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Improvement of the Thermostability of Xylanase from *Thermobacillus composti* through Site-Directed Mutagenesis S

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Thermostability is an important property of xylanase because high temperature is required for its applications, such as wood pulp bleaching, baking, and animal feedstuff processing. In this study, *XynB* from *Thermobacillus composti*, a moderately thermophilic gram-negative bacterium, was modified via site-directed mutagenesis (based on its 3D structure) to obtain thermostable xylanase, and the properties of this enzyme were analyzed. Results revealed that the half-life of xylanase at 65°C increased from 10 to 50 min after a disulfide bridge was introduced between the α-helix and its adjacent β-sheet at S98 and N145. Further mutation at the side of A153E named XynB-CE in the C-terminal of this α-helix enhanced the half-life of xylanase for 60 min at 65°C. Therefore, the mutant may be utilized for industrial applications.

Keywords: Xylanase thermostability, *Thermobacillus composti*, disulfide bridge, structure

Introduction

Xylans are the second-most abundant natural polysaccharides in cellulose and are considered renewable resources [1, 2]. Xylan hydrolysis is an essential step for the degradation of lignocellulosic materials [3], and complete xylan degradation requires the concerted action of various glycosyl hydrolases (GHs). The most important GH is endo-1,4-β-xylanase (E.C. 3.2.1.8), which catalyzes the random hydrolysis of β-1,4-glycosidic bonds via a double displacement mechanism, thereby converting xylans to generate soluble xylooligosaccharides and xyloses [4]. According to amino acid sequence homologies and hydrophobic cluster analysis, xylanases have been grouped mainly into glycoside hydrolase families GH10 and GH11 [5, 6], and most of the industrially applied xylanases belong to GH11 [7]. The structure of GH11 xylanases consists of two large β-sheets and one α-helix, which form a structure resembling a partially closed right hand.

Xylanases are used in several high-temperature biotechnological processes, such as wood pulp biobleaching, baking, and animal feeding. However, these applications depend on the thermal stability of enzymes. Several thermostable families in GH11 xylanases have been cloned from thermophilic organisms to satisfy the demands of harsh conditions [8–13]. Another approach is to improve the thermal stability of xylanases by protein engineering to withstand extreme conditions.

The thermostability of xylanases has been largely enhanced by potential factors, such as charged amino acids, disulfide bridge introduction, and chimeric alterations near the N-terminal region of a protein. Xylanase stability can be

improved by introducing disulfide bridges in the N-terminal of the protein [14–18].

In this work, the thermostability of *Thermobacillus composti* XynB was increased when a novel disulfide bridge was engineered into the N-/C-terminal ends of the α-helix of the protein. Our results suggested that the mutant may be used for industrial applications.

Materials and Methods

Strain, Vector, and Chemicals

Pichia pastoris strain GS-115 (His-Mut+) was purchased from Invitrogen, the expression vector pYPX88 (GenBank Accession No. AY178045) was prepared in our laboratory, and the pGEM-T Easy vector was obtained from Promega. *Taq* DNA polymerase, T4 DNA ligase, restriction endonucleases, and endoglycosidase H (Endo H) were procured from Takara and New England Biolab. Xylan from beechwood and xylose were purchased from Sigma. All other chemicals were of analytical grade.

Synthesis and Modification of *XynB*

On the basis of the amino acid sequence (GenBank Accession No. CP003255), we optimized *XynB* from *T. composti* by utilizing codon usage bias and tagged this gene with an additional C-terminal 6× His sequence. *XynB* was produced through a successive PCR-based two-step DNA synthesis method [19], and 14 primers were used (Table S1). The PCR was performed under the following conditions: 5 min at 94°C, followed by 25 cycles of 20 sec at 94°C, 20 sec at 58°C, 30 sec at 72°C, and a final extension at 72°C for 10 min. The mutant genes *XynB-C* and *XynB-CE* were obtained through site-directed mutagenesis [20]. The oligonucleotide sequences of *XynB-C* and *XynB-CE* are shown in Table S2.

Expression of *XynB* Gene by *P. pastoris*

The amplified fragment was digested with BamHI and SacI and then ligated into the eukaryotic expression vector pYPX88, which contains a 357 bp fragment of α-factor prepro-leader MF4I (GenBank Accession No. AY145833) with *P. pastoris*-preferred codon usage. MF4I is a substitute for a wild-type α-signal sequence to enhance expression levels [21], and a standard recombinant method was used for routine DNA manipulations.

Recombinant pPIC9K-XynB was linearized with BglII to favor transformation into *P. pastoris* strain GS115 competent cells through electroporation. The His^+-Mut^+ phenotype of the transformants was screened on a histidine-deficient Synthetic Dropout medium and incubated at 30°C for 3 days. Test expression experiments were performed to detect the expression of the target recombinant protein. The transformants with His^+ phenotype were streak cultivated on BMGY at 30°C for 24 h. Two 96-well plates containing 60 μl of BMMY medium with 1% (v/v) methanol were incubated at 30°C for 12 h. After 12 h of induction, positive colonies that contain xylanase activity were measured according to standard methods [22]. For large-scale protein production, the single isolated colony with a high expression level was cultured in 30 ml of BMGY until the optical density at 600 nm reached 3.0; that is, *P. pastoris* reached log-phase growth. Subsequently, the cells were harvested and resuspended in an equal volume of BMMY medium at 30°C for 3 days. Xylanase expression was continuously induced by maintaining the concentration of methanol at 1% (v/v) every 24 h. Samples were taken at 0, 24, 48, and 72 h to examine protein production through SDS-PAGE, and the xylanase activity was assayed concurrently.

Protein Purification and Enzyme Assay

The culture, which was induced with methanol for 72 h, was harvested through centrifugation. Recombinant protein with additional six histidine residues at the C-terminal was purified using a Ni^{2+}-NTA agarose affinity column (Sigma, China). The total protein concentration of the samples was determined via Bradford's dye-binding method, and bovine serum albumin was used as a standard [23]. The purified protein (10 μg) was deglycosylated with 100 U endoglycosidase H for 2 h at 37°C. Deglycosylation production was conducted on 12% SDS-PAGE involving stacking and separating gels with 5% and 12% polyacrylamide, respectively. The spots of proteins after staining were visualized with Coomassie Brilliant Blue R-250.

Activity was assayed using 1% (w/v) beechwood xylan in different buffers as substrates, and reducing sugars were determined with the dinitrosalicylic acid assay method [24, 25]. One unit of xylanase activity was defined as the amount of enzyme that catalyzed the formation of 1.0 μmol reducing sugar equivalent to xylose from xylan per minute under the optimal assay conditions. All of the assays were carried out with an enzyme dilution appropriate for activity measurement. Each assay was repeated three times.

Analysis of Kinetic Parameters for Xylanase

The optimal pH was assayed in the following buffers: Na_2HPO_4–citric acid buffer (pH 2.0–7.0) and Tris–HCl buffer (pH 7.0–10.0), at optimal temperature of 60°C. pH stability was examined after incubation in buffers of pH varying from 2.0 to 10.0 at 37°C for 3 h. The activity under standard reaction conditions (60°C, pH 6.0) without preincubation at different pH values was defined as 100%.

The kinetic parameters (K_m) of xylanase activity were calculated from initial velocities at beechwood xylan concentrations ranging from 2 to 24 mg/ml. The K_m values of xylanase were graphically determined by Lineweaver–Burk plotting.

Analysis of Xylanase Thermostability

The optimum temperature was determined at temperatures ranging from 20°C to 90°C. Thermostability was obtained after preincubation in the absence of substrate for 30 min at varying temperatures. The half-life was measured at 65°C and 70°C for the preincubation of the enzyme solution for 10–60 min. Subsequently, the remaining activity was determined in each case.

Sequence Analysis and Homologous Modeling

The deduced protein sequence was analyzed using the NCBI BLAST. Several xylanase amino acid sequences that belong to the published GH11 family were chosen for the alignment using DNAMAN. The active site of xylanase was identified on the basis of the alignment and known catalytic residues. The tertiary structural homology model and three-dimensional structures of xylanases were analyzed using SWISS-MODEL (http://swissmodel.expasy.org) and DSViewerPro60, respectively. Putative N-linked glycosylation sites were located with the NetNGlyc 1.0 program (http://www.cbs.dtu.dk/services/NetNGlyc/).

Results

Homologous Alignment and Structural Assay

Compared with other known characterization methods for xylanase, the amino acid sequence alignment of XynB indicated the presence of conserved residues at the active site. Some studies that have investigated the members of xylanase family G have demonstrated that the two conserved glutamate residues Glu76 and Glu169 can reach this cleft from opposite sides [22, 26, 27]. *Aspergillus niger* and *T. composti* xylanases were highly similar in three-dimensional structure (Fig. 1A), but their sequence identity was only 35%. Crystallography studies on *A. niger* xylanase I have revealed that Glu79 and Glu170 are possibly the active nucleophile and the projected acid–base catalyst, respectively, and Gln129, Tyr70, Tyr81, and Asp37 residues may be involved in substrate binding [28]. Glu76 and Glu169 in XynB likely function as a nucleophile and an acid–base residue, respectively. The authenticity of the active-site glutamate residues Glu76 and Glu169 was identified using the PROSITE database (http://prosite.expasy.org). Gln124, Tyr67, Tyr78, and Asn33 were located in the XynB active site, and the stability of the catalytic site was maintained by hydrogen bond interactions. Asn33 is well correlated with optimum pH shifting [29], and *N*-bromosuccinimide, which is an amino acid modifier, restrains enzyme activity, thereby indicating the role of tryptophan residues in the catalytic function of xylanase [30].

Design of Mutants

The mutants of the N- and C-terminals of the α-helix were constructed to investigate their effects on the thermal stability and other characteristics of XynB-WT. The mutation sites in the *T. composti* XynB structure are shown in Fig. 1C. Ser98 and Asn145 of the N-terminal were replaced with cysteine (S98C and N145C) and named XynB-C. Furthermore, Ala153 of the C-terminal of XynB-C was substituted with glutamic acid (S98C, N145C, and A153E) and labeled XynB-CE.

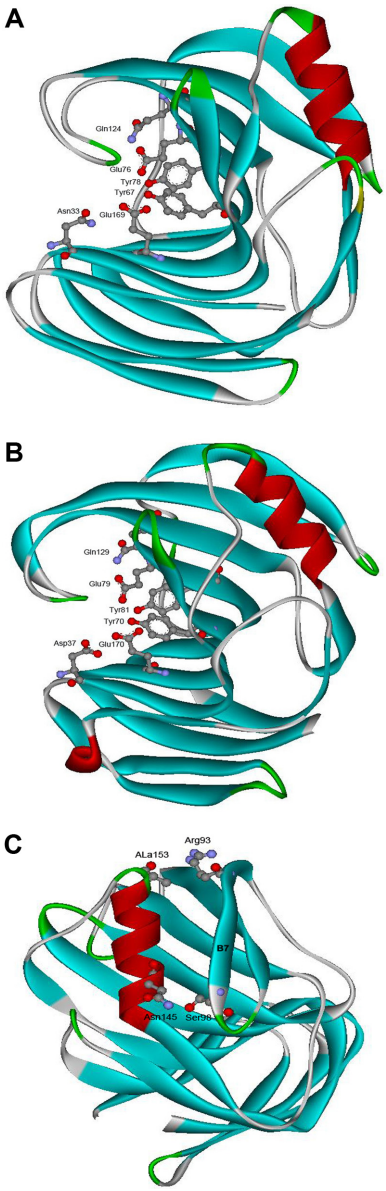

Fig. 1. Stereo-views of the structure of *T. composti* XynB (**A**) and xylanase I from *Aspergillus niger* (**B**), and (**C**) the location of stabilizing mutations in the *T. composti* XynB structure.
The α-helix is shown in red, and β-sheets are in blue. The sites for the active site and mutations are indicated in black. The tertiary structural homology modeling and the three-dimensional structure of xylanases were analyzed by SWISS-MODEL (http://swissmodel.expasy.org) and DSViewerPro60.

The open reading frame of xylanase was 561 bp in length, and it encoded a protein of 182 amino acids. SDS-PAGE revealed that XynB-WT and its mutants had an apparent molecular mass of approximately 29 kDa. The size of

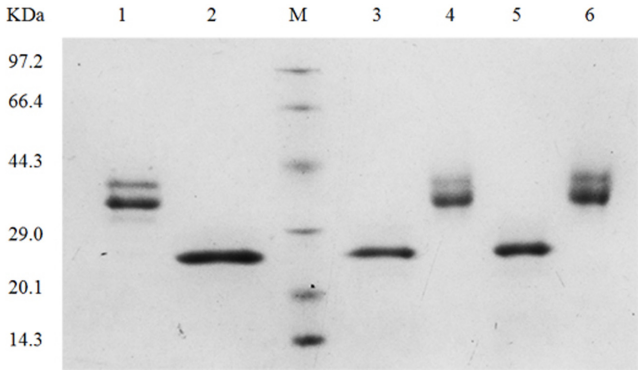

Fig. 2. SDS-PAGE patterns of the crude extract and deglycosylated xylanase.

Lane M: protein markers, Lane 1, 4, and 6: crude extract of xylanase; Lanes 2, 3 and 5: deglycosylation of xylanase with Endo-H. Lanes 1 and 2: XynB; Lanes 3 and 4: XynB-C; Lanes 5 and 6: XynB-CE.

xylanase molecules was theoretically 20 kDa (Fig. 2), but native xylanase produced in *T. composti* was glycosylated under actual conditions. The possible glycosylation sites are shown in Fig. S1.

Kinetic Parameters and Effect of pH

Enzyme activity as a function of pH was determined using Na_2HPO_4–citric acid buffer at pH 2.0–7.0 and Tris–HCl buffer at pH 7.0–10.0. The pH activity of the mutants was higher in the neutral–alkaline (7–10) regions than in Xyn-B-WT (Fig. 3A). The optimum pH values of the mutants and Xyn-B-WT were 8 and 6, respectively (Fig. 3A). The pH stability of xylanase was tested by incubating the enzyme solution in the absence of a substrate under diverse pH conditions for 3 h at 37°C. XynB-CE was predominantly in the acidic region (5.5–6.5). However, XynB-C showed further stabilized activity in a wide pH range of 5–8 (Fig. 3B).

The K_m of XynB-WT was roughly 3.3 mg/ml (Table 1 and Fig. 2) when beechwood xylan was used as a substrate. The K_m of the mutant of XynB-C was similar to that of XynB-WT. The K_m of XynB-CE was higher than those of XynB-C and XynB-WT. Moreover, the specific activities of XynB-WT and XynB-C were 5,039 and 4,990 U/mg, respectively. However, the specific activity of XynB-CE (3,993 U/mg) was lower than those of XynB-WT and XynB-C. This finding indicated that E153 in combination with the disulfide bridge weakened the catalytic function.

Temperature-Dependent Activity

The resistance of XynB-WT, XynB-C, and XynB-CE to high-temperature inactivation was determined to investigate

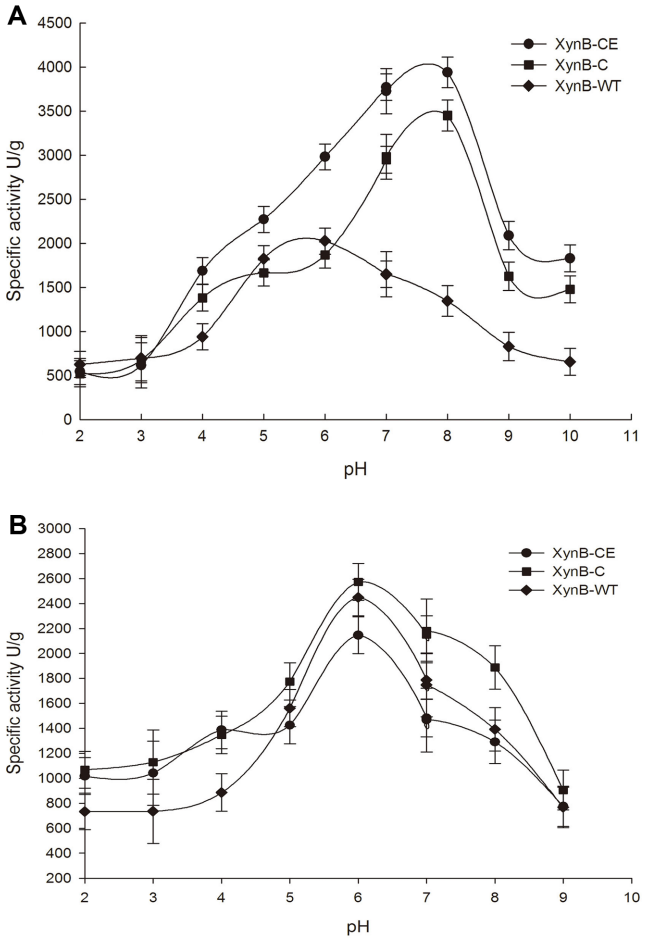

Fig. 3. Optimal pH (**A**) and pH stability (**B**) of the wild-type and mutant xylanases.

The optimal pH was assayed at various pH values (2–10) at 60°C. The pH stability, assayed at 37°C for 3 h, was examined after incubation without substrate in buffers with pH varied from 2.0 to 10.

Table 1. Half-lives (T_{half}) at 65°C and 70°C and kinetic parameters of xylanases.

Enzyme	T_{half} (min)		K_m (mg/ml)	Specific activity (U/mg)
	65°C	70°C		
XynB	10	~4	3.3	5,039
XynB-C	50	~12	3.5	4,990
XynB-CE	>60	~18	13.3	3,993

the contribution of the disulfide bond to thermostability. To compare the resistance of wild-type and mutant xylanases to inactivation at 65°C and 70°C, we determined the half-lives (T_{half}) of inactivation from the time-dependent inactivation curves. T_{half} in mutations reached more than 60 min at 65°C compared with that of XynB-WT at 10 min. At

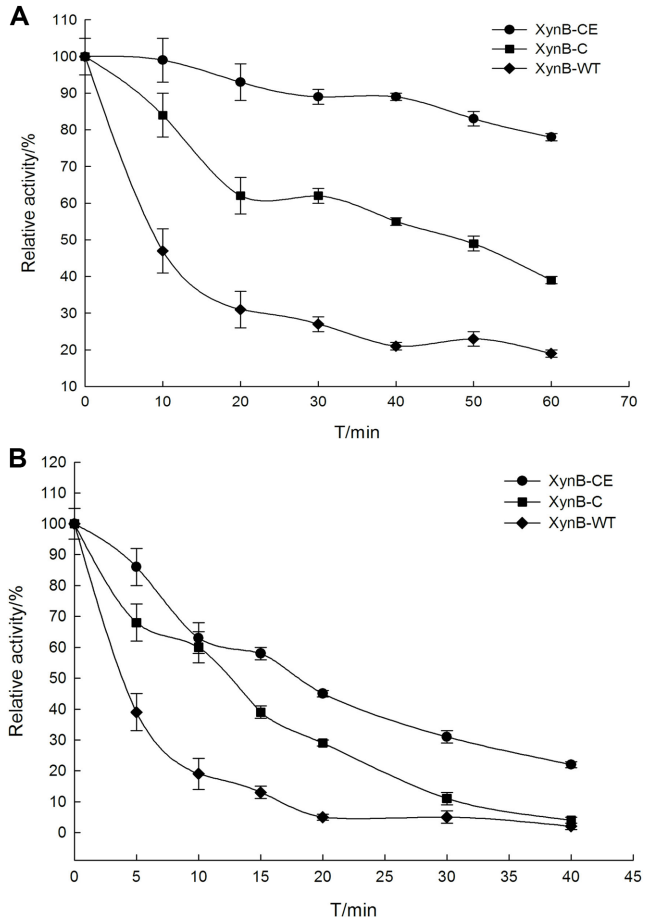

Fig. 4. Half-lives of wild-type XynB and mutants at 65°C (**A**) and 70°C (**B**).
The half-life was examined at the optimum pH after incubation without substrate.

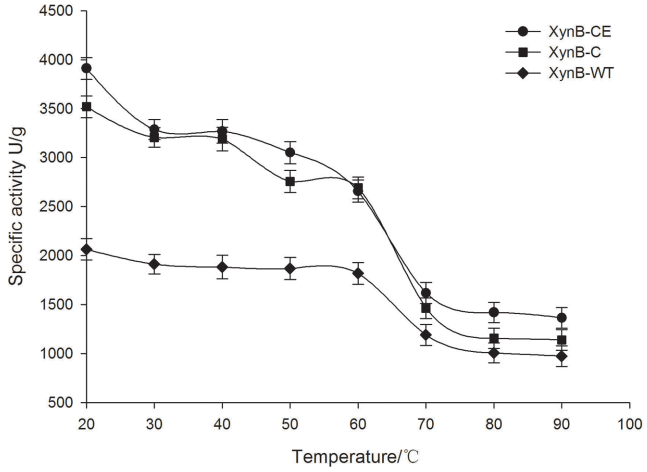

Fig. 5. Thermostability of the wild-type and mutant xylanases.
The enzyme activity was measured at the optimum pH after 30 min incubation in the absence of substrate at different temperatures.

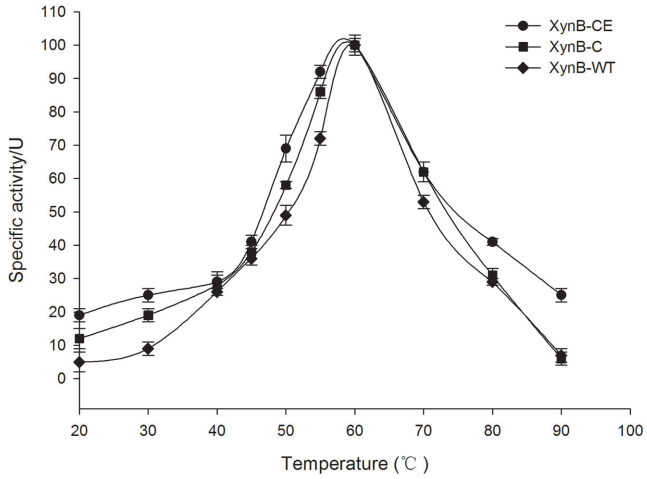

Fig. 6. Effect of temperature on the activity of wild-type and mutant xylanases.
Xylanase activity was determined at the optimum pH after 10 min pre-incubation in the presence of substrate at each temperature.

70°C, T_{half} decreased to less than approximately 4 min in XynB-WT and roughly 12 and 18 min in the mutants (Table 1). XynB-C and XynB-CE that showed a 3-fold to 6-fold enhancement in T_{half} indicated a stabilizing effect at 65°C and 70°C (Fig. 4).

To confirm the heat tolerance of mutant xylanase, we incubated the enzymes at various incremental temperatures in the absence of substrate for 30 min and measured the residual activity. Temperature-dependent inactivation curves suggested that the enzyme activity was more stable in the mutants than in the wild type (Fig. 5). The temperatures at which 50% of xylanase was inactivated for 30 min of incubation were 50°C and 60°C for the mutants. Conversely, the residual activity of XynB-WT was only 40%. At 20°C–60°C, the residual xylanase activity of XynB-WT was between 30% and 40%, whereas the average activity of the mutants was more than 50%. The residual activity of the mutants was enhanced at increased temperatures because of the increase in thermal resistance. The thermostability of XynB-CE was slightly higher than that of XynB-C because the combination of weakly stabilizing mutations (A153E) synergistically affected the thermostability of xylanase with a disulfide bridge (S98C–N145C). However, the optimal temperature of the mutants was similar to that of the wild type (Fig. 6). These results revealed that the disulfide bridge in the mutants greatly improves the relative activity of xylanase at the same temperature.

Discussion

The use of xylanase in several industrial applications is dependent on the thermostability of the enzyme. Therefore, the thermostability of the enzyme was improved by protein engineering. For example, the thermostability of a *Streptomyces* xylanase was improved by introducing proline and glutamic acid residues [31]. The properties of the disulfide bridge are crucial factors for the increased thermostability of family 11 xylanases. A disulfide bridge introduced at the N-terminal in xylanase II enhanced thermostability [17, 26, 32–34].

Thermal stability can be increased by modifying a few residues in the primary sequence, possibly because the net free energy of the stabilization of native proteins is unexpectedly small [35]. New disulfide bonds inside proteins can be designed by comparing the xylanase II sequence from *Trichoderma reesei* with the xylanase B sequence from *T. composti*, and this design shows that the presence of a disulfide bridge between these sequences corresponds to positions 98 and 145. Introducing a disulfide bond at the N-terminal regions of the α-helix improved the thermal stability of xylanases because the presence of a disulfide bond will restrict the motional freedom of the protein backbone in the unfolded state, thereby decreasing conformational entropy [17, 35, 36]. The combination of the mild stabilizing mutation with the disulfide bridge (S98C–N145C) elicits a cooperative effect on enzyme stability. In our study, the thermostability of *T. composti* XynB was enhanced when the binding of the α-helix (amino acids 143–152) to the protein core was improved at two separate sites (N- and C-terminals of the α-helix) compared with one site. A bulky side chain of the α-helix in the C-terminal can possibly improve the packing of the nearby β-strand [17], and all of the amino acids with increased stability at positions 145 and 153 (cysteine, glutamic acid) can possibly improve the binding of the α-helix to the nearby β-sheet B7 (amino acids 91–97, serine 98, arginine 93; Fig. 1B). The results of thermostability analysis indicated that XynB-CE is more stable than XynB-C. The C-terminal region of α-helix Glu153 from the hydrogen bond over the cavity to the main chain β-sheet B7 (Arg93) may also play a role in the stabilization.

Although the α-helix region strongly affects the thermostability of xylanase, excessive rigidity in this region may disturb the catalytic activity [17]. Certain factors are known to indicate a lower activity of XynB-CE: lower specific activity, limited pH stability (pH 5.5–6.5), and higher K_m. These findings suggest that the high rigidity of the α-helix region decreases the catalytic activity of the enzyme, and the α-helix region is correlated with thermal stability. Only introducing a disulfide bridge in XynB-C does not evidently affect the catalytic activity, but this part increases the thermal stability of the enzyme. Hence, this finding provides the possibility of introducing other minor modifications reported to be critical factors that contribute to the thermophilic nature of family 11 xylanases. The optimum pH of the mutants increased to 8, and the enzyme stability of XynB-C was higher than that of XynB-WT (Fig. 3). No studies have reported changes in the optimum pH of a mutant with a disulfide bridge, and the mechanism is also unclear. This result provides a novel basis for investigations on the alkali resistance of xylanase.

In general, a disulfide bridge and minor mutations induce an additive increase in stability that is much greater than their individual effects. This experiment demonstrated that the introduction of disulfide bridges does not influence the expression of mutant xylanase. The strategic combination of disulfide bridges and minor mutations is suitable for the creation of xylanases for industrial applications.

Acknowledgments

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