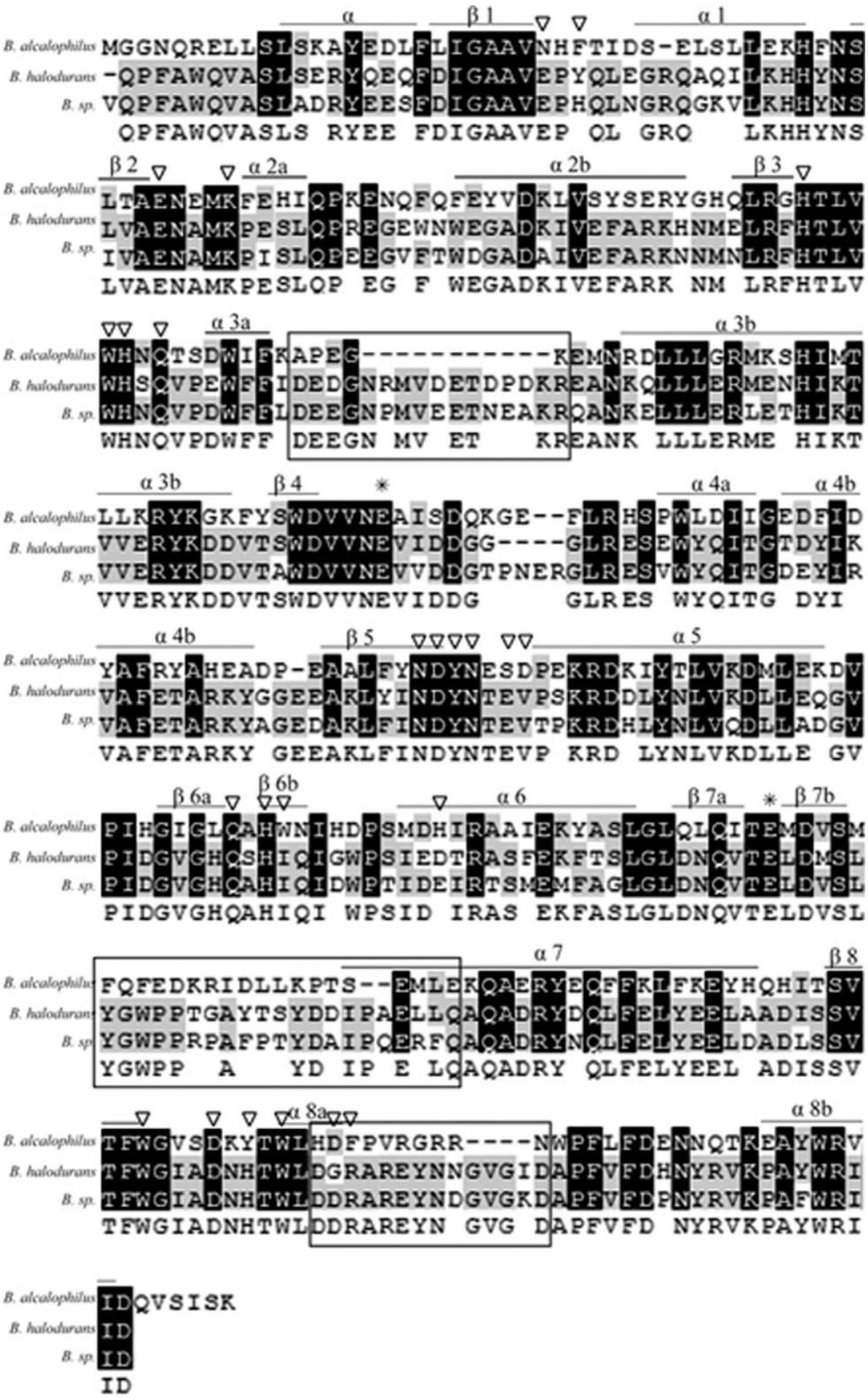

Fig. 1 Comparison of the amino acid sequence of endo-β-1,4-xylanase (XynT) from *Bacillis alcalophilus* AX2000 with alkalophilic xylanases from *B. halodurans* (GenBank accession no. AF534180) and *Bacillus* sp. Ng-27 (GenBank accession no. 2FGL_A). *Inverted open triangles* Critical amino acids comprising subsites of the XynT active site, *asterisks* key amino acids (E137 and E244) of the acid–base residue and nucleophile, respectively, *horizontal line* α-helix and β-sheet, *boxes* amino acid stretches unique to alkalophilic xylanase

Table 2. XynT activity was enhanced by 39 and 50 % in the presence of 10 mM Co^{2+} and Mn^{2+}, respectively. Activity of XynT was strongly inhibited by the addition of 10 mM Hg^{2+} and Ni^{2+} ions.

Discussion

The endo 1-4 xylanase gene (*xynT*) from *B. alcalophilus* AX2000 that produces an xylan-degrading enzyme (XynT)

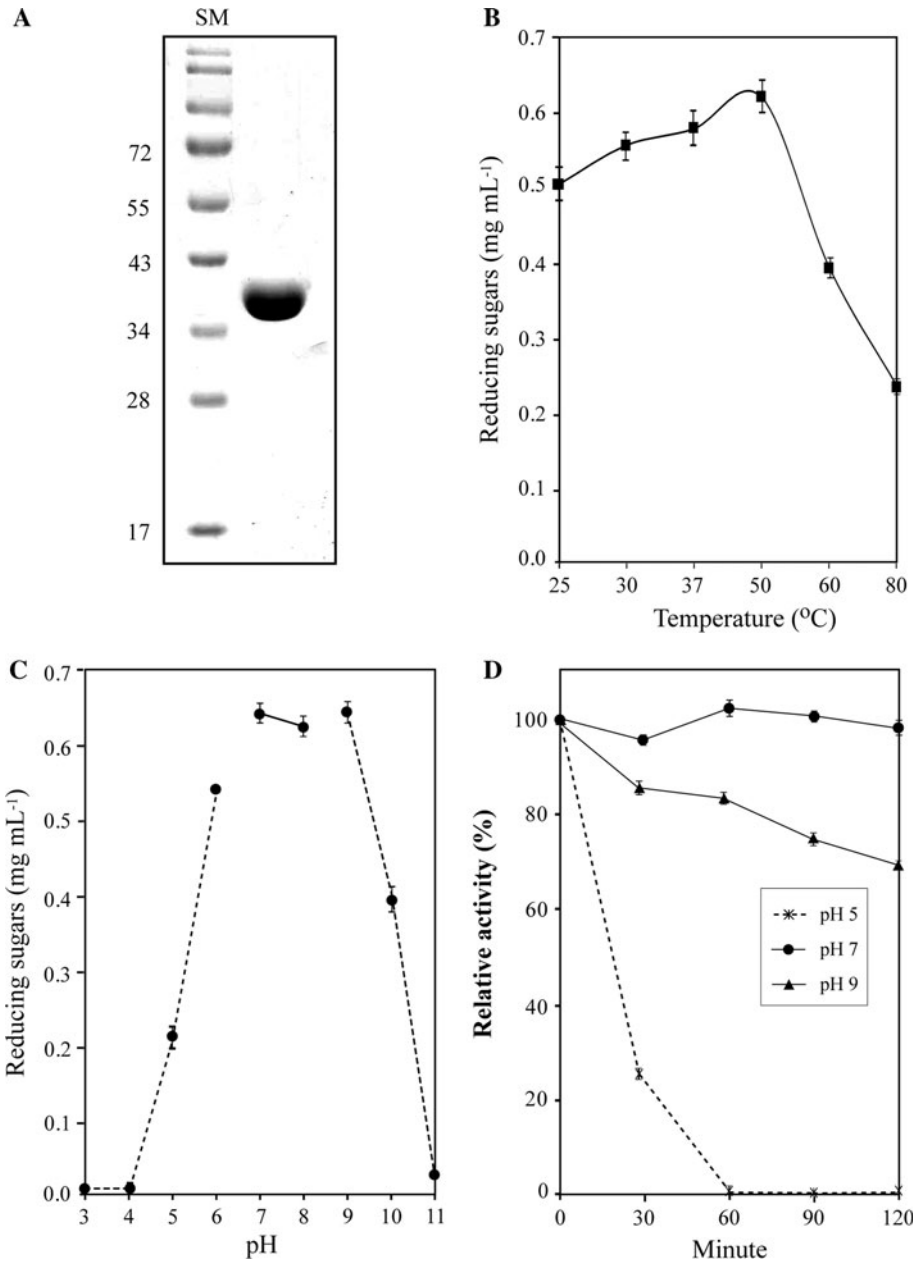

Fig. 2 Effects of pH and temperature on the activity of the recombinant XynT. **a** The purified enzyme was separated by 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis, revealing one protein band with a molecular weight of 37 kDa. **b**, **c** Effect of temperature and pH on XynT activity. **d** pH stability of XynT was determined by preincubating the enzyme at different pH (pH 5, 7, and 9) without substrate before measuring its activity at pH 7 and 50 °C for 10 min. In the reactions, 1 % birchwood xylan was used as substrate. Enzyme activity under optimal conditions prior to preincubation was taken as 100 %

Table 1 Kinetic parameters of endo-β-1,4-xylanase toward each substrate

Substrate	K_m (mg mL^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mg^{-1} mL)
Wheat arabinoxylan	23.7	315.2	13.3
Beechwood xylan	4.9	2,344.7	479.5
Birchwood xylan	4.5	2,254.6	504.4
Oat spelt xylan	16.4	1,235.6	75.4

K_m, Absolute maximum reaction rate; k_{cat} turnover number [direct measure of the catalytic production of product under optimum conditions (saturated enzyme)]

with a high activity at pH 8–11 was isolated. The recombinant enzyme was highly expressed in *E. coli* in soluble form. The expression level and specific activity of the purified enzyme were equivalent to 180,000 µmol min^{-1} mL^{-1} and 25,000 µmol min^{-1} mg^{-1}, respectively. The activity of XynT was even higher than that reported for Xyn2 (*T. reesei* Rut C-30) expressed in *E. coli* and *Pichia pastoris* [15] and for xylanases from *Aspergillus sulphureus* [5], *Thermomyces lanuginosus* [9], *Aspergillus niger* [22], and *Bacillus licheniformis* [23] expressed in *Pichia pastoris* and xylanase Umxyn10A [26] expressed in the *E. coli* BL21strain.

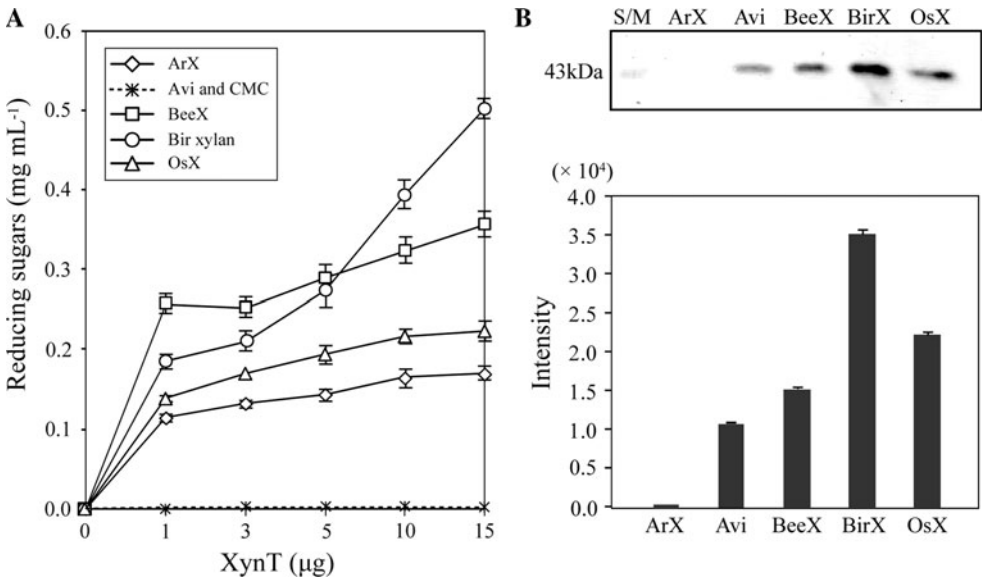

Fig. 3 Activity of XynT depending on enzyme quantity (**a**) and binding ability (**b**) of XynT toward insoluble substrates. *ArX* Wheat arabinoxylan, *Avi* avicel, *BeeX* beechwood xylan, *BirX* birchwood xylan, *OsX* oat spelt xylan

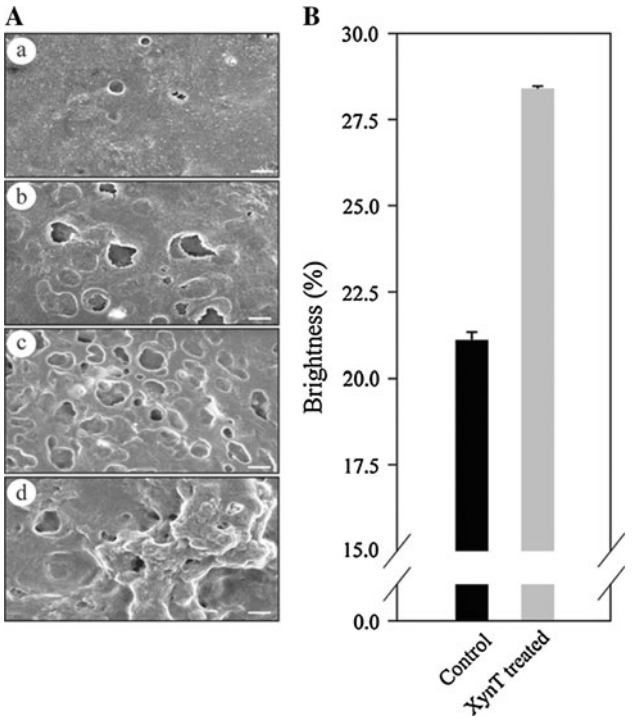

Fig. 4 Scanning electron microscopy photomicrographs (**a**) and bleaching effect (**b**) of XynT. **a** Results of birchwood xylan hydrolysis were determined at the following time points: *a* 0 min, *b* 10 min, *c* 30 min, *d* 60 min. *Bar* 1 µm. **b** Bleaching effect of XynT toward *Pinus densiflora* kraft pulp. Brightness increased by up to 7.3 % ISO in relation to the control pulp. The control was incubated without the enzyme

The kinetic parameters of XynT on diverse substrates indicate that *B. alcalophilus* XynT had a higher activity on beechwood and birchwood xylan than on oat spelt xylan and wheat arabinoxylan, likely due to oat and wheat xylan containing 10 and 40 %, respectively, more side chains substituted by arabinose. This corresponds to the binding affinity and enzyme dosage results. The lower activity of XynT toward xylan containing side chains indicates that debranching enzymes, such as α-L-arabinofuranosidase and feruloyl esterase, are required to enhance the accessibility and hydrolysis of heterogeneous xylan [20, 34, 38].

An analysis of the amino acid sequence and three-dimensional structure indicates that XynT has a typical GH family 10 structure. GH family 10 enzymes have a $(\beta/\alpha)_8$-barrel and highly conserved amino acid residues that are involved in the formation of an acid–base catalyst (E137) and nucleophile (E244) and substrate binding interactions (W88, Y182, W294, and W302); subsites form at their catalytic clefts [8, 13]. Schmidt et al. [33] and Charnock et al. [6] reported on the patterns of interactions of xylanase subsites with xylooligomers, and on the mode of action of XYLA from *Pseudomonas fluorescens* and Cex from *Cellulomonas fimi* belonging to GH family 10 toward each xylooligomer, respectively. Our data suggest that the action mode of XynT toward xylotriose and xylohexaose is similar to that of XYLA from *P. fluorescens* and that the action mode of XynT toward xylotetraose is similar to that of Cex from *C. fimi*. However, we found that XynT has a unique mode of cleavage toward xylopentaose, producing intermediate products such as xylobiose, xylotriose, and xylotetraose. Protein stability, solubility, recognition, and catalysis are critically affected by electrostatic interactions, and many important conformational transitions in proteins are induced physiologically by changes in bulk ion composition and pH [3]. The kinetic parameters of Cex, a family 10 xylanase from *C. fimi*, were determined at

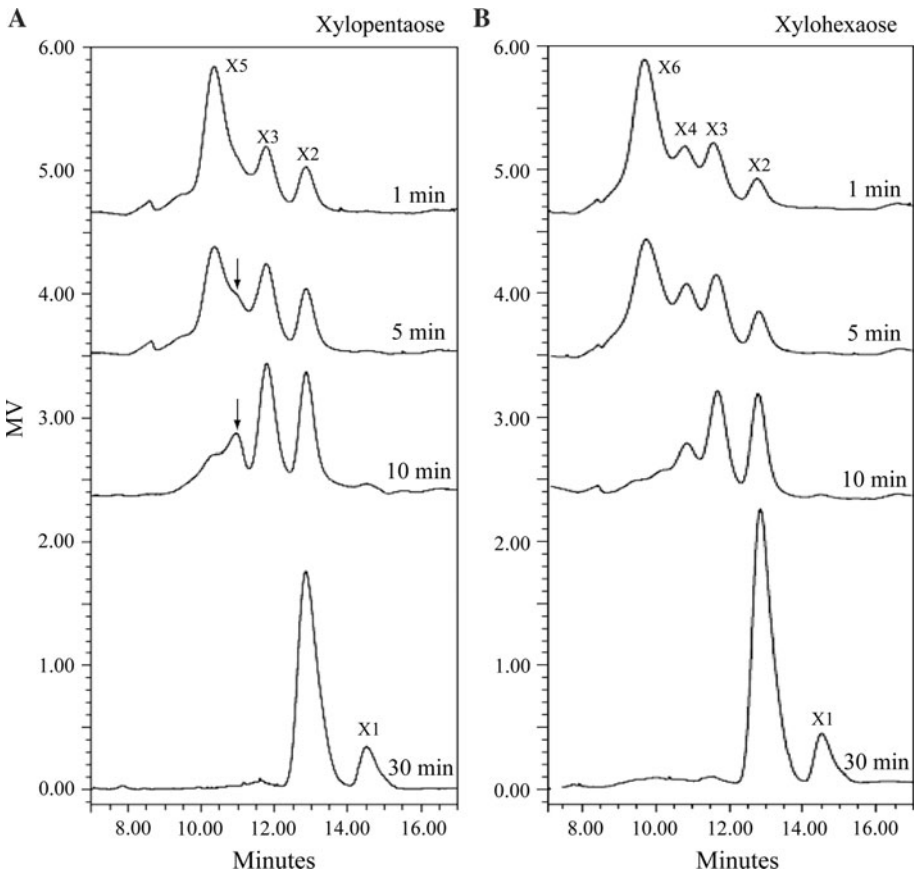

Fig. 5 Mode of action of XynT toward xylopentaose and xylohexaose. **a**, **b** 120 U of the enzyme was added to 0.5 mL phosphate buffer (pH 7) containing either 1 mg mL^{-1} xylopentaose or 1 mg mL^{-1} xylohexaose. The reactions were carried out at 50 °C for 1, 5, 10, and 30 min. *Arrow* Xylotetraose intermediate. *X1* Xylose, *X2* xylobiose, *X3* xylotriose, *X4* xylotetraose, *X5* xylopentaose, *X6* xylohexaose

various pH values with soluble birchwood xylan and three different synthetic substrates [16]. The chimeric enzyme Stx15, mutated by error-prone PCR, had a reduced K_m value, and specific activity was increased at alkaline pH ranges [35]. In addition, XynT was active toward xylotetraose as well as xylopentaose and xylohexaose (hydrolyzed into xylotriose and -biose) at pH 5, but it had a much lower activity on xylotriose at the same pH condition. Based on these results we suggest that xylanase substrate specificity was affected by changes in the critical amino acid residues in the active site of the enzyme under acidic conditions. Joshi et al. [17] reported that a single amino acid residue substitution (N35D) in the active site of xylanase from *Bacillus circulans* shifted its optimum pH from 5.7 to 4.6, with an approximate 20 % increase in activity. In addition, conformational changes, such as flipping of the aromatic ring of Tyr80 by 180° and the moving away of Asn35 from the protonated Glu172 in the active site of *B. circulans* xylanases have been observed when the enzyme was exposed to acidic conditions (pH 4) [3]. These results indicate that the amino acid residues near the catalytic residues in the active site play a critical role in the effect of pH on activity [24]. Analysis of the amino acid sequence and three-dimensional structure of XynT showed that the critical amino acids at subsite −1 (Asn138, Asp133, and Trp88) and subsite +1 (Asp181, Tyr182, and Trp302) were located near catalytic residues (E137 and E244) of the active site of XynT. According to previous studies [3, 17], XynT weakly interacts with xylotriose and the soluble form of xylooligomer released from beechwood xylan through conformational changes of amino acid residues in the active site under weakly acidic conditions, resulting in lower activity and altered kinetic parameters. Further systematic evaluations are required to confirm the pH–substrate specificity of XynT at various pH conditions.

In terms of the effect of ions on xylanase activity, it has been reported that Mg^{2+} induces structural stability of *Bacillus* sp. BSX xylanase and increases its activity [25], while Mn^{2+} increases the activity of *Aspergillus awamori* PXII-1 xylanase [39] and *Nesterenkonia xinjiangensis* Xyn11NX xylanase [18]. The activity of XynT was stimulated by as much as 39–50 % in the presence of Mn^{2+} or Co^{2+}. However, Hg^{2+} and Ni^{2+} strongly inhibited XynT activity due to oxidization of the indole ring in four tryptophan residues (W88, W216, W294, and W302) or interaction with the imidazol group of four histidine (H84, H89, H215, and H225) residues consisting of subsites in the active site of XynT [18].

Biobleaching of pulp with xylanase has been reported to effectively increase the brightness and reduce the kappa

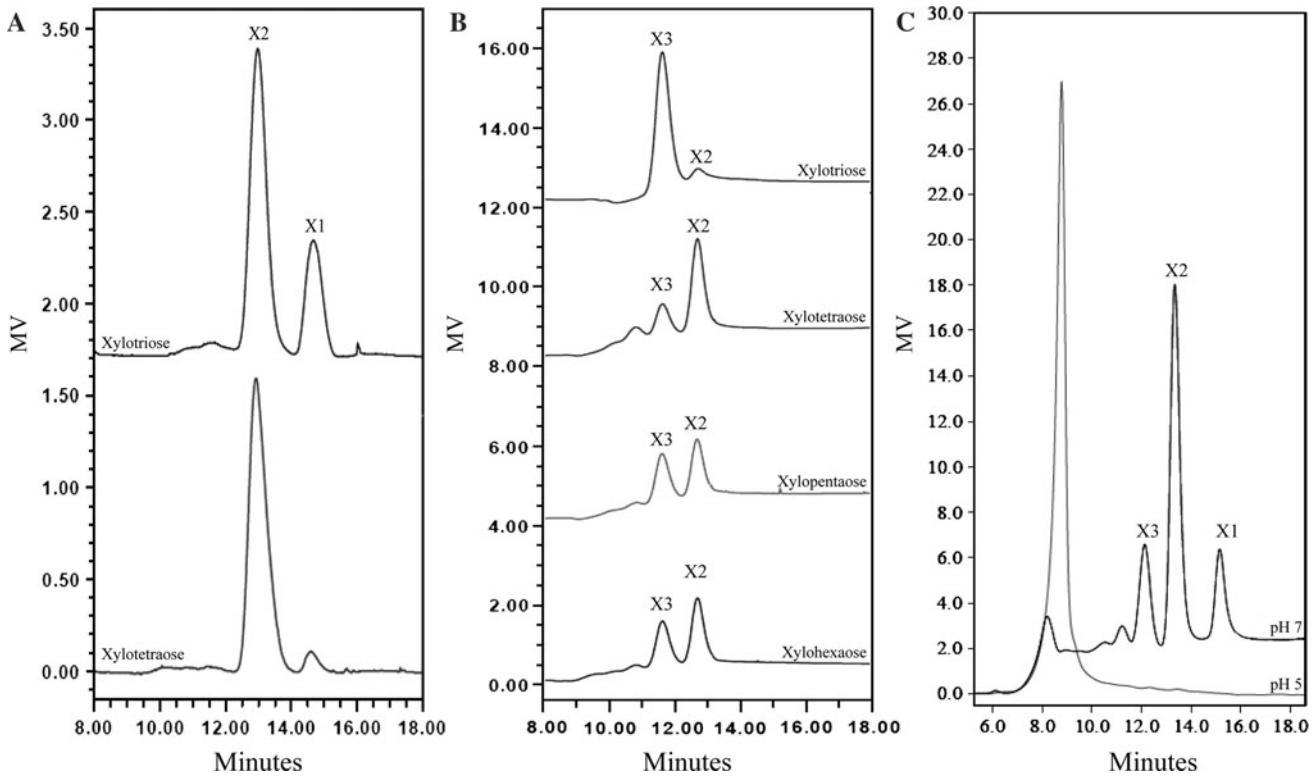

Fig. 6 Effect of pH on substrate-specific XynT activity. **a** Cleavage patterns of the enzyme toward xylotriose (0.2 mg mL^{-1}) and xylotetraose (0.4 mg mL^{-1}) at pH 7. **b** Cleavage patterns of the enzyme towards xylotriose (0.2 mg mL^{-1}), xylotetraose (0.4 mg mL^{-1}), xylopentaose (1 mg mL^{-1}), and xylohexaose (1 mg mL^{-1}) at pH 5. **c** HPLC profiles of beechwood xylan hydrolyzed by the enzyme at pH 5 and pH 7. The 8.621 min oligosaccharide peak is estimated as longer xylooligomer than xylohexaose

Table 2 Effect of metal ions and detergents on endo-β-1,4-xylanase activity

Reagent (10 mM)	Activity ratio (%)	Reagent (10 mM)	Activity ratio (%)
Control	100.00	KCl	99.87 ± 0.54
$HgCl_2$	0.00	$CoCl_2$	139.2 ± 10.73
$CuSO_4$	73.67 ± 2.54	$NiSO_4$	0.00
$ZnCl_2$	105.45 ± 2.10	$MnSO_4$	149.6 ± 3.15
$FeCl_2$	102.13 ± 1.03	Urea	101.86 ± 0.57
$CaCl_2$	101.06 ± 0.11	Tween-80[a]	84.31 ± 2.35
NaCl	99.07 ± 1.84	Tween-100[a]	94.02 ± 2.84
$MgCl_2$	96.28 ± 1.95	Glycerol[a]	83.51 ± 3.90

[a] 0.2 % (v/v) final concentration

number of wheat straw pulp [21], bagasse pulp [4], eucalypt kraft pulp [40], and softwood kraft pulps [1]. In this study, the brightness of pine tree pulp bleached with XynT showed a significant improvement—up to 7.3 % ISO—in relation to the control pulp. XynT exhibited a high binding affinity and hydrolytic activity toward soluble and insoluble xylans, resulting in a pronounced improvement in brightness.

Conclusions

The high cost associated with large-scale enzyme production remains the principle hurdle to the industrial application of xylanase. Even though xylanases of fungal or bacterial origin possess high activity and thermostability, they are expressed at relatively low levels in yeasts or *E. coli* due to depletion of the supply of specific tRNAs through the repetitive use of degenerate codons and the presence of an unfavorable N-terminal coding sequence [19]. XynT from *B. alcalophilus* AX2000 exhibited high activity over a broad range of pH, a high yield in an *E. coli* expression system, and high hydrolysis activity on insoluble substrates. These properties are advantageous for the biobleaching application in the pulping industry. XynT also exhibited pH-dependent substrate-affinity for xylotriose at pH 5, which explains its lower activity under weakly acidic conditions. Overall, the properties of XynT make it an ideal candidate for mutagenesis strategy aimed at enhancing enzyme activity in suboptimal pH ranges, with the ultimate aim of preparing an enzyme cocktail able to efficiently hydrolyze lignocellulosic materials.

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