Characterization of a Salt-Tolerant and Cold-Adapted Xylanase from *Bacillus cellulosilyticus*

Jianzhong Sun, Sailing Shi, Jian Wu,* Rongrong Xie, Alei Geng, and Dao Chen Zhu

A xylanase (Xyn10A) gene from the saline-alkali-tolerant microorganism *Bacillus cellulosilyticus* DSM 2522 was cloned and expressed in *Escherichia coli* BL21 (DE3). The open reading frame was composed of 1008 base pairs, and it encoded 335 amino acid residues belonging to glycosyl hydrolase family 10. The optimal temperature and pH of the purified Xyn10A were 40 °C and 8.0, respectively. The Xyn10A was sensitive to heat and showed obvious cold-adapted activity, retaining 38.3%, 55.7%, and 82.9% of the optimal activity at 4, 20, and 30 °C, respectively. Xyn10A also showed a high level of NaCl tolerance. The highest activity was observed with 1.5 M NaCl. The specific enzyme activity of Xyn10A was as much as 163.8 U/mg. Kinetic assays showed that K_m, V_{max}, and K_{cat} were 2.56 mg/mL, 202.5 μM/min/mg, and 132.6 /s, respectively. Additionally, the main hydrolysis products using birchwood xylan as substrate were xylobiose, xylotriose, and xylotetraose, as determined by thin layer chromatography analysis. As a cold-adapted and salt-tolerant enzyme, Xyn10A is an ideal candidate for further research and biotechnological applications.

Keywords: Xylanases; Bacillus cellulosilyticus; Cold adapted; Salt tolerant

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INTRODUCTION

Endo-1,4-β-xylanases (EC3.2.1.8), which randomly hydrolyze the β-1,4-xylosidic bonds of xylan, have gained much attention in biotechnological fields because of their potential value. They have been used widely in the conversion of lignocelluloses into fermentable sugars and prebiotic oligosaccharides (Juturu and Wu 2011; Shi *et al.* 2013; Amel *et al*. 2016). They can also be used to improve the quality of breads and beverages, enhance the digestibility of animal feeds, and improve the bleaching and deinking of paper (Huang *et al*. 2006; Dhiman *et al*. 2008; Garg *et al*. 2009; Maity *et al.* 2012; Singh *et al*. 2012).

Many xylanases have been reported from various microbes, such as bacteria, fungi, and yeasts (Polizeli *et al*. 2005; Dhiman *et al*. 2008; Rao *et al.* 2008; Khandeparker *et al*. 2011; Tenkanen *et al*. 2013). Xylanases originating from the *Bacillus* strains are intrinsically interesting because of their characteristics, such as alkali and salt tolerance, cold adaptation, or thermostability; they are widely employed in industrial applications (Chang *et al.* 2004; Huang *et al*. 2006; Mamo *et al*. 2006; Ruller *et al*. 2008; Khandeparker *et al*. 2011; Haddar *et al*. 2012; Thomas *et al.* 2014).

The genome for *Bacillus cellulosilyticus,* a soil microorganism that is both alkaliphilic and halophilic, has recently been revealed (GenBank accession number: GCA_000177235.2) (Mead *et al.* 2013). *B. cellulosilyticus* possesses rich biomass-degrading enzymes besides those for crystalline cellulose. However, research on the

enzymes from *B. cellulosilyticus* is still limited. Among the enzymes researched, three predicted xylanases have been denoted as Bcell_0541, Bcell_0537, and Bcell_0547, respectively. Bcell_0541 was predicted to belong to the GH8 family. Bcell_0537 and Bcell_0547 were classified in the GH10 family, with the former composed of a catalytic domain and carbohydrate-binding module (CBM), and the latter lacking the CBM domain. In this study, Bcell_0547 was studied and designated as Xyn10A.

Salt-tolerant xylanases can be applied in marine products, wastewater processing, and bioethanol production from seaweeds (Bai *et al*. 2012; Liu *et al*. 2013, 2014). The cold-adapted enzymes are most active at low and medium temperatures, which has obvious advantages in the bioprocess, such as saving energy, reducing production costs, and improving food flavor (Cavicchioli *et al*. 2002; Vester *et al*. 2014).

However, in the past, cold-adapted and salt-tolerant xylanases have rarely been published. In this study, the Bcell_0547 gene was heterologously expressed and characterized. The Xyn10A displayed high salt tolerance and cold-adapted activity. Thus, Xyn10A has widespread application prospects in biotechnology fields and especially in the food industry.

EXPERIMENTAL

Materials

Birchwood xylan, Avicel, carboxymethylcellulose (CMC), and cellobiose were purchased from Sigma Chemical Company (St. Louis, USA). Xylo-oligosaccharides (XOs) were purchased from ADHOC International Technologies Co., Ltd. (Beijing, China). HisTrap HP and HiTrap Q HP columns were obtained from GE Life Sciences (Piscataway, NJ, USA). The primers were synthesized by Sangon (Shanghai, China), and the pEASY-E2 expression kit and the protein assay kit were provided by TransGen Biotech (Beijing, China). All other chemicals were of analytical grade commercially available unless otherwise specified.

The *B. cellulosilyticus* DSM 2522 was purchased from DSMZ (Braunschweig, Germany). The pEASY-E2 Expression Vector (Transgen Biotech, China) and *E. coli* BL21 (DE3) (Novagen, Darmstadt, Germany) were employed as the expression vector and host cells, respectively.

Methods

Strain culture

The *B. cellulosilyticus* DSM 2522 strain was maintained in ATCC medium 661 (alkaline *Bacillus* medium, peptone 10.0 g, yeast extract 5.0 g, glucose 10.0 g, K_2HPO_4 1.0 g, NaCl 5.0 g, agar 15.0 g, distilled water 900.0 mL, autoclaved at 115 °C for 15 min, cooled to 50 °C, and 100.0 mL of filter-sterilized 10% Na_2CO_3 solution added). *E. coli* BL21 (DE3) was grown at 37 °C in a Luria-Bertani medium (LB) and supplemented with ampicillin (100 μg/mL).

DNA manipulation

DNA was extracted from *B. cellulosilyticus* with the extraction kit (Takara, Dalian, China), and the Xyn10A gene was amplified by PCR using forward primer 5'-AAGCAAAAGCTAGAAGAAAC-3' and reverse primer 5'-AAAACGAGTAATG-TTCCA-3'. Direct ligations of the PCR products to the pEASY-E2 expression vector were

performed according to the manufacturer's instructions. The recombinant plasmid was named pEASY-E2-Xyn10A, and the sequence was confirmed by Sangon Biotechnology Inc. (Shanghai, China).

Expression and purification of the Xyn10A

Plasmid pEASY-E2-Xyn10A was transformed into *E. coli* BL21 (DE3) cells. The transformed strains were grown in an LB medium containing 100 μg/mL of ampicillin at 37 °C until the OD600 reached 0.8. Expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM and incubation at 25 °C for 12 h. The cells were harvested by centrifugation at 8,000 × g for 15 min at 4 °C, washed with sterile distilled H_2O, and resuspended in buffer A (20 mM sodium phosphate, 0.5 M NaCl, pH 7.4). The cells were disrupted by sonication on ice and centrifuged at 10,000 × g for 10 min at 4 °C. The supernatant was loaded onto a pre-equilibrated 5-mL Histrap HP Ni^{2+}-NTA affinity column for purification. The proteins were eluted by a buffer solution containing stepwise increases of imidazole (50, 100, 200, and 300 mM), desalted, and further purified by Histrap Q HP. The purified recombinant proteins were confirmed by sodium dodecyl sulfate-polycrymide gel electrophoresis (SDS-PAGE). Protein concentrations were determined by the BCA method using bovine serum albumin as the standard.

Determination of enzyme activities

To measure Xyn10A activity, the reaction mixture containing 450 μL of birchwood xylan (1%, w/v) in 50 mM sodium phosphate buffer (pH 8.0) and 50 μL of enzyme was incubated for 10 min at 40 °C. The reaction was stopped by the addition of 1.0 mL of 3,5-dinitrosalicylic acid (DNS), followed by boiling for 5 min. The amount of reducing sugar released was quantified by the 3,5-dinitrosalicylicacid (DNS) method using xylose as a standard. One unit (U) of enzyme activity was defined as the amount of enzyme required to release 1 μmol of reducing sugars per min under the assay conditions. Specific activities were expressed as units per mg of protein.

Enzyme characterization

To determine the optimal pH, the activity of Xyn10A was measured at 40 °C in the following buffers (50 mM) of different pH: citrate acetate buffer (pH 4.0 to 6.0), sodium phosphate buffer (pH 6.0 to 8.0), Tris-HCl buffer (pH 8.0 to 9.0), and NaOH-glycine buffer (pH 9.0 to 11.0). To determine the optimal temperature, the activity of Xyn10A was measured at different temperatures (4 to 60 °C) in the presence of 50 mM phosphate buffer (pH 8.0). The value obtained at the optimum temperature or pH was defined as 100%, and the results were expressed as relative activity.

The pH stability was determined by pre-incubating the enzyme at 30 °C for 1 h at various pH levels from 4.0 to 11.0. The residual activity was assayed by the standard assay methods. Thermal stability was measured by assaying the residual activity after incubation of the enzyme at 30, 40, and 50 °C for various time periods (0, 10, 20, 40, and 60 min) in 50 mM phosphate buffer (pH 8.0). The value from the sample without pre-incubation was defined as 100%.

Substrate specificities were studied by measuring enzyme activity using various polysaccharides as substrates in place of the xylan. The enzyme reactions were carried out in the 50 mM phosphate buffer (pH 8.0) at 40 °C.

The effects of metal ions and chemicals on Xyn10A activity were investigated. To study the effects, 1 mM metal salts (NaCl, KCl, $CaCl_2$, $FeCl_2$, $NiCl_2$, $CoCl_2$, $MnCl_2$, $CuCl_2$, $MgCl_2$, and $ZnCl_2$) and 5 mM chemicals (Na_2EDTA, SDS, and Tween-20) were added to the reaction mixture, respectively. The residual activities were measured at 40 °C in 50 mM phosphate buffer (pH 8.0), and the activity without the addition of metal ions and chemicals was set as 100%. To assess the effect of sodium chloride on xylanase activity, various concentrations of NaCl (0.5 to 3.0 M) were added to the reaction mixture. Then, the activity was measured under standard assay conditions, and the residual activity (%) was calculated. The enzyme activity without the addition of NaCl was set as 100%.

To estimate the kinetic parameters, the enzyme reaction was carried out at 40 °C for 10 min using different substrate concentrations (0% to 1.0% birchwood xylan) in 50 mM phosphate buffer (pH 8.0). The Michaelis-Menten constant (K_m) and maximal reaction velocity (V_{max}) of Xyn10A were determined using the Lineweaver–Burk plots.

Hydrolysis products analysis by thin layer chromatography (TLC)

A 500-μL reaction solution containing 50 μL of Xyn10A in 50 mM phosphate buffer (pH 8.0) and 450 μL of birchwood xylan (1%) was incubated at 30 °C for 90 min. After incubation, the solution was boiled for 5 min and then centrifuged at 10,000 × g for 10 min. The supernatant was filtrated through a 0.22-μm cellulose acetate membrane. Analysis of the hydrolytic products (2 μL) was performed on a silica gel-coated glass plate (50 mm × 100 mm) with a mixture consisting of xylose, xylobiose, xylotriose, and xylotetraose as standards. A solvent system composed of n-butanol/acetic acid/H_2O (10: 5: 1, v/v/v) was used. After the plate was dried at room temperature, the TLC plate was sprayed with a mixture of diphenylamine/aniline, phosphoric acid/acetone reagents (2 g: 2 mL: 10 mL: 100 mL) and then heated at 85 °C for 10 min for coloration.

Sequence and data statistics

The open reading frame (ORF) was obtained from KEGG (http://www.genome.jp/kegg). The signal peptide was predicted using the Signal P 4.0 server (http://www.cbs.dtu.dk/services/SignaIP). The amino acid sequence alignment was performed using the DNAMAN program or by using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The theoretical molecular weight and isoelectric point were predicted by online software (http://web.expasy.org/compute_pi/). All of the values shown in the figures were averaged from three replicates. The figures were created with origin 8.0 software.

RESULTS AND DISCUSSION

Expression and Purification of the Recombinant Xyn10A

One of the predicted xylanases (Bcell_0547, Xyn10A) belonging to the GH10 family from *B. cellulosilyticus* was examined in this study. The ORF of the Xyn10A gene consisted of 1008 base pairs, and it encoded a protein consisting of 335 amino acid residues with a theoretical molecular weight of 39.3 kDa. There was no obvious signal peptide sequence found in Xyn10A using SignalP 4.0 analysis. In addition, the multiple sequence alignment (Fig. 1) revealed two highly conserved residues, Glu48 and Glu138, which are considered to be crucial for the catalytic activity of family 10 glycosyl hydrolases. Xyn10A showed the highest sequence similarity (67%) to the xylanase from *Bacillus akibai*

(Accession number WP_035668525.1) and the highest identity (61%) to the xylanase from *Bacillus* sp. SN5, among the characterized xylanases (Bai *et al*. 2012).

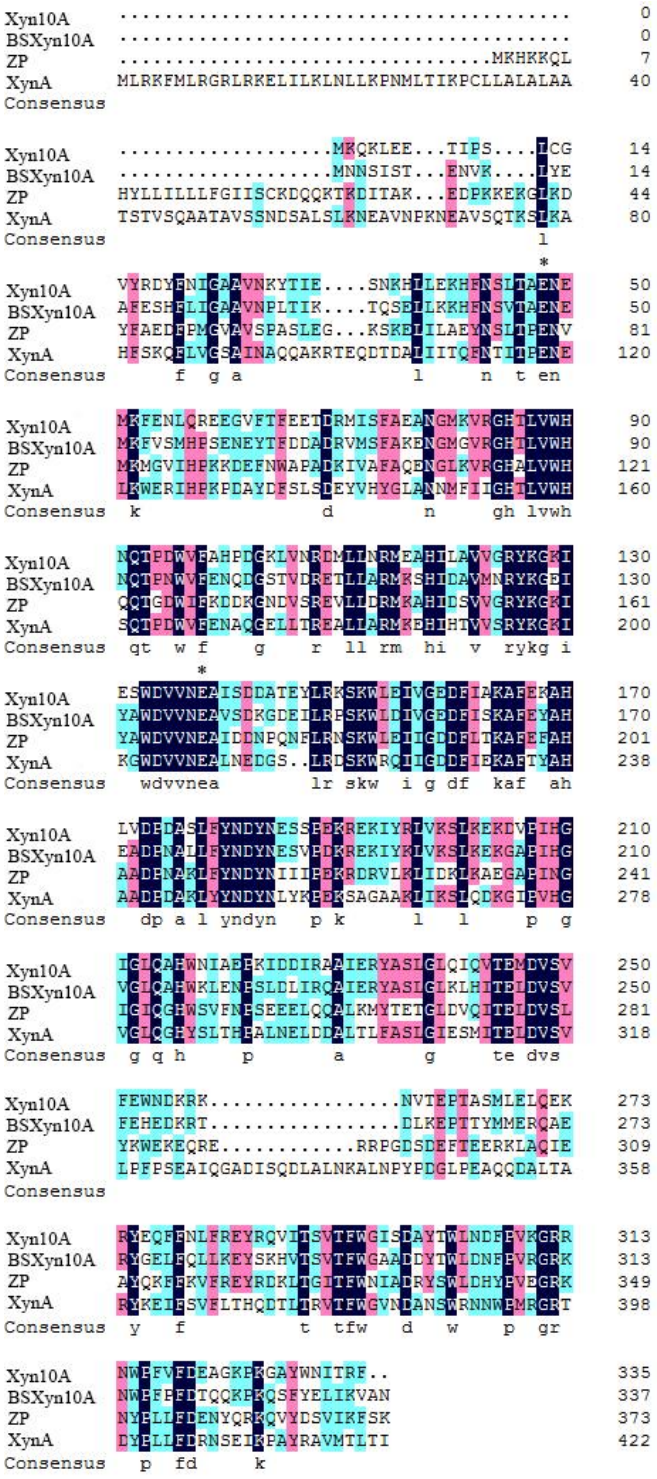

Fig. 1. Sequence alignment of Xyn10A to other cold-adapted xylanases belonging to GH10. Sequence alignment was performed using DNAMAN8.0. Stars above residues indicate the conserved catalytic amino acids. Identical residues are shaded. Xyn10A, in this study (CP002394); BS Xyn10A, from *Bacillus sp.* SN5 (AGA16736); XynA, from G. *mesophila* KMM241 (FJ715293); ZP: from *Z. profunda* (WP_013072455).

The Xyn10A gene was cloned from the genomic DNA of *B. cellulosilyticus.* The amplified gene was transferred into the pEASY-E2 Expression Vector using the pEASY-E2 expression kit. The Xyn10A gene was successfully expressed in *E. coli* BL21 (DE3) as the his_6 tagged fusion protein. The recombinant Xyn10A protein was purified to homogeneity by Ni-NTA affinity and anion exchange chromatography, and the single bands were exhibited by SDS-PAGE analysis (Fig. 2). It showed a molecular weight of about 39 kDa, which was consistent with the theoretical molecular weight.

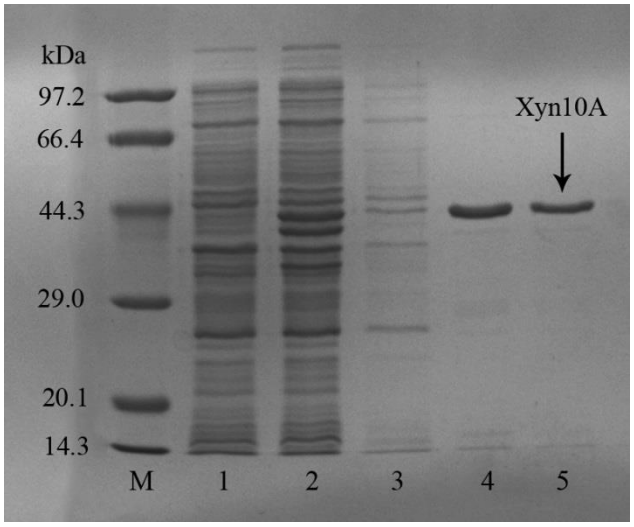

Fig. 2. SDS–PAGE analysis of purified recombinant Xyn10A. Lanes: M, protein markers; 1, total protein from *E. coli* without IPTG induction; 2, total protein from *E. coli* by induction with 0.5 mM IPTG for 12 h; 3, the supernatant from *E. coli* after cell disruption; 4, purified protein after Ni-affinity; 5, purified protein after anion exchange Q column

The Properties of Xyn10A

Effect of pH and temperature on activity

The results showed the highest enzyme activity of Xyn10A at a pH of 8.0. Almost no activity was found at pH 4.0 and 10.6. However, the enzyme retained more than 50.0% of the relative activity in the pH range from 6.0 to 9.0 (Fig. 3a). When Xyn10A was pre-incubated for 1 h at 30 °C with pH values ranging from 4.0 to 11.0, it showed good stability, retaining at least 90.0% of the original activity in the pH range from 6.0 to 9.0. When the pH value was less than 5.0 or above 9.0, however, the pH stability decreased sharply (Fig. 3b). These results indicated that Xyn10A was stable in moderate and slightly alkaline conditions. This was similar to the xylanases from *Bacillus mojavensis* A21 (Haddar *et al.* 2012), as its best activity was also found at pH 8.0. Moreover, several alkaliphilic xylanases from the same genus (*Bacillus*) showed an optimum activity at pH 9.0 to 10.0, such as *B. pumilus* SV-85S (Nagar *et al*. 2010), and *B. halodurans* MTCC 9512 and S7 (Mamo *et al*. 2006; Mamo *et al*. 2009; Garg *et al*. 2009). These xylanases retained more than 50% activity even at pH 12.0 after 1 h incubation. The xylanases from *Bacillus* sp. SN5(Bai *et al*. 2012) showed the highest similarity (61%) with Xyn10A among characterized xylanases. Its optimum pH was 7.0 and not especially alkaline, though *Bacillus* sp. SN5 (Bai *et al*. 2012) was the alkaliphilic strain. Compared with the other reported cold-adapted xylanases, only Xyn10A displayed the optimum activity at alkaline conditions.

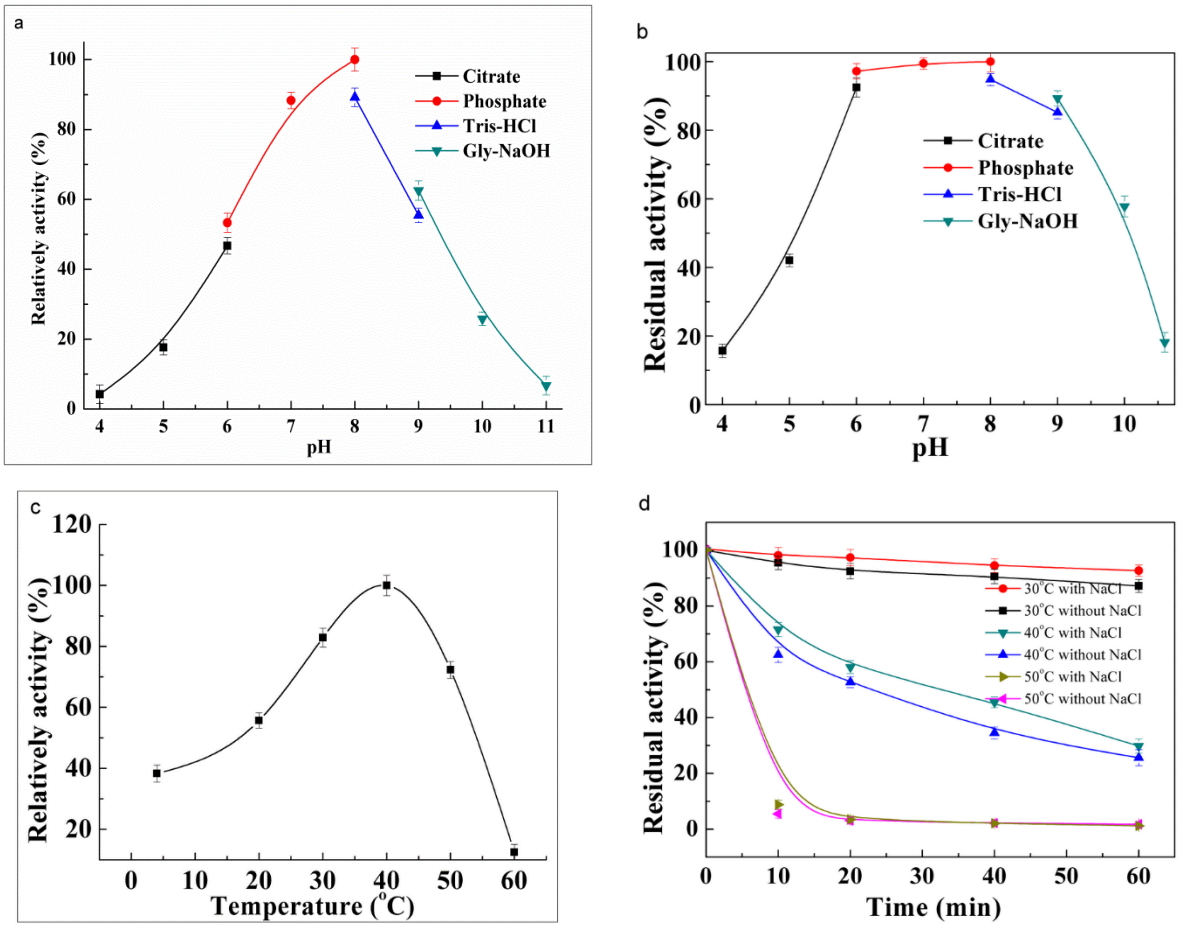

Fig. 3. Optimal pH and temperature and their stabilities for Xyn10A. a, Effect of pH on Xyn10A activity. Activities at various pHs were assayed at 40 °C; b, pH stability of Xyn10A. Residual activities were assayed at pH 8.0, 40 °C after incubation in buffers of different pH at 30 °C for 1 h; c, Effect of temperature on enzyme activity. The assay was performed at pH 8.0 in phosphate buffer. d, Thermostability of Xyn10A with and without NaCl. The enzyme was incubated at 30, 40, and 50 °C for different periods of time (10, 20, 40, 60 min), and then the residual activity was assayed at standard conditions. Each value represents the average of triplicate experiments. Error bars represent the standard deviation.

The results of testing the optimal temperature for Xyn10A showed the highest activity at a temperature of 40 °C. It is noteworthy that Xyn10A showed high relative activity at low temperature, and even retained significant activity at 4 °C. The relative activity was 38.3%, 55.7%, and 82.9% at 4 °C, 20 °C, and 30 °C, respectively (Fig. 3c). When the temperature was above 50 °C, the catalytic activity was lost quickly. Meanwhile, Xyn10A exhibited low thermostability at elevated temperature. The Xyn10A retained nearly 87.2% residual activity after being incubated at 30 °C for 60 min. However, more than 90% of the activity was lost after 10 min of incubation at 50 °C, and all activity was lost at 60 °C (Fig. 3d). According to previous reports, the typical cold-adapted enzyme exhibited the optimum activity at lower temperatures and was more sensitive to heat (Cavicchioli *et al*. 2002; Siddiqui and Cavicchioli 2006), which was a sharp contrast with the thermophilic xylanases (Petegem *et al.* 2003; Chang *et al*. 2004; Shi *et al*. 2013; Vester *et al*. 2014). Therefore, Xyn10A can be considered a cold-adapted xylanase. Recently, the research about cold-adapted enzymes has been given much attention, as the use of cold-

adapted enzymes has many advantages, such as reducing energy consumption and maintaining the original food flavor (Liu *et al*. 2014; Vester *et al*. 2014).

Various kinds of cold-adapted enzymes have been reported, such as esterases (Novototskaya-Vlasova *et al*. 2012), lipases (Tian *et al*. 2014), chitinases (Ramli *et al*. 2011), β-glucosidases (Vester *et al*. 2014), and proteinases (Kredics *et al*. 2008). However, there has been only limited study of cold-adapted xylanase. The xylanases from *Glaciecola mesophila* (Guo *et al*. 2009), *Bacillus* sp. SN5 (Bai *et al*. 2012), *Zunongwangia profunda* (Liu *et al*. 2014), and goat rumen contents belonging to the GH10 family showed obvious cold-adapted activity (Wang *et al*. 2011). Additionally, several xylanases from *G. mesophila* KMM241 (Guo *et al*. 2013), *Pseudoalteromonas haloplanktis* (Petegem *et al*. 2003), and the environmental DNA library of lagoons (Lee *et al*. 2006) belonging to the GH8 family also exhibited the cold activity. The temperature characteristics of these xylanases were similar to the Xyn10A results. The molecular mechanism has been explored with an increasing number of primary sequences and three-dimensional structures of cold-adapted enzymes. The relationship between stability, flexibility, and activity in these enzymes may be of crucial importance. It is generally accepted that cold-adapted proteins are more flexible than their mesophilic counterparts, with a reduced number of weak interactions. However, more details still need to be elucidated (Petegem *et al*. 2003; Papaleo *et al*. 2011; Zheng *et al*. 2016).

Effects of metal ions and chemicals on enzyme activity

The effects of metal ions and chemicals on Xyn10A activity were also tested (Fig. 4). The results revealed that the enzyme activity was positively stimulated by Ca^{2+}, Mn^{2+}, Mg^{2+}, K^{+}, and Tween-20. The activity was enhanced by nearly 20% in a solution of 1 mM Mn^{2+} and Mg^{2+}. Moreover, Xyn10A activity was inhibited by Cu^{2+}, Zn^{2+}, Ni^{2+}, EDTA, and SDS. The most obvious decreases (-25%) were observed with Zn^{2+} and SDS. The effects of Fe^{2+}, Co^{2+}, and Na^{+} on the enzyme activity were insignificant. The results showed differences from the other cold-adapted xylanases, such as xylanases from *Bacillus sp.* SN5 and *G. mesophila* KMM241 (Guo *et al*. 2009; Bai *et al*. 2012), which showed that Mg^{2+} had no evident effect on activity. On the contrary, the activity of XynB from *G. mesophila* KMM241 was obviously inhibited by Mn^{2+} (Guo *et al*. 2009).

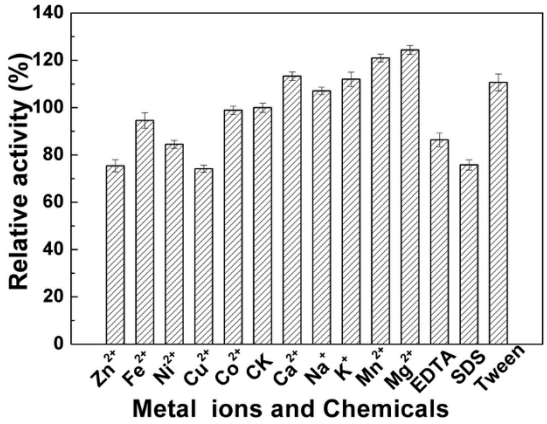

Fig. 4. Effects of ions and chemicals on the activity of purified Xyn10A. The final concentration of the ions and chemicals was 1 mM and 5 mM, respectively. The activity was measured at pH 8.0 and 40 °C, and activity without adding ions or chemicals was defined as 100%. Each experiment was performed in triplicate.

Effects of NaCl tolerance on enzyme activity

The halophilic bacteria and enzymes are very interesting (Khandeparker *et al*. 2011). Because the *B. cellulosilyticus* was isolated from a saline-alkali soil, the effect of NaCl on Xyn10A activity was evaluated. As shown in Fig. 5, the highest enzyme activity for Xyn10A was obtained by adding 0.5 M NaCl, which is approximately equivalent to the salinity of seawater. Compared to the control, the relative activity reached 127.3%. When the salt concentration reached 3 M, the residual activity remaining was still 66.5%. Additionally, compared to the absence of NaCl, the temperature stability was improved by adding the 0.5 M NaCl into the reaction solution (Fig. 5). The results were similar to xylanases from *Bacillus sp.* SN5 and *G. mesophila* KMM241 (Guo *et al*. 2009; Bai *et al*. 2012). The highest level of NaCl tolerance for a xylanase was found from *Z. profunda* (Liu *et al.* 2014), and its optimal activity was found with 3 M NaCl. The activity of XynB from *G. mesophila* KMM241 was not obviously affected by NaCl, though it showed some extent of salt-tolerant ability (Guo *et al*. 2013). Notably, several reported cold-adapted xylanases also showed the NaCl tolerance (Guo *et al*. 2009; Bai *et al.* 2012; Guo *et al*. 2013; Liu *et al*. 2014). Previous molecular dynamics research has shown that the salt-tolerant and cold-adapted properties may have some relevance, as the enzymes exhibit more flexible loop regions at the appropriate concentration of salt (Benrezkallah *et al*. 2015). In general, NaCl-tolerant enzymes originated from saline-alkaline land and marine environments, and they had to change their molecular structure in order to adapt to the corresponding environments.

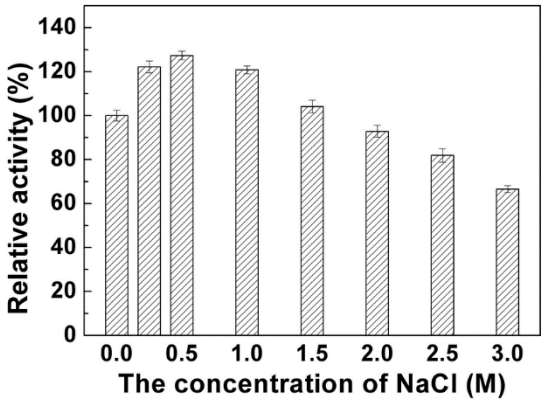

Fig. 5. Effect of NaCl on the activity of Xyn10A. The enzyme activity was measured at 40 °C in phosphate buffer (pH 8.0) containing 0 M to 3.0 M NaCl. The activity in 0 M NaCl was set as 100%.

Specific enzyme activity and kinetic parameters

Under the optimal conditions (pH 8.0 and temperature 40 °C), the Xyn10A exhibited the highest specific enzyme activity of 163.8 U/mg of protein. This activity was slightly higher than that of other NaCl-tolerant and cold-adapted xylanases. The specific activity toward beechwood of xylanase from *Bacillus sp.* SN5 and *G. mesophila* KMM241 were 105 U/mg and 143 U/mg, respectively (Bai *et al* 2011; Guo *et al*. 2013). The specific activity was also lower than that of some xylanases. The specific activity of the WSUCF1 endo-xylanase from *Geobacillus sp.* WSUCF1 was 461 U/mg (Bhalla *et al*. 2014), and the XYN-LXY from rumen contents of Hu sheep showed a specific activity of 664.7 U/mg (Wang *et al*. 2015). Even higher specific activity was reported for xylanases originating

from *Paenibacillus campinasensis* and *Neocallimastix patriciarum* (Liu *et al*. 2008; Ko *et al*. 2010), with activity reaching 2392 U/mg and 1982.8 U/mg, respectively. The commercial xylanases from *Trichoderma viride* have been reported to have activity of 100 U/mg to 300 U/mg (Shi *et al*. 2013).

The kinetic parameters K_m and V_{max} of the Xyn10A were determined at 40 °C using various concentrations of birchwood xylan as the substrate and calculated by Lineweaver-Burk double-reciprocal plots. The K_m, V_{max}, and K_{cat} of Xyn10A were 2.56 mg/mL, 253.1 μM/min/mg, and 165.8 /s, respectively. The K_m value of Xyn10A using birchwood xylan as the substrate was lower than that of some other xylanases, such as the xylanases from goat rumen contents, the rumen contents of Hu sheep, and *Malbranchea cinnamomea*, which had K_m values of 3.2, 4.39, and 7.1 mg/mL, respectively (Fan *et al*. 2014; Wang *et al*. 2015). Furthermore, the K_m of salt-tolerant and cold-adapted xynlases from the *Bacillus sp.* SN5, *Z. profunda*, goat rumen contents, and *G. mesophila* KMM 241 (XynA and XynB) were 0.6, 2.98, 1.8, 0.78, and 1.22 mg/mL, respectively, toward beechwood xylan (Guo *et al*. 2009; Bai *et al*. 2012; Guo *et al*. 2013; Liu *et al*. 2014). From the literature, the K_m for birchwood xylan was higher than that for beechwood as the substrate. It can be concluded that the salt-tolerant and cold-adapted xylanases showed high affinity to various xylan sources.

Substrate specificity and analysis of hydrolytic products

The activity of the purified xylanase towards various substrates was studied. The Xyn10A had almost no activity on CMC-Na, Avicel, CMC, cellobiose, and xylobiose (data not shown). This result indicated that Xyn10A had no cellulase, glucosidase, or xylosidase activity, and is a strict endoxylanase. Furthermore, the birchwood xylan was incubated with purified Xyn10A, and the hydrolytic products were confirmed by TLC methods using xyloligosaccharides (XOs) as standards (Fig. 6). The results showed that xylobiose, xylotriose, and xylotetraose were the main products in the hydrolytic mixture. No xylose was detected.

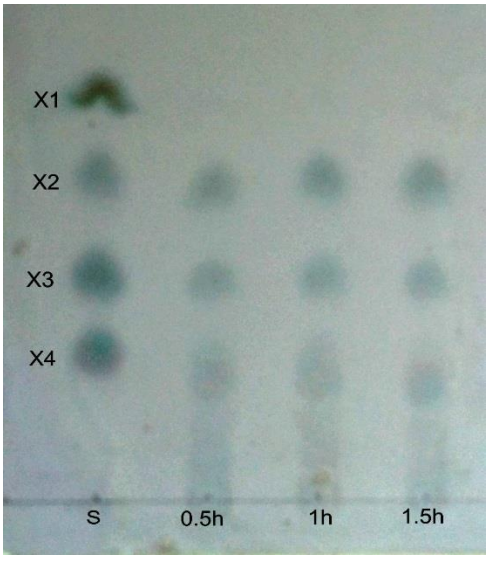

Fig. 6. TLC analysis of the hydrolysis products of birchwood xylan (1%) by Xyn10A.The reactions were performed at 30 °C and pH 8.0, and the samples were taken for analysis at 0.5, 1, and 1.5 h. S, Standard of XOs: X1, xylose; X2, xylobiose; X3, xylotriose; X4, xylotetraose.

The XOs could not be hydrolyzed further to form xylose after prolonged hydrolysis time. This is in accordance with the results of the xylanases from *B. mojavensis* A21 (Haddar *et al*. 2012), *G. mesophila* KMM241 (Guo *et al*. 2013), *Geobacillus thermoleovorans* (Verma and Satyanarayana 2012), *M. cinnamomea* (Fan *et al*. 2014), *etc.* While some other xylanases showed xylosidase activity, the end product was xylose, such as originated from *Thermoascus thermarum* (Shi *et al*. 2013), *Thermoascus aurantiacus* (Zhang *et al.* 2011), the rumen contents of Hu sheep, and *Caldicoprobacter algeriensis* (Amel *et al*. 2016). The hydrolysis properties could enable Xyn10A to be used as an effective and powerful enzyme for large-scale production of XOs, which can be used as prebiotics.

CONCLUSIONS

1. A GH10 family xylanase, the Xyn10A from *B. cellulosilyticus*, was heterologously expressed and biochemically characterized successfully.
2. Xyn10A displayed obvious cold-adapted characteristics and promising activity in the presence of weak alkalinity and a broad range of NaCl concentrations. Therefore, Xyn10 would be a useful candidate for biotechnology applications at low/room temperature conditions and/or a high salinity environment.
3. Xyn10A hydrolyzed xylans to yield mainly XOs (xylobiose, xylotriose, and xylotetraose). It can be used as excellent biocatalyst for production of XOs.

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