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*Full Length Research Paper*

# Acidic xylanase II from *Aspergillus usamii*: Efficient expression in *Pichia pastoris* and mutational analysis

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**The *xyn II* cDNA encoding the Xylanase II (Xyn II) of *Aspergillus usamii* E001 was cloned into the pPIC9K vector and expressed in the methylotrophic yeast *Pichia pastoris* under the control of the alcohol oxidase I gene promoter and secreted via the α-mating factor leader of *Saccharomyces cerevisiae*. In shake-flask culture induced with methanol, the supernatant from a 96 h culture of Xyn II had specific activity of 1373.37 U/mg. The three-dimensional model and mutational analysis of the *xyn II* gene products showed that Glu 79 and Glu 170 were the important catalytic amino acid residues in the active site and Asp37 played a significant role in its low pH optimum. When Asp37 was mutated to asparagine, the optimum pH shifted to 5.3 and the maximum specific activity decreased to about 20% of that of the wild-type enzyme. This is the first report of functional expression and mutational analysis of *A .usamii* xylanase in *P. pastoris*.**

**Key words:** Xylanase, *Aspergillus usamii*, expression; *Pichia pastoris*, mutational analysis

## INTRODUCTION

Xylan is a complex low-molecular-weight polysaccharide with a backbone of β-1,4-linked β-D-xylose residues that carries various substituents. Complete breakdown of this complex polymer requires the action of several enzymes. Among them, the most important class is endo-β-1,4-xylanases (EC 3.2.1.8) which cleave internal glycosidic bonds at random or specific positions of the xylan backbone and thus hydrolyze xylan into xylooligo-saccharide and xylose. Xylanases have been attracting worldwide research interests over the past two decades due to its great potential in industrial applications, such as in the food, feed, pulping and papermaking industries. On the basis of sequence similarities and hydrophobic cluster analysis, endo-β-1,4-xylanases have been mainly grouped in families 10 and 11 of the glycosyl hydrolases (Collins et al., 2005; Jeya et al., 2009; Sibtain et al., 2009). Family 10 xylanases exhibit eightfold α/β barrel structures and have molecular masses>30,000, whereas family 11 xylanases have an all β-strand sandwich fold structure resembling a partly closed “right hand” and a lower molecular mass of ~20,000 (Hakulinen et al., 2003; Zhou et al., 2009). Xylanases of both families hydrolyze xylan by a double displacement mechanism involving two glutamic acids (Sinnott, 1990). These two carboxylic acid residues suitably located in the active site are involved in the formation of the intermediate. One acts as a general acid catalyst by protonating the substrate, while the second performs a nucleophilic attack, which results in the departure of the leaving group and the formation of the α-glycosyl enzyme intermediate (Collins et al., 2005).

It has been reported that fungal Aspergillus usamii E001 produced an acidic xylanase (Xyn II) with an optimum pH 4.6 (Fu et al., 2006). Xyn II was a family 11 xylanase with a molecular mass of 21 kDa. We have previously succeeded in cloning Xyn II gene (*xyn II*) from *A. usamii* E001 and obtaining the recombinant protein using *Escherichia coli* expression system (Zhou et al., 2008). Though Xyn II was secreted into the periplasm and was soluble, the *expression* level was low. The methylotrophic yeast *Pichia pastoris* has several advantages over *E. coli* as a host system for heterologous

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**Table 1. PCR** primers used in this study.

| Primer name | Sequence (5′→3′) | Primer name | Sequence (5′→3′) |
|---|---|---|---|
| XynII-F | C*G*GAATTCAGTGCCGGTATCAACT ATG | XynII-R | ATTTGCGGCCGCTTAAGAAGATATCGT GAC |
| D37N-F | CAGTTCC**AAC**TTCGTCGTTG | D37N-R | CGACGAA**GTT**GGAACTGACT |
| D37S-F | CAGTTCC**TCT**TTCGTCGTTG | D37S-R | CGACGAA**AGA**GGAACTGACT |
| D37E-F | CAGTTCC**GAA**TTCGTCGTTG | D37E-R | CGACGAA**TTC**GGAACTGACT |
| E79Q-F | CAGGCC**CAG**TACTACATCG | E79Q-R | TGTAGTA**CTG**GGCCTGAGG |
| E79D-F | CAGGCC**GAT**TACTACATCG | E79D-R | TGTAGTA**ATC**GGCCTGAGG |
| E170Q-F | TGGCGGTG**CAA**GCATGGAAC | E170Q-R | TCCATGC**TTG**CACCGCCATGA |
| E170D-F | TGGCGGTG**GAT**GCATGGAAC | E170D-R | TCCATGC**ATC**CACCGCCATGA |

Restriction enzyme and mutagenesis sites are underlined and bold, respectively.

expression because of its high secretion efficiency, high cell densities attained in inexpensive culture media, and the relative ease of scale-up to industrial processes (Delroisse et al., 2005; Manuel et al., 2005; Xu et al., 2009; Li et al., 2010). Therefore, this study was aimed to functionally express the *A. usamii* Xyn II in *P. pastoris*. Furthermore, the three-dimensional model and mutational analysis of the xylanase have facilitated identification of key amino acid residues responsible for the catalytic mechanism and its low pH optimum, along with their locations.

## MATERIALS AND METHODS

### Strains, plasmids and culture media

*E.* coli DH5α (Invitrogen, Carlsbad, CA) was used as host for plasmid cloning experiments. Bacteria were grown in Luria-Bertani (LB) medium [contained (in g/l): yeast extract 5, tryptone 10, NaCl 10, pH7.0] at 37℃.

P. pastoris GS115 (Invitrogen, Carlsbad, CA) was used as host for heterologous expression of the xylanase. For *P. pastoris* cultivations, the following media were used at appropriate steps. It was grown and maintained in yeast peptone dextrose (YPD) medium [contained (g/l): yeast extract 10, peptonel 10 and dextrose 20] at 30℃; $His^+$ transformants were recovered on minimal dextrose (MD) plates [contained (g/l): yeast nitrogen base without amino acids (YNB) 13.4, biotin 0.0004, dextrose 20, agar 15]; For Xyn II expression, recombinant strains were cultured in buffered minimal glycerol (BMGY) medium [contained (g/l): YNB 13.4, biotin 0.0004, glycerol 10 and was supplemented with 0.1 M potassium phosphate, pH 6.0] to generate biomass and buffered minimal methanol (BMMY) medium [identical to BMGY, except that the glycerol was replaced by 0.5% (v/v) methanol] for induction.

The plasmid pMD19-T-*xyn II* containing Xyn II gene (*xyn II*) from *A.* usamii E001 was prepared by our laboratory. Plasmid pPIC9k used as expression vector, was purchased from Invitrogen (Carlsbad, CA).

### Reagents

DNA Gel Extraction Kit, Taq polymerase, restriction enzymes and protein marker were purchased from TaKaRa Biotechnology (Dalian, China) Co. Ltd. Birchwood xylans, D-xylose and bovine serum albumin were purchased from Sigma Chemical Co., USA. Culture media were obtained from Invitrogen and Shanghai Sangon Co., Ltd. All other chemicals were of analytical grade.

### DNA manipulations and analyses

Standard molecular cloning techniques were performed as described by Sambrook (Sambrook et al., 1989). Polymerase chain reactions (PCRs) were done in a thermal cycler (GeneAmp PCR system 2400; PE Applied Biosystems, Foster City, CA, USA). The nucleotide sequences were sequenced by Invitrogen (Shanghai, China) Co., Ltd.

### Generation of Xyn II mutants

In planning the mutations, Swiss-Pdb Viewer (http://www.expasy.ch/spdbv/) and Insight II Molecular modeling software package version 2000 (Accelrys Inc., USA) were used as tools to examine the xylanase structure and to ensure that the mutations are not likely to cause conformational change.

### Construction of yeast expression plasmids

### Plasmid containing the xyn II gene

The plasmid pMD19-T-*xyn II* vector was used as a template to amplify Xyn II cDNA by PCR with the forward primer XynII-F and reverse primer XynII-R (Table 1). PCR was performed in 50 µl volume containing 2 ng of template DNA, 10 mM of each dNTP, 1 µM of forward primer XynII-F, 1 µM of reverse primer XynII-R and 2.5 units of DNA polymerase. Amplification conditions were: denaturation at 94℃ for 2 min firstly; 30 cycles of (30 s at 94℃, 30 s at 55℃, 45 s at 72℃); followed by 10 min at 72℃. The amplified

fragment was gel-purified and digested with *Eco*RI and *Not*I, and then inserted into the *Eco*RI/*Not*I site of pPIC9K resulting in the recombinant plasmid pPGX which was confirmed by restriction analysis and nucleotide sequence analysis.

**Mutant plasmids**

Site-directed mutagenesis was performed using the overlap extension PCR methods as described previously (Ho et al., 1989). For the first-round PCR, the XynII-F forward or XynII-R reverse external primer was used along with equimolar amounts of reverse or forward internal mutagenic primer (Table 1), respectively. Mutant fragments were amplified using plasmid pMD19-T-*xyn II* as a template, through 30 cycles of (30 s at 94°C, 30 s at 52°C, 30 s at 72°C). Two amplified fragments having overlapping ends were precipitated by ethanol, redissolved, and combined. Subsequent 3′extension of the complementary strand consisted of 10 cycles of (30 s at 94°C, 30 s at 55°C, 45 s at 72°C). Each product was amplified further by the second round PCR using the XynII-F forward and XynII-R reverse primers. The amplified fragments, each carrying mutation that corresponded to D37N, D37S, D37E, E79D, E79Q, E170D and E170Q, were digested with *Eco*RI and *Not*I and cloned into pPIC9K to yield mutant plasmid pPGX-D37N, pPGX-D37S, pPGX-D37E, pPGX-E79D, pPGX-E79Q, pPGX-E170D and pPGX-E170Q, respectively. DNA sequencing was again performed to verify that there were no other changes other than at the described codons.

**Transformation of P. pastoris**

The resulting plasmids were linearized by *Sac*I to favor integration via homologous recombination at the *AOX1* locus. The linearized plasmids were purified by phenol-chloroform extraction and ethanol precipitation. The purified DNA fragments, about 200 ng, were dissolved in 10 µl of $ddH_2O$, respectively. Electrocompetent *P. pastoris* strain GS115 was prepared according to the method described in the Manual Version A of the *Pichia* Multi-Copy Expression Kit (Invitrogen, Carlsbad, CA). Eighty microliters of competent cells were mixed with 10 µg linearized recombinant plasmids in a 0.1 cm electroporation cuvette. The mixtures were incubated on ice for 5 min, and the electroporation was carried out on a Bio-Rad Gene Pulser II (Hercules, CA, USA) with the following settings: 1.5 kV voltage, 25 µF capacitance, and 200 Ω resistance. After pulsing, 1.0 ml 1 M ice-cold sorbitol was added immediately to the cuvette. The cells were transferred into a 1.5 ml sterile tube and incubated at 30°C without shaking for 1 h. Then the transformed cells were plated on MD plates and incubated at 30°C for at least 2 days. As the corresponding controls, yeast cells were also transformed with the vector pPIC9K using the same methods. For xylanase production in shake-flask cultures, a single-copy transformant of each construct was selected according to Brunel et al. (2004).

**Recombinant protein expression**

Single colonies of the transformants were initially inoculated into a flask containing 25 ml BMGY medium. After 24 h at 30°C and 250 rpm, the cultures were centrifuged at 3000 rpm for 5 min and resuspended by 50 ml BMMY medium to induce expression. The cells were allowed to grow for 96 h at 30°C, and methanol was added every 24 h to a final concentration of 0.5% (v/v) for inducing expression of the target protein. Culture aliquots were collected and cells were removed by centrifugation at 3000 rpm for 10 min. The supernatant fluids were assayed for the xylanase activity.

**Enzyme and protein assays**

The reaction mixture consisted of 2.4 ml of a 0.5% (w/v) suspension of birchwood in deionized water and 0.1 ml of a suitably diluted enzyme solution in 50 mM $Na_2HPO_4$-citrate. After incubation at 50°C for 15 min, reducing sugars were determined by the dinitrosalicylic acid methods (Miller, 1959). The absorbance of the supernatant was measured at 540 nm. As standard, different concentrations of monomeric D-xylose, ranging from 0–4 mM prepared in the same conditions were used. One unit (U) of xylanase activity was defined as the amount of enzyme that liberated 1 µmol of xylose equivalents from xylan per minute. Protein was measured by the method of the Bradford assay (Bradford, 1976) using bovine serum albumin as a standard. The results were means of duplicate determination on triple independent measurements.

**Enzyme purification**

After 96 h growth, cultures were centrifuged at 10,000 rpm for 10 min and the supernatant was used as enzyme source. The crude xylanase was precipitated with ammonium sulfate (60% saturation) followed by centrifugation at 10,000 rpm for 15 min at 4°C. The precipitated proteins were then resuspended in $Na_2HPO_4$-citric acid buffer (0.2 mol/L $Na_2HPO_4$, 0.1 mol/l citric acid; pH 6.0). Desalting was performed on a Sephadex G-25 column. The fractions containing xylanase were applied onto a Sephadex G-100 column and eluted with the same buffer, and then fractions containing the enzyme were pooled and concentrate. No significant loss of activity was seen even after 6 months′ storage.

**Enzyme properties**

The pH profile of purified xylanase was evaluated by incubating the enzyme for 15 min in the presence of appropriate buffers: $Na_2HPO_4$-citric acid buffer (pH 3.0–7.0), $KH_2PO4$-NaOH buffer (pH 8.0) and glycine-NaOH buffer (pH 9.0). The activity of each sample was then quantified using the DNS assay. Further study on the pH stability of the recombinant xylanase was carried out at 50°C by pre-incubation of the enzyme solutions in the aforementioned buffer systems in the absence of substrate at 40°C for 1 h. The pH values of various reaction solutions were adjusted to the optimum pH. Then they were subjected to xylanase activity assay.

The optimal temperature of purified xylanase was determined by incubating the enzyme for 15 min at different temperatures ranging from 30 to 60°C. For thermostability, the enzyme solutions were incubated at 50°C in the absence of substrate for various times. The residual xylanase activity was measured using the DNS assay.

Kinetic parameters were determined by non-linear regression, using the Michaelis-Menten equation, of initial rates between 1.25 and 30.0 mg/ml of soluble birchwood xylan. The inability to measure activity at higher concentrations due to substrate interference and high viscosity meant that substrate saturation was not achieved and, thus, extrapolation was used in the determination of the kinetic values, giving rise to apparent values.

**SDS-PAGE**

SDS-PAGE was done according to the method of Laemmli (Laemmli, 1970) using 15% acrylamide separating gel and 5.0% acrylamide stacking gel. Gels were stained for protein with Coomassie Brilliant Blue R-250. Molecular weight of test protein(s) was compared with standard protein marker.

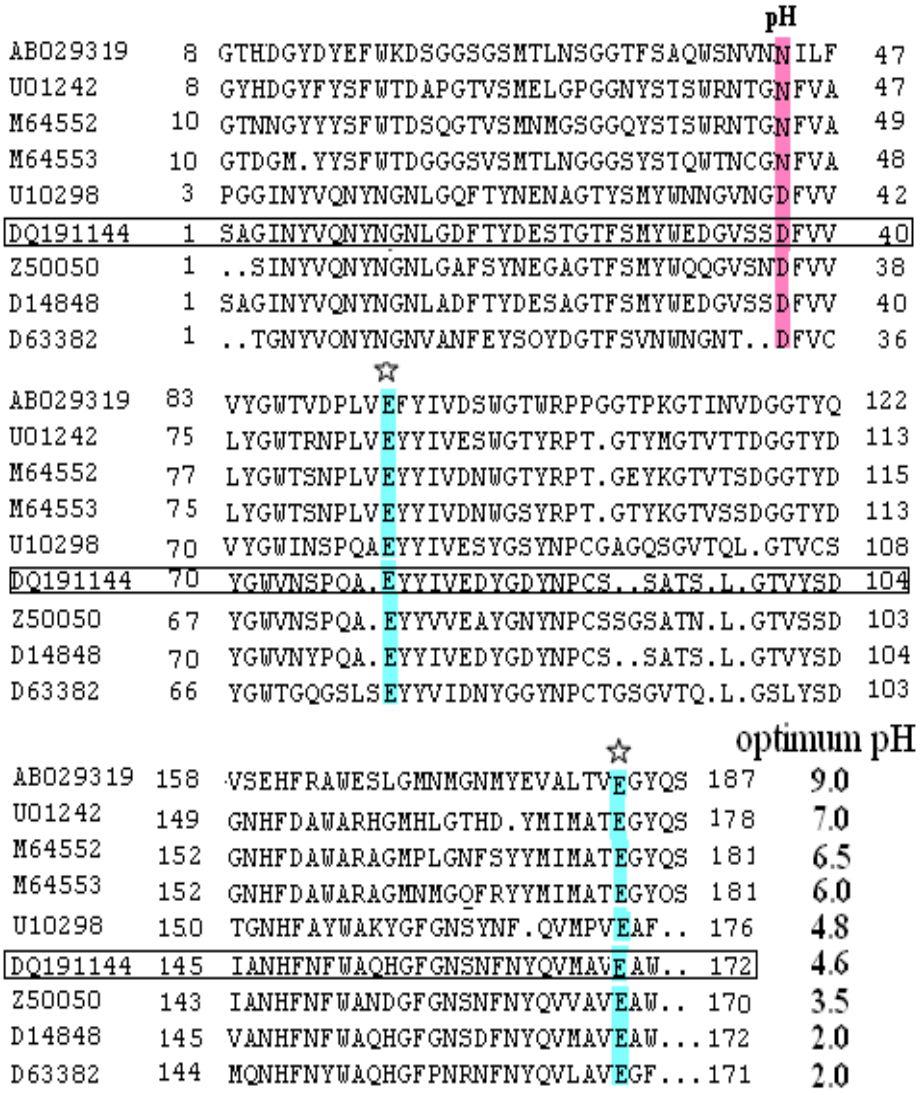


**Figure 1.** Alignment of *xyn II* (DQ191144) with other family 11 xylanases. The genes were indicated by Genbank accession number, which included AB029319 from *Bacillus* sp. 41M-1 (Nakai et al., 1994), U01242 from *Thermomonospora fusca* (Irwin et al., 1994), M64552 from *Streptomyces lividans* (Shareck et al., 1991), M64553 from *Streptomyces lividans* (Shareck et al., 1991), U10298 from *Aureobasidium pullulans* (Li and Ljungdahl, 1994), DQ191144 from *Aspergillus usamii* (Zhou et al., 2007), Z50050 from *Penicillium purpurogenum* (Diaz et al., 1997), D14848 from *Aspergillus kawachii* (Ito et al., 1992), D63382 from *Cryptococcus* sp.S-2 (Lefuji et al., 1996).

**Amino acid numbering**

The standard amino acid numbering used in the text started at the N-termini of the precursor proteins (Figure 1).

## RESULTS AND DISCUSSION

### Design and generation of Xyn II mutants

The amino acid sequences of the catalytic domains of eight family 11 endo-β-1, 4-xylanases have been aligned with Xyn II (Figure 1), which indicated that only two glutamic acid residues were absolutely conserved in this family of xylanases. The enzymes showing a pH optimum below 5 had an aspartate at position 37, while an asparagine was present in those with pH optima of 5 or more. Analysis of the three-dimensional structures showed that in other xylanases with low pH optima, Asp37 (Figure 2) was located at hydrogen-bonding distance from the acid-base catalyst. On the other hand, in the structures of xylanases with relatively high pH optima, this residue was replaced with asparagine and located relatively far away from the acid-base catalyst, glutamic acid. Hence Asp37 was expected to critically

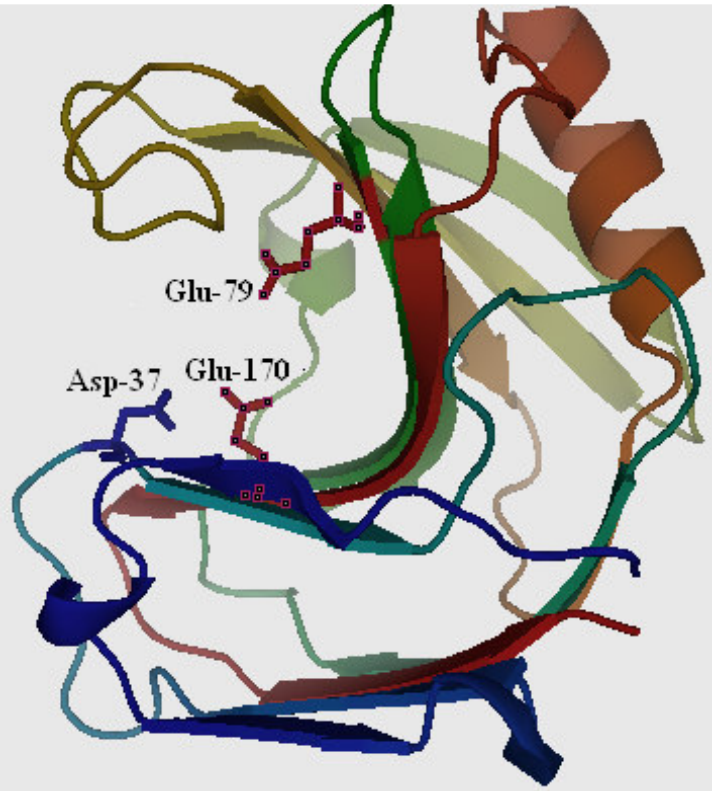


**Figure 2.** Structural prediction of Xyn II.

influence the pH dependence of xylanase activity. So the two conserved glutamic acid residues, Glu79 ad Glu170, which could be involved in catalysis of Xyn II and Asp37 were targeted for site-directed mutagenesis

## Structural prediction of Xyn II

In order to generate xylanase mutants, the three-dimensional structure of *A. usamii* xylanase II was determined by using Insight II Molecular modeling software (Figure 2). The Xyn II structure consisted of a single domain comprising two β-sheets and a single α-helix, which was unique for family 11 xylanases. The overall structure of the molecule resembled a “right hand”. In Figure 2, the Xyn II structure was represented as “fingers” at the bottom, the “palm” at the right-hand side, and a “thumb” at the top. The two conserved residues (Glu79 and Glu170) and the residue Asp37 studied in this research were shown in the cleft.

## Expression of Xyn II in *P.* pastoris

The gene encoding Xyn II from *A. usamii* was successfully engineered for expression into the heterologous host *P. pastoris*. Recombinant Xyn II, expressed in *P. pastoris* by fusing to the *Saccharomyces cerevisiae* α-factor secretion signal peptide, accumulated as active enzyme in the culture broth. The supernatant from a 96 h culture of Xyn II had specific activity of 1373.37 U/mg. No activity was detected in the control GS115 (pPIC9K) transformed with the vector only. SDS-PAGE of purified recombinant Xyn II revealed a single band with a molecular mass of 21 kDa (Figure 3) which was greater than that of *E. coli* (Zhou et al., 2008), but consistent with that of *A. usamii* (Fu et al., 2006). It is probable that the recombinant enzyme was *O*-linked glycosylated since there was no putative N-linked glycosylation site in the deduced amino acid sequence of *A. usamii* Xyn II (Zhou et al., 2008).

## Properties of recombinant Xyn II

Purified recombinant Xyn II was used to evaluate its biochemical properties. Under assay conditions used, the recombinant enzyme showed maximal activity at pH 4.2 (Figure 4a), and the pH-activity profile was similar to that of the native enzyme with an optimum pH of 4.6 (Fu et al., 2006). There are small differences in pH optimum between the recombinant Xyn II expressed by *P. pastoris* and the native Xyn II from *A. usamii* E001, probably due to the differences in posttranslational modifications of the two expression hosts. At 40℃, in the absence of substrate, the enzyme was stable between pH 3.0 and pH 7.5 for 1 h (Figure 4b). The apparent optimal

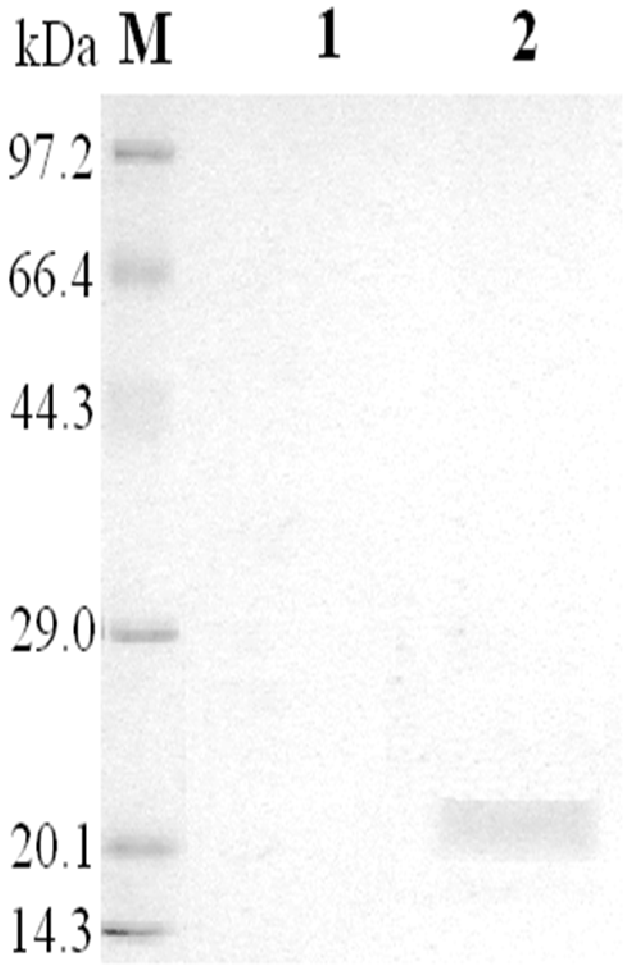


**Figure 3**. SDS-PAGE analysis of recombinant Xyn II secreted by *P. pastoris*. Lane 1: culture supernatants of negative control; Lane 2: purified Xyn II; Lane M: protein markers with the following molecular weight standards: phosphorylase b (97,200), bovine serum albumin (66,400), ovalbumin (44,300), carbonic anhydrase (29,000), soybean trypsin inhibitor (20,100), lysozyme (14,300).

temperature of the recombinant enzyme at pH 4.2 was 50℃ (Figure 5a) which was consistent with the native enzyme. At pH 4.2, in the absence of substrate, the enzyme was stable under 50℃ for 15 min, but lost 50% of its original activity in 40 min (Figure 5b).

**Mutagenic analysis of Glu79 and Glu170**

An analysis of a primary sequence alignment of nine family 11 xylanases indicated that only two glutamic acid residues were absolutely conserved in this family of xylanases (Figure 1). When the glutamic acid residues of Xyn II were mutated to glutamine (E79Q and E170Q), no enzyme activity could be detected; however, when the carboxylate side chain was maintained but the side-chain length was shortened in E79D and E170D, some residual activity was observed. The large decrease in activity seen with the mutant enzymes E79D and E170D, 0.05 and 0.19% residual activity respectively (Table 2), strongly suggested that E79 and E170 were the catalytic amino acid residues. There was only a minor increase in the $K_m$ for the E170D enzyme and no change in the $K_m$ for the E79D enzyme (Table 2). This indicated that the active site structure of the enzyme must be maintained in these mutants and the major effect of these mutations was on catalysis rather than substrate binding. Compared to the two conserved glutamic acid residues which have been shown to be involved in catalysis in other members of family 11 xylanases (Bray and Clarke, 1994; Wakarchuk et al., 1994), we would propose that Glu 79 was the nucleophile and Glu170 was the acid-base catalyst in the

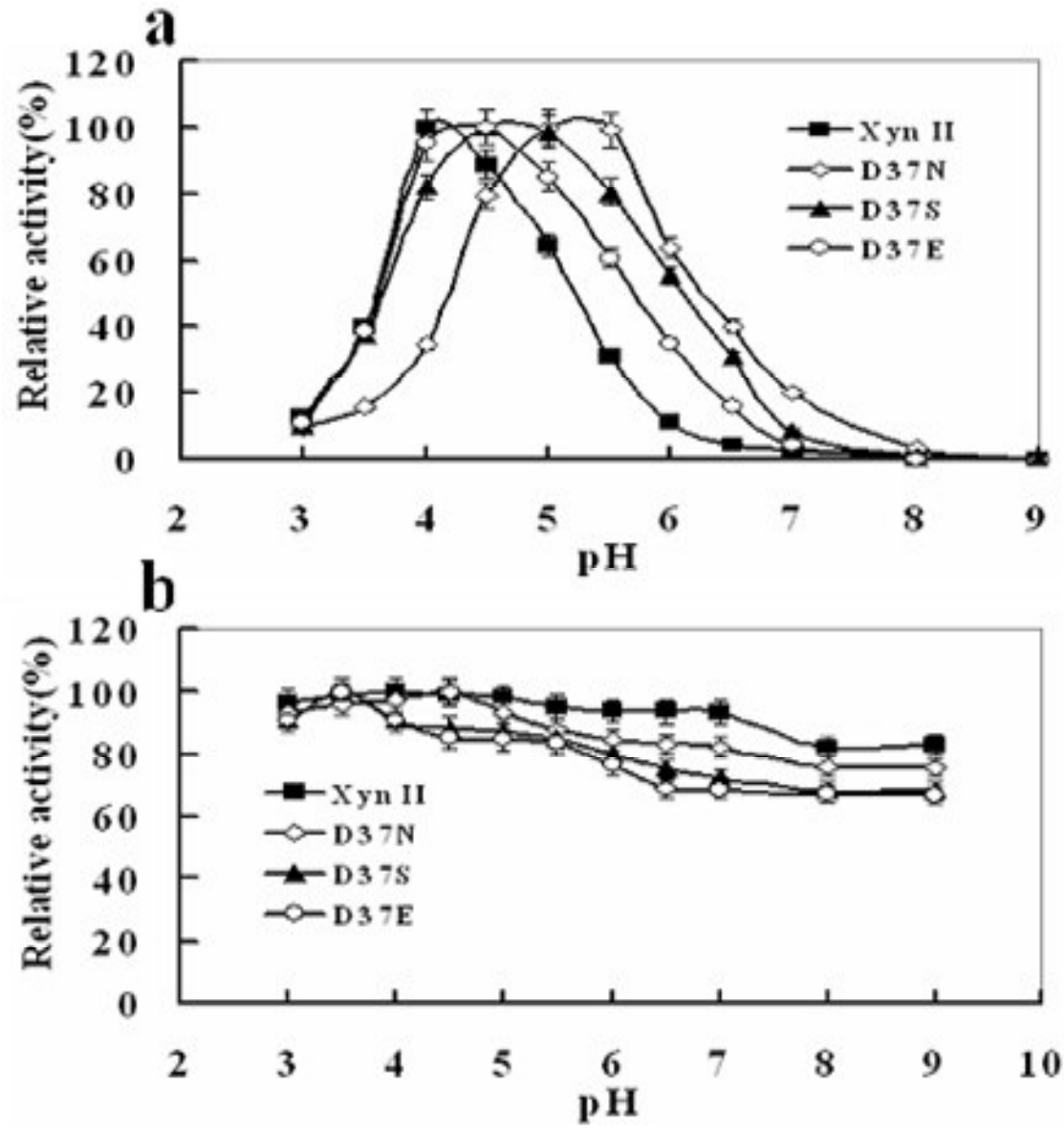


**Figure 4.** Effect of pH on the activities (a) and stabilities (b) of the wild-type and mutant xylanases. Relative activity was calculated at indicated pH using the maximal activity as 100%.

catalytic reaction. The two catalytic residues are shown in red in Figure 2, and Glu79 was shown on the upper side and Glu170 on the lower side. Both residues extended their side-chains to the bottom of the cleft from opposite sides.

## Mutagenic analysis of Asp37

An Asp37 residue of the *A. usamii* Xyn II was suggested to be critical for its low pH optimum. Availability of the *P. pastoris* expression system and the three-dimensional structure of the *A. usamii* Xyn II allowed us to determine whether the corresponding Asp37 residue was responsible for the acidophilicity by means of site-directed mutagenesis and to map its position at the bottom edge of the active site cleft (Figure 2).

Asp37 of Xyn II was replaced with Asn (D37N), Ser (D37S) and Glu (D37E) by site-directed mutagenesis. The activities of the mutant enzymes were shown in Table 2. Although, the maximum specific activity of the D37N mutant was decreased to about 20% of that of the wild-type Xyn II, its optimum pH was shifted to 5.3, as expected (Figure 4a). It is likely that in the enzymatic reaction, the acid-base catalyst Glu170 acted as a general acid by donating a proton to the substrate oxygen and the protonated state of Glu170 was stabilized through a hydrogen bond with Asp 37. When the

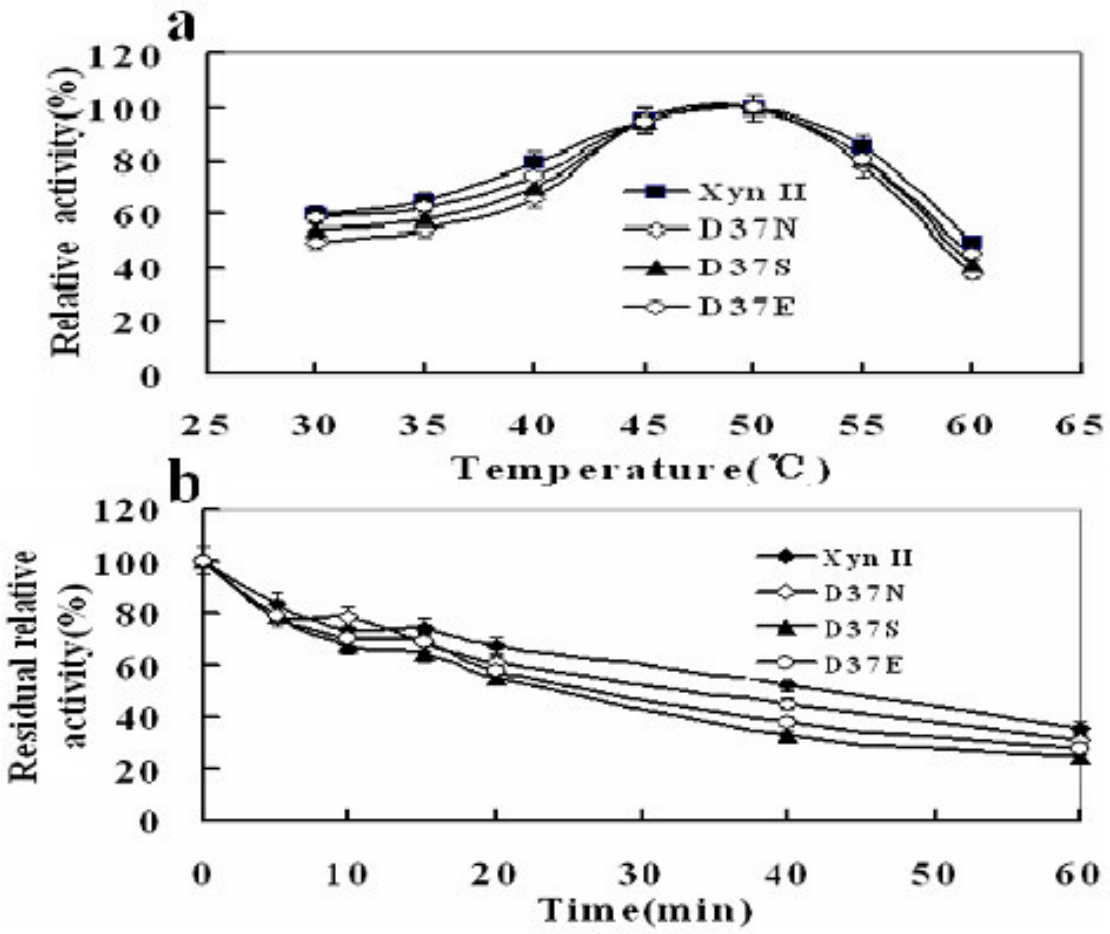


**Figure 5.** Effect of temperature on the activities (a) and stabilities (b) of the wild-type and mutant xylanases. Relative activity was calculated as enzymatic activity at indicated temperature using the maximal activity as 100%. Residual relative activity was determined as enzymatic activity at indicated time using the activity at time zero as 100%.

**Table 2.** Enzymatic properties of various mutants.

| Enzyme | Enzyme activity (%)[a] | $K_m$ (mg ml$^{-1}$) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$mg$^{-1}$ml) |
|---|---|---|---|---|
| Xyn II (wild type) | 100 | 4.5±0.31[b] | 455±5.4 | 101 |
| E79Q | 0 | n.d.[c] | n.d. | n.d. |
| E79D | 0.05 | 4.7±0.48 | 0.24±0.0020 | 0.051 |
| E170Q | 0 | n.d. | n.d. | n.d. |
| E170D | 0.19 | 5.5±0.30 | 1.1±0.015 | 0.20 |
| D37N | 20 | 4.6±0.52 | 6.3±0.50 | 1.37 |
| D37S | 12 | 5.1±0.43 | 4.8±0.20 | 0.94 |
| D37E | 2.5 | 6.1±0.46 | 2.1±0.15 | 0.34 |

[a] 100% activity was 1373.37 U/mg.[b] The confidence levels are the averages of the standard deviation.[c] Not detected.

aspartate was replaced by asparagine which was located relatively far away from Glu170, their interaction prevented the proton of Glu170 from dissociating. The D37S mutant also showed decreased activity (about 12%) and a higher pH optimum 4.7 (Figure 4a). Although, a glutamic acid residue has a carboxyl moiety, the D37E mutant exhibited very low activity (about 2.5%) and a pH optimum of 4.3 (Figure 4a). It is likely that the longer side-chain of the glutamic acid residue could not interact with the acid-base catalyst and/or caused steric hindrance at

the edge of the cleft that interfered with the substrate binding. Furthermore, this residue was important for the pH stability of the protein, as shown in Figure 4b. At 40°C, in the absence of substrate, the mutants maintained 90% activity for 1 h between pH 3.0 and pH 5.5 which was narrower than that of the wild-type Xyn II (Figure 4b). However, the replacements of Asp37 had little effect on the optimal temperature and thermostability of the enzyme (Figure 5). Compared with the wild-type, only the $K_m$ of mutant D37N remained unchanged, or very slightly increased, while all other mutants appeared to have an apparently increased $K_m$ and all mutations resulted in large decreases in catalytic efficiency (Table 2). Therefore, mutational analysis of Asp37 of Xyn II revealed that the amino acid residue at position 37 had to be aspartate for the expression of high activity at low pH.

## Conclusions

In conclusion, this is the first report of the functional expression of *A.* usamii xylanase in *P. pastoris*. Comparative analysis of Xyn II and other family 11 xylanase sequences and their three dimensional structures, several amino acids were targeted for site-directed mutagenesis. The mutational analysis of the recombinant xylanase suggested that Glu 79 and Glu 170 were the important catalytic amino acid residues and we would propose that Glu 79 was the nucleophile and Glu170 was the acid-base catalyst in the enzymatic reaction. Subsequent studies showed the Asp37 residue, at the edge of the active site cleft, played a significant role in its low pH optimum.

## ACKNOWLEDGEMENTS

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