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# Residue mutations of xylanase in *Aspergillus kawachii* alter its optimum pH

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ABSTRACT

*Aspergillus kawachii* and *Aspergillus niger* have been traditionally used as molds for commercial microbial fermentation because of their capability to grow in extremely acidic environments and produce acid-stable enzymes. Endo-1,4-β-xylanase cleaves the glycosidic bonds in the xylan backbone, consequently reducing the degree of polymerization of the substrate. The amino acid sequences of xylanases from *A. kawachii* and *A. niger* only differ in one amino acid residue. However, the xylanases from *A. kawachii* and *A. niger* show different optimum pH values of 2.0 and 3.0, respectively. In this study, we synthesized the *A. kawachii* xylanase gene (*XynC*) on the basis of the bias codon of yeast and mutated the gene in the dominating region related to optimum pH shifting during gene synthesis. After the overexpression of this gene in *Pichia pastoris* G115, the mutant (Thr64Ser) enzyme (XynC-C) showed an optimum pH of 3.8, which indicated partial alkalinity compared with the original xylanase from *A. kawachii*. Similar to that of the enzyme with one residue mutation (Asp48Asn), the optimum pH of the enzyme with two residue mutations (Thr64Ser and Asp48Asn) shifted to 5.0. The result indicated that mutation Asp48 was more important than mutation Thr64 in optimum pH shifting. We proposed a model that explains the lower optimum pH of XynC-C than other members of the xylanase family G. XynC-C showed similar proteolytic resistance and $K_m$ and $V_{max}$ values for beechwood xylan to other xylanases.



## 1. Introduction

The common hemicellulose xylan accounts for up to one-third of the total dry weight of plants (Thomson, 1993). Enzymes such as endoxylanase, β-xylosidase, acetyl xylan esterase, α-glucuronidase, α-arabinofuranosidase, feroryl, and *p*-coumaroyl esterases are involved in xylan degradation. Endo-β-1,4-xylanase catalyzes the hydrolysis of the main backbone of xylan (Biely, 1985). On the basis of amino acid sequence similarities, the majority of endoxylanases fall into families 10 and 11 of glycosyl hydrolase (Finn et al., 2008; Gouda and Abdel-Naby, 2002).

Xylanases are found in bacteria, actinomycetes, fungi, and protozoa. Fungal xylanases have attracted considerable attention because of their special properties, such as broad pH adaptability, good thermostability, strong proteolytic resistance, and high specific activity. Xylanases are mainly applied as bleaching agents replacing toxic chlorine-containing chemicals in the paper and pulp industries (Bajpai, 1999). Moreover, incorporating xylanase into lignocellulosic feeds for animal nutrition reduces intestinal viscosity and enhances feed conversion efficiency (Murphy et al., 2009). Several acidophilic xylanases from fungi have been reported to date (Kimura et al., 2000; Luo et al., 2009b; Zhang et al., 2011). Acidophilic and acidic-stable xylanases benefit processes that require low pH condition to avoid microbial contamination.

Certain *Aspergillus* strains can grow in extremely acidic environments and produce acid-stable enzymes that are peculiar to them (Mimura et al., 1998). These enzymes display properties appropriate for potential industrial applications. *Aspergillus kawachii*, used in the shochu industry in Japan, produces a large quantity of citric acid and several interesting acid-stable enzymes, such as α-amylases, glucoamylases (Mikami et al., 1987), xylanases (Ito et al., 1992), and acidophilic proteinases (Yagi et al., 1986). *A. kawachii* is phylogenetically close to *Aspergillus niger*, which is also used indus-

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trially to produce citric acid (Yamada et al., 2011). However, the former is distinct from the latter because the endo-β-1,4-xylanase from *A. kawachii* shows more acidophilicity than the homo-enzyme from *A. niger* (Fushinobu et al., 2011).

*A. niger* Xylanase I, whose optimum pH is 3.0, has one residue difference from *A. kawachii* XynC; however, the optimum pH of XynC is 2.0. To elucidate the mechanism underlying the optimum pH determination of fungal family 11 xylanase, we studied two mutations in *A. kawachii* xylanase. One mutation was near the region that has one residue difference from *A. niger*, and the other mutation was near the catalytic residues. We then compared the optimum pH and other characteristics, such as substrate specificity, temperature stability, and proteolytic resistance, of the mutant enzyme (XynC-C) with those of its origin. We elucidated the catalytic mechanism under low pH conditions on the basis of the 3D structure of the enzyme.

## 2. Materials and methods

### 2.1. Microorganism and chemicals

All chemicals used were of analytical grade. *Pichia pastoris* strain G115 (His-Mut+), which was used as a host for heterologous expression of xylanase, was purchased from Invitrogen. (Carlsbad, CA, USA). Vector pYPX88 (GenBank Accession No: AY178045) which was used as an expression vector was prepared in our laboratory. Genomic DNA and plasmid isolation and purification kits were purchased from TIANGEN (Beijing, China). Restriction endonucleases, T4 DNA ligase, DNA polymerase, dNTPs and GC buffer I were purchased from TaKaRa (Otsu, Japan). Beechwood xylan (X4252-25G) and proteases including pepsin and trypsin, were purchased from Sigma (St. Louis, MO).

### 2.2. Gene synthesis and sequence analysis

In consideration of the amino acid sequence, the mutational xylanase gene *XynC-C* with an additional C-terminal 6× histidine tag sequence at the 3′ end was optimized using codon usage bias and was synthesized via successive polymerase chain reaction (Xiong et al., 2004). The amplification reaction mixture (25 μL) was composed of 2.5 μl of 10 × PCR buffer, 2.5 μl of 25 mM $Mg^{2+}$, 2 μl of 10 mM dNTPs, 1 μL of each primer (10 mM), 10 ng of each inner primers, 100 ng of outer primers, and 0.5 U of *Pyrobest*™ DNA polymerase. The PCR cycling parameters were an initial denaturation step at 94 °C for 5 min, 25 cycles of amplification (denaturation at 94 °C for 20 s, annealing at 58 °C for 20 s, extension at 72 °C for 30 s), and a final elongation step at 72 °C for 10 min. The resulting PCR product was separated by electrophoresis in a 1% (w/v) agarose gel and recovered. The amplified fragment was digested with *Bam* HI and *Sac* I and then transformed into *Bam* HI/*Sac* I site of pYPX88 vector to obtain a genomic library. Errors in the synthetic gene were corrected by the overlap extension PCR method (Xiong et al., 2006). The vector pYPX88 contains a 357-bp fragment of α-factor prepro-leader MF4I (GenBank accession No: AY145833) with *P. pastoris* preferred codon usage, which substituted for the wild-type α-signal sequence to enhance the expression level (Xiong et al., 2003). Routine DNA manipulations were performed by standard recombinant method (Sambrook et al., 1989).

### 2.3. Electroporation and screening transformants

The synthetic plasmid was linearized with *Bgl* II and transformed into *P. pastoris* strain G115 cells by electroporation method. *P. pastoris* strains were grown in YPD broth (1% tryptone, 0.5% yeast extract and 0.5% NaCl) at 30 °C with vigorous aeration to an $OD_{600}$ of 1.5–2.0. Cells were harvested and washed three times in sterile cold de-ionised water by centrifugation at 3000 g for 10 min and finally resuspended in 0.6 mol/l sorbitol. Electroporation was performed using a Bio-Rad Gene Pulser and Pulse Controller as described by Dower et al. (Dower et al., 1988). Cells to be transformed were first thawed on ice, 500 ng DNA in 10 μ1 of water was added, gently mixed and transferred to a pre-chilled 0.2 cm gap electroporation cuvette. Cells were then plated out onto SD-his medium and grown at 28 °C for 2 days. The transformants were inoculated from a single colony into a broth for overnight culture. The transformants were screened for their ability to grow on histidine deficient medium. Small-scale expression experiments were performed to detect expression of the recombinant protein. The $his^+$ transformants were streak cultivated on BMGY (2% peptone, 1% yeast extract, 1.34% YNB, 0.4 μg/ml biotin, 1% glycerol) at 28 °C with constant shaking at 225 rpm for 24 h. Cells grown in BMGY were inoculated into 96-well plates containing 50 μl BMMY medium and 1% methanol respectively and incubated for 10 h at 28 °C, 225 rpm. Subsequently, the positive colonies were selected by enzymatic activity determination. One strain showing the highest xylanase activity was selected for further analysis. The strain was cultivated in 50 ml BMGY until the $OD_{600}$ reached 3.0. The cells were harvested and resuspended in equal volume of BMMY. To maintain induction, methanol was added to the culture to a final concentration of 1% every 24 h. After 3 days of cultivation, the xylanase activity of the cultures reached their highest level. All purification procedures were performed at 4 °C. Aliquots of culture supernatant were taken daily and examined for protein production by SDS-PAGE, and the xylanase activity was assayed at the same time.

### 2.4. Protein purification and enzyme activity assay

The culture was induced with methanol for 72 h ($OD_{600}$ = 5 – 6) and then centrifuged at 3000× *g* for 10 min at 4 °C to remove cell debris and purify the enzyme. The His-tagged protein was purified using $Ni^{2+}$-NTA Agarose (Qiagen, Valencia, CA). After purification, the production was analyzed using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized with Coomassie Brilliant Blue R-250 staining. The concentrations of soluble proteins in the filtered fermentation culture samples were determined using a Bradford assay kit.

Xylanase activity was determined by measuring the release of reducing sugar from beechwood xylan using the 3, 5-dinitrosalicylic acid (DNS) reagent (Miller, 1959). The standard assay for xylanase activity was performed at 45 °C for 10 min in citrate buffer (pH 3.8). The reaction mixture (200 μl) contained 50 μl of the diluted enzyme solution and 100 μl of 1% beechwood xylan. The reaction was stopped by the addition of 150 μl of DNS reagent. Absorbance of samples was measured at 540 nm against the substrate blank. One unit of xylanase activity was defined as the amount of enzyme required to liberate 1 μmol of xylose from xylan in 1 min under its optimal assay conditions.

### 2.5. Optimal pH and stability of xylanase

The optimal pH of the purified synthetic enzyme was determined at 45 °C in buffers with pH ranging from 2.6 to 8.0. The pH stability was estimated by measuring the residual enzyme activity after pre-incubating the enzyme in different buffers of pH 2.6–8.0 at 37 °C for 3 h; The buffers used were: 0.2 M glycine-HCl (pH 2.6–3.0), and 0.2 M $Na_2HPO_4$-citric acid buffer (pH 3.0–8.0). The xylanase activity was assayed under standard conditions.