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Improvement of the Optimum pH of *Aspergillus niger* Xylanase towards an Alkaline pH by Site-Directed Mutagenesis

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In an attempt to shift the optimal pH of the xylanase B (XynB) from *Aspergillus niger* towards alkalinity, target mutation sites were selected by alignment between *Aspergillus niger* xylanase B and other xylanases that have alkalophilic pH optima that highlight charged residues in the eight-residues-longer loop in the alkalophilic xylanase. Multiple engineered XynB mutants were created by site-directed mutagenesis with substitutions Q164K and Q164K+D117N. The variant XynB-117 had the highest optimum pH (at 5.5), which corresponded to a basic 0.5 pH unit shift when compared with the wild-type enzyme. However, the optimal pH of the XynB-164 mutation was not changed, similar to the wild type. These results suggest that the residues at positions 164 and 117 in the eight-residues-longer loop and the cleft's edge are important in determining the pH optima of XynB from *Aspergillus niger*.

Keywords: *Aspergillus niger*, alkaline, site-directed mutagenesis, xylanase B

Introduction

Xylan is a major component of hemicellulose, which constitutes about 20-40% of the total plant biomass and accounts for approximately one third of all the renewable organic carbon on Earth [1]. Xylan is composed of a backbone of β-1,4-linked xylopyranose residues with branches containing acetyl, arabinose, and 4-*O*-methylgluconic acid [19]. Its complete degradation requires a whole suite of xylanolytic enzymes, of which endo-β-1,4-xylanase play a major role [12]. Owing to this ability, xylanases have a wide range of potential biotechnological applications in the pulp and paper industry [8]. Pretreating the paper pulp with xylanases is a very important technological improvement as it can enhance the chemical reagents' bleaching effect, thereby cutting costs and, more importantly, considerably diminishing the pollution associated with bleaching [26].

The xylanases that are used in the biobleaching application are usually family 11 xylanases. This is because a low-molecular-mass enzyme is desirable for fiber penetration, and a lack of cellulose activity is required so as to maintain pulp yield and strength [29]. Because pulp bleaching requires both high temperatures (60−80°C) and a high pH (8−10), the optimum xylanase for this application would have both a high level of activity and possess certain critical characteristics that are not typically found in native enzymes: that is, thermophilic, thermostable, alkaliphilic, and would remain stable in an alkaline environment [6, 7, 11]. Therefore, efforts have been focused on improving existing enzymes to achieve stability and activity at high temperatures and extreme pH.

Endo-β-1,4-xylanase can be produced by a broad range of organisms, including fungi, bacteria, and yeasts. Members of family 11 xylanases are derived from both eukaryotic and bacterial species that share a 40% to 90% sequence identity. All members of this family consist of similar three-dimensional structures and active-site geometries, but the pH optima vary widely from acidic values as low as 2 to alkaline values as high as 11 [14]. Fungi are widely used as xylanase producers and are generally considered as more potent xylanase producers than bacteria and yeasts, since fungi secrete a much higher amount of

xylanolytic enzymes into the medium than either bacteria or yeasts do [1, 5, 23]. Commercially available fungal endoxylanases display an optimum activity over a pH range of 4 to 6; however, these enzymes are inactive in the alkaline pH range. Furthermore, their optimum temperature varies between 40°C and 60°C. Additionally, fungal xylanases are generally less thermostable than bacterial xylanases [15]. As such, the application of endoxylanases in the pulp and paper industry is quite limited. Despite the extensive research conducted on the isolation, enzymatic properties, reconstruction, and application of the alkaline endoxylanases from bacteria, the bacterial enzyme yield has not been sufficient for industrial purposes [3, 9]. However, protein engineering offers hope for the eventual optimization of fungal xylanases; this approach provides a powerful tool through the use of rational protein design to enhance the pH optima of fungal xylanase.

Protein engineering has been frequently used to enhance the enzymatic properties of enzymes. To accomplish this enhancement, there are two main protein engineering approaches used: directed evolution and rational protein design [28]. Rational design involves precise, preconceived amino acid sequence changes based on the knowledge of the protein structure, function, and mechanism. However, directed evolution offers the significant advantage that neither structural information nor catalytic mechanisms are required to guide the enzymatic evolution [20].

In the present study, the xylanase-encoding *xynB* gene from *Aspergillus niger* nl-1 has been cloned, optimized, and synthesized to match the codon preference of *Pichia pastoris*, and the recombinant enzyme obtained had high-level expression and was characterized [16, 18]. The structure and catalytic mechanisms of Xylanase B (XynB) from *Aspergillus niger* nl-1 are presented. The amino acid sequence of XynB indicates that it belongs to the family 11 xylanases, with an optimal pH of 5.0. The enzymes consist of an α-helix and two β-sheets lined against each other to form a structure resembling a partly closed right hand [11]. XynB catalyzes hydrolysis through a double-displacement mechanism that retains an anomeric configuration. Two glutamic acid residues, E121 and E212, function as the nucleophile and acid/base catalyst located in the active site and are involved in an intricate network of hydrogen bonding contributed by the highly conserved neighboring residues.

Much of the previous research focused on improving the alkalophilic pH stability of bacterial xylanases [10, 14, 25, 27, 30], whereas improving the fungal xylanase's pH optima received scant attention. Several studies indicated that there is a correlation between the alkaliphilic adaptation of xylanase and salt bridges and charged residues in the catalytic cleft, particularly in the thumb and at the edge of the cleft [25]. Some alkaline xylanases exhibit broader pH activity profiles with optima located near a neutral pH [10]. In this paper, we report the use of a site-directed mutagenesis technique to enhance the pH optima of xylanase B from the filamentous fungus *Aspergillus niger* nl-1. In addition, the relationship between the shift in the optimum pH and the charged residues in the eight-residues-longer loop was analyzed by molecular modeling.

Materials and Methods

Strains, Plasmids, and Growth Media

Escherichia coli TOP10F′ was used for the general DNA manipulations. *P. pastoris* GS115 (his4) was used for the heterologous expression of the mutant xylanase, *xynB*. The plasmid pPICzαA-xynB was constructed as described in a previous paper published by our laboratory and is used as a template in Inverse-PCR reactions [18].

Escherichia coli TOP10F′ was grown overnight at 37 °C on Luria-Bertani (LB) medium containing zeocin (25 μg/ml). *P.pastoris* mutants were cultured and selected on YPDS medium containing zeocin (100 μg/ml and 500 μg/ml). Then, *P. pastoris* mutants were induced and expressed in a buffered glycerol complex (BMGY) medium, which had been prepared according to the *Pichia* Expression Kit manual (Invitrogen 2002).

Site-Directed Mutagenesis

From comparing XynB with other xylanases that have an alkalophilic pH optima, two residues, one located in the edge of the catalytic cleft and one in the eight-residues-longer loop, were selected for mutation. The Swiss-PdbViewer (http://www.expasy.ch/spdbv/) was used to model the XynB structure with the structure template (PDB accession code: 1te1B) used. The mutations were generated by inverse-PCR using synthetic oligonucleotide primers, as shown in Table 1. The inverse-PCR with primer Q164K was carried out using the Prime STAR HS DNA Polymerase with pPICzαA-xynB as the template, which then generated the plasmid pPICzαA-xynB-164. The inverse-PCR with primer D117N was carried out with pPICzαA-xynB-164 as template, which then generated the plasmid pPICzαA-xynB-117. The amount of template DNA (10 ng) and the primers (10 pmol) were kept constant in the PCRs, resulting in a total volume of 50 μl. The PCR products were isolated through gel purification and were then treated with T4 Polynucleotide Kinase and ligated by T4 DNA ligase (TaKaRa). The resulting plasmids were transformed into *Escherichia coli* TOP10F′. Successful mutagenesis was verified by sequencing.

Protein Expression, Enzyme Assay, and SDS-PAGE Analysis

Plasmids pPICzαA-xynB-164 and pPICzαA-xynB-117 were

Table 1. Sequences of oligomers used in site-directed mutagenesis of XynB.

Target sites	Oligonucleotide sequence (5′-3′)
Q164K	GCTGCCTCCATC**AAG**GGTACAGCAACCTTT
	GTTAGTTCTGGTGGCTGTGTAG
D117N	**AAC**CCACTTATCGAATACTACATCG
	TGTACTCCAACCATAAACTGACAA

The mutated sites are underlined in bold.

linearized using *Bst*XI and then transformed into *P. pastoris* GS115 competent cells by electroporation according to the *Pichia* expression vectors manual (Invitrogen 2002), resulting in the variants XynB-164 and XynB-117, respectively. The transformed cells were selected by zeocin resistance using YPDS plates containing 500 μg/ml of zeocin at 28°C until colonies appeared. The variant was induced to express recombinant xylanase by adding methanol to a final concentration of 0.5% at an OD_{600} of about 1.0 and were then further incubated at 28°C for approximately 13 days. The culture filtrates were collected, and cells were removed *via* centrifugation and then treated with ammonium sulfate in a one-step procedure. The precipitate was collected at 75% saturation. The solution that was obtained from dialysis was loaded onto an anion-exchange column and pre-equilibrated with buffer A (30 mM NaCl–20 mM Tris-HCl buffer, pH 7.9). The column was then washed with buffer A to remove any unadsorbed protein. Proteins were eluted with a 1 L gradient of buffer B (buffer A supplemented with 1 M NaCl) at a 1 ml/min flow rate. The active fractions were pooled to characterize the xylanase mutation.

Xylanase activity was determined by measuring the increase in reducing sugar formation according to the DNS method, from 1% birchwood xylan in 50 mM sodium citric acid buffer, pH 5.0, at 50°C [21]. One unit of endoxylanase activity was defined as the amount of enzyme that releases 1 μmol of reducing sugar per minute.

Protein concentrations were determined using the Bradford method with bovine serum albumin as a standard [4].

SDS-PAGE was done according to Laemmli's method [16]. Gels were stained for protein with Coomassie Brilliant Blue R-250.

Properties of the Mutant Xylanases

To determine the optimal pH, the mutant xylanases were purified and assayed at different pHs, ranging from 3.0 to 7.0, in a 50 mM sodium citrate buffer. The enzyme's pH stability was determined by measuring the remaining enzymatic activity after a pre-incubation of the enzyme at 4 °C for 24 h at various pH levels (pH 4–10). The enzyme activity without a pre-incubation was defined as 100%.

The optimal temperature was determined by incubating the purified enzyme at different temperatures, ranging from 40°C to 65°C in 50 mM sodium citric acid buffer (pH 5.0). To estimate the thermal stability, the enzyme was pre-incubated at 60°C for various periods of time. The residual activity of the treated enzymes was then measured according to the standard assay method.

The xylanases' kinetic parameters were determined by measuring the enzymatic activity towards birchwood xylan at various concentrations (3–10 mg/ml in 50 mM sodium citric acid buffer, pH 5.0) [24] under standard reaction conditions.

Results and Discussion

Comparison of XynB with Other Alkalophilic Enzymes and Selection of the Target Mutation Sites

XynB and other xylanases that are present in an eight-residues-longer loop located in the substrate binding area are involved in the alkaliphilic adaptation that would lead to the creation of more salt-bridges in alkalophilic enzymes. The amino acid sequences of nine family 11 xylanases located in the eight-residue-longers loop, which are optimally active in neutral or in the alkaline pH range, were aligned with XynB from *Aspergillus niger* nl-1, as shown in Table 2.

The charged residue was exclusively found in or near the thumb of alkalophilic xylanases. The alignment shows that there is a conserved charged residue, "Lysine," that is located at the thumb's edge from the two alkalophilic enzymes that would be involved in alkalophilic pH optima, whereas it is "Glutamine" in the XynB. The positive-charged or polar side group near the catalytic cleft would make the microenvironment more alkaline [22]. The mutant XynB-164, in which Glutamine is substituted for Lysine, was successfully obtained in order to shift the pH optima towards alkaline. Additionally, we found that the

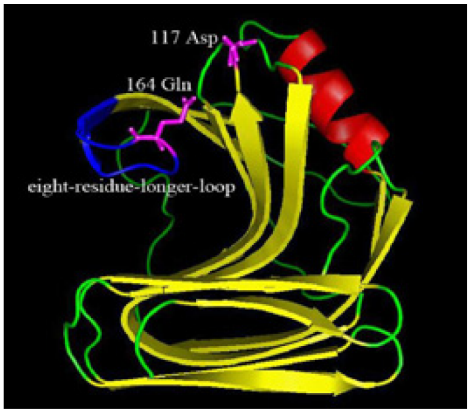

Fig. 1. Three-dimensional structure model of *A. niger* nl-1 XynB.

The sites of the mutational substitutions (Q164K, Q164K+D117N) are indicated by the corresponding sites. The picture of the enzyme structure was created by Swiss-Pdb Viewer and Pymol Molecular modeling software.

Table 2. Sequence comparison of the eight-residues-longer loops of family 11 xylanases.

Organism	Xylanase	Protein sequence	pH optimum
Bacillus sp.141M-1	Xyl J	QPSIKGTA	9.0
Bacillus agaradhaerens	Xynl1	QPSIKGIA	8.0
Bacillus pumilus A-30	XYNA	QPS I I GIA	6.7
Dictyoglomus thermophilum Rt46B.1	XynB	QPSIVGTA	6.5
Streptomyces lividans	XlnB	KPSVEGTR	6.4
Streptomyces sp. S38	Xyl1	APSVEGTK	6
Trichoderma reesei	XYNII	QPS I I GTA	5. 5
Bacillus circulans	XynA	APSIDGDR	6
Bacillus sp. D3	XynA	APSIDGTQ	6
Aspergillus niger nl-1	XynB	AASIQGTA	5.0

The key charged residues involved in the alkaliphilic adaptation are shaded.

Q164K substitution and D117 located at the cleft's edge are connected by a salt-bridge, which may have influenced the microenvironment of the active site [22], as seen in the three-dimensional structure shown in Fig. 1 (PDB accession code: 1te1B).

Protein Expression and SDS-PAGE Analysis

The mutants XynB-164 and XynB-117 were successfully expressed in *P. pastoris* GS115 and purified. SDS-PAGE analysis of the mutant xylanases revealed three apparent molecular sizes of about 21, 22.5, and 24 kDa, as shown in Fig. 2, which was the same as the native recombinant xylanase. The different bands appearing on the electrophoresis

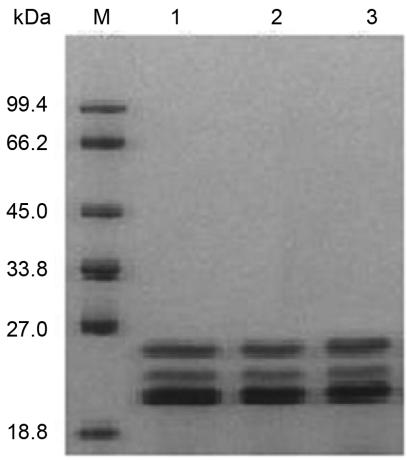

Fig. 2. SDS-PAGE analysis of different mutant xylanase samples.

The culture supernatants were collected at day 13. Lane M: Protein molecular weight standards. Lane 1 is the native recombinant xylanase as control. Lane 2 is the mutant XynB-164 sample. Lane 3 is the mutant XynB-117 sample.

are due to a difference in the degree of glycosylation of the polypeptide chains [17].

Optimum pH and pH Stability

The pH activity profiles of the mutants XynB-164 and XynB-117 were determined and compared with that of the wild type (Fig. 3). Wild-type XynB showed a broad pH profile, with its highest activity occurring at pH 5.0. Conversely, both the mutants XynB-164 and XynB-117 demonstrated a narrow pH profile that was shifted more towards an alkaline pH than XynB. Although mutant XynB-164's optimum pH did not significantly change, the relative activity was obviously decreased in a lower pH and increased in a higher pH, greater than pH 6.0, when compared with XynB. On the other hand, mutant XynB-117 had a higher optimum pH (pH 5.5) and corresponded to a basic 0.5 pH unit shift when compared with the wild-type enzyme, as shown in Fig. 3A. A surprising finding was that the mutation Q164K had no effect, whereas the charge inversion obtained by the D117N substitution shifted XynB's optimum pH activity towards alkaline. When Q164K and 117D were combined, the optimum pH value was similar to that of the wild type. The results identified that the lysine located at the thumb's edge clearly impacted the pH-dependent activity profile. One study indicated that the thumb possesses an opening and closing capability that modifies both the topology and the binding capacity of the active site, and the thumb's precise position determines the width of the catalytic cleft. The loop's position may change when a larger substrate binds in a slightly different conformation, which then affects the distance from the nucleophile and acid/base catalyst to the substrate and, thus, impacts the enzyme's catalytic performance [14]. The

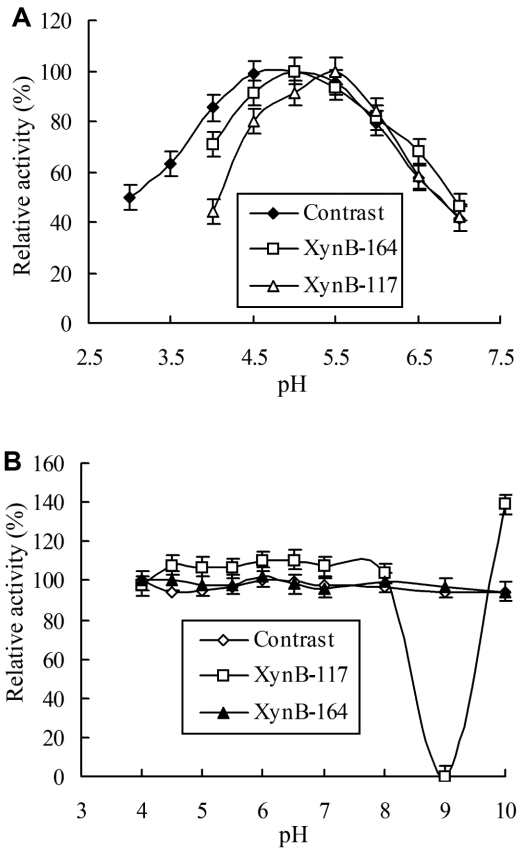

Fig. 3. pH activity profiles for the mutants and wild type.
(**A**) The optimum pH of the mutants and wild type. The reaction was carried out under different pH values ranging from 3.0 to 7.0, at 50°C for 30 min. The highest xylanase activity was taken as 100% in an optimum pH assay. (**B**) The pH stability of the mutants and wild type. The enzymes were treated at 4°C for 24 h at various pH levels (pH 4–10). The remaining activity was determined under pH 5.0 at 50°C for 30 min. The enzyme activity without pre-incubation was defined as 100%. All these assays were performed as described above using 1% birchwood xylan as the substrate.

thumb is connected to the main protein scaffold *via* β-strands B8 and B7, and at the edge of this thumb is K164. Owing to the enzyme's flexibility, it is likely that an interaction between this lysine residue and the substrate would occur. However, this putative interaction may be eliminated when the Q164K substitution and the D117 located at the cleft's edge are connected by a salt-bridge. The D117N mutation was carried out and was then used to further identify this interaction. The characterization of the two mutant proteins bearing the loop elongation and the elongated loop combined with the Q164K+D117N substitution has the potential to yield interesting information on the structural role of this longer β-stand in the alkalophilic adaptation.

The residual enzyme activity was analyzed in order to study the pH stability of the mutants and the wild type, the results of which are shown in Fig. 3B. Above 90% of the initial activity remained for both the mutant XynB-164 and the wild type after being incubated at a pH of 4.0 to 10 for 24 h at 4°C. The mutant XynB-117 lost nearly all of its activity when treated at pH 9.0 at 4°C for 24 h, whereas its stability increased significantly under pH 10.0 in comparison with XynB-164 and the wild type. Additionally, the pH stability of XynB-117 was further studied. As expected, the XynB-117 activity quickly diminished, and only 75% of the activity remained after pre-incubating the enzyme at 4°C for 3 h at pH 9.0. XynB-117's pH stability decreased after being incubated at pH 9.0, primarily due to the irreversibility of the intermediate conformation or molten globule-like protein state at pH 9.0. The enzyme might be in a fully unfolded state at pH 10.0, and after the incubated enzyme was subjected to an assay condition (pH 5.0, 50°C), the fully unfolded protein (treated at pH 10.0) could be readily refolded to its active form. At pH 9.0, however, the mutant XynB-117 is likely to be in an intermediate conformation or molten globule-like state, which commonly exposes more hydrophobic surfaces to the external environment and, thus, is susceptible to aggregation; as a result, its ability to recovery its active form is very low during an assay at pH 5.0. Therefore, only a very low enzyme activity amount could be recovered [2, 13]. The characteristic salt-bridge formed by Q164K and 117D was re-inforced, so the cleft structure's stability at an alkaline pH increased, which consequently improved the alkaliphily. Therefore, the mutant XynB-164 had good pH stability under the same condition. Establishing the salt-bridge is not significant for XynB's optimum pH, but it does obviously improve the pH stability. According to the above results, which were observed by replacing Q164K and D117N, it can be concluded that K164 and N117 might be the key residues involved in determining the optimal pH and pH stability of XynB from *Aspergillus niger* nl-1.

Temperature Optimum Profiles

Like the wild-type, the optimal activity temperature of the mutants XynB-164 and XynB-117 did not change, as shown in Fig. 4. Every enzyme was most active at 45°C, but the mutant XynB-164 exhibited higher relative activity at the high temperature range of 50–60°C. Additionally, the thermostability of XynB-164 was further studied. After

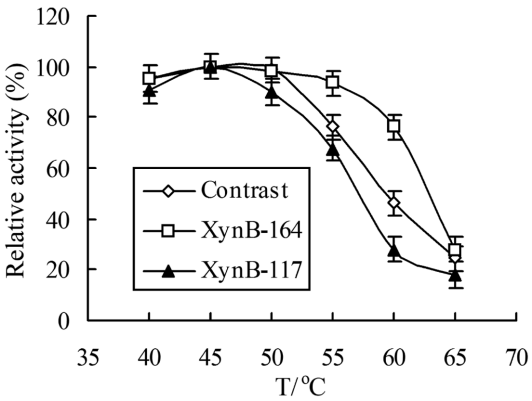

Fig. 4. Apparent optimum temperature profiles.
The reaction was carried out at each temperature under pH 5.0 for 30 min, using 1% birchwood xylan as a substrate. The highest xylanase activity was taken as 100% in an optimum temperature assay.

Table 3. Kinetic constants for mutants and wild-type XynB.

Enzyme	K_m (mg/ml)	V_{max} (mg·ml^{-1}·min^{-1})
XynB	16.7 ± 0.1	188.68 ± 6.4
XynB-164	7.56 ± 0.3	112.36 ± 5.9
XynB-117	21.42 ± 0.7	227.78 ± 9.7

incubation at 60°C for 10 min, XynB-164 retained 77% activity, higher than that of the wild type. This result indicates that the 164K-117D salt-bridge increased the XynB-164's thermostability. Furthermore, XynB's thermostability has also been studied, the results of which will be reported in other paper.

Kinetic Parameters of the Xylanases

When using birchwood xylan at a concentration range of 3 to 10 mg/ml, the enzymatic reaction was found to follow Michaelis-Menten kinetics with K_m and V_{max} values of 7.56 ± 0.3 mg/ml and 112.36 ± 5.9 mg·ml^{-1}·min^{-1} for XynB-164 and 21.42 ± 0.7 mg/ml and 277.78 ± 9.7 mg·ml^{-1}·min^{-1} for XynB-117, respectively. This is in comparison with 16.7 ± 0.1 mg/ml and 188.68 ± 6.4 mg·ml^{-1}·min^{-1} for the wild type, as shown in Table 3. XynB-164 had a lower K_m, indicating higher substrate affinity. Additionally, the K_m value of XynB-117 was slightly higher than that of the wild type, indicating that mutant XynB-117 was nearly as functional as the wild-type enzyme in combining with the xylan.

Xylanase activities were determined at 50°C using birchwood xylan in a 50 mM sodium citric acid buffer, at pH 5.0. The xylan concentration ranged from 3–10 mg/ml. Each value represents the average of triplicate experiments.

Acknowledgments

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