Heterologous Expression of Xylanase II from *Aspergillus usamii* in *Pichia pastoris*

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Summary

To efficiently produce xylanase for food processing industry, a gene encoding xylanase II (XynII) from *Aspergillus usamii* has been cloned into the vector pPIC9K and integrated into the genome of *Pichia pastoris* KM71 by electroporation. By means of minimal dextrose (MD) plates and PCR, the recombinant *P. pastoris* strains (His^+Mut^s) have been obtained. Activity assay and SDS-PAGE demonstrate that XynII was extracellularly expressed in *P. pastoris* with the induction of methanol. In shake flask culture, the xylanase activity was up to 1760 U/mL, with the specific activity of 3846.83 U/mg. The optimal pH and temperature of the recombinant XynII were pH=4.0 and 50 °C, respectively. The xylanase was stable below 50 °C and within pH=3.0–5.0. The molecular mass of the recombinant protein was estimated to be 21 kDa by SDS-PAGE. This enzyme had K_m of 4.55 mg/mL, v_{max} of 15.15 mM/s and k_{cat} of 455 s^{-1}. Its activity was increased by EDTA and Ca^{2+} ions, but strongly inhibited by Mn^{2+} and Fe^{2+} ions. This is the first report demonstrating the possibility of mass production of *A. usamii* protein using *P. pastoris*.

Key words: Aspergillus usamii, characterization, expression, xylanase, *Pichia pastoris*

Introduction

Xylan, a polymer consisting primarily of β-1,4-linked xylose residues, is the main constituent of plant hemicellulose. Several enzymes such as endoxylanase, β-xylosidase, α-glucuronidase, α-arabinofuranosidase and esterase are involved in the hydrolysis of xylan polymers. Among them, the most important one is the β-1,4--xylanase (EC 3.2.1.8), which cleaves internal glycosidic bonds at random or specific positions of the xylan backbone and thus hydrolyzes xylan into xylooligosaccharide and xylose (*1*). The glycoside hydrolases have been classified based on amino acid sequence similarities among the catalytic domains (*2*). Until now, at least 110 different families have been identified, with xylanase belong-

ing to families 5, 7, 8, 10, 11 and 43 (*3*). Xylanases have attracted considerable research interest because of their potential applications in various industries. For instance, in the animal feed industry, xylanases are used to increase the body mass of the animals (*4–6*). In food industry, xylanases are used to clarify beer or juices, decrease the dough viscosity while increasing the bread volume and shelf life (*7–10*). Xylanases are found in plants, algae, insects, protozoans, and microorganisms (*11*). In recent years, many kinds of xylanases have been cloned, and expressed in heterologous hosts (*12–14*).

We have previously succeeded in cloning the xylanase gene (*xynII*) from *Aspergillus usamii* E001 and obtaining the recombinant protein using *E. coli* expression system (*15*). However, the expression level of this xyla-

nase is too low for industrial application. The methylotrophic yeast *Pichia pastoris* has been developed into a highly successful system for the large-scale production of a variety of recombinant proteins (16–21). Generally, the exogenous gene is expressed under the control of alcohol oxidase 1 (*AOX1*) promoter, which is highly inducible by methanol. As an eukaryote, it has many advantages of higher eukaryotic expression systems such as protein processing, protein refolding and posttranslational modifications, while being as easy to manipulate as *E. coli* (17). Besides, it has the added advantage of 10- to 100-fold higher heterologous protein expression levels. Heterologous expression in *P. pastoris* can be either intracellular or secreted (22). Thus, the objective of this study is to determine if *P. pastoris* can be used to produce recombinant xylanase in an inducible or constitutive way, and to characterize the properties of the expressed protein.

So far, to our knowledge, the direct expression of a xylanase from *A. usamii* in *P. pastoris* using either a secreted or an intracellular expression system has never been reported, so this is the first report on the expression of a xylanase gene from *A. usamii* in *P. pastoris*.

Materials and Methods

Strains, plasmids and culture media

Escherichia coli DH5α (Invitrogen, Carlsbad, CA, USA) was used as a host for plasmid cloning experiments. Bacteria were grown in Luria-Bertani (LB) medium, containing (in g/L): yeast extract 5, tryptone 10 and NaCl 10, at pH=7.0 and 37 °C.

Pichia pastoris KM71 (Invitrogen, Carlsbad, CA, USA) was used as a host for heterologous expression of xylanase. It was grown and maintained in yeast peptone dextrose (YPD) medium, containing (in g/L): yeast extract 1, peptone 1 and dextrose 2, at 30 °C. For XynII expression, recombinant strains were cultured in buffered minimal glycerol medium (BMG), containing (in g/L): biotin 0.0004, yeast nitrogen base without amino acids (YNB) 13.4 and glycerol 10, supplemented with 0.1 M potassium phosphate, pH=6.0, to generate biomass, and in buffered minimal methanol medium (BMM), containing (in g/L): biotin 0.0004, YNB 13.4, supplemented with 20 mL/L of methanol and 0.1 M potassium phosphate, at pH=6.0 for induction.

The plasmid pMD19-T-*xynII* containing *xynII* from *A. usamii* E001 was prepared in our laboratory. Plasmid pPIC9K, used as expression vector, was purchased from Invitrogen (Carlsbad, CA, USA).

Nucleic acid manipulation

DNA was purified and manipulated essentially as described by Sambrook *et al.* (23).

Construction of expression plasmid

The plasmid pMD19-T-*xynII* vector was used as a template to amplify xylanase by PCR with the forward primer: 5′-CGGAATTCAGTGCCGGTATCAACTATG-3′ with *Eco*RI site (underlined) and reverse primer: 5′-ATTTGCGGCCGCTTAAGAAGATATCGTGAC-3′ with *Not*I

site (underlined). The PCR parameters were: denaturation at 94 °C for 2 min, 30 cycles (30 s at 94 °C, 30 s at 55 °C and 45 s at 72 °C), followed by 10 min at 72 °C. The PCR reaction mixture contained 30 pmol of each primer, 2 units of Taq DNA polymerase, 20 mM Tris/HCl (pH= 8.4), 50 mM KCl, 2.5 mM MgCl$_2$, and 100 μM of each nucleotide (dNTP) in 50 μL of total volume. PCR reaction was carried out in a GeneAmp PCR system 2400 (Perkin Elmer, Norwalk, CT, USA). 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 and transformed into *E. coli* DH5α. The resultant plasmid pPGKX contained *xynII* gene under the control of *AOX1* promoter. The cloned gene was sequenced with 5′-*AOX1* promoter primer (5′-GACTGGTTCCAATTGACAAGC-3′) and 3′-*AOX1* termination primer (5′-GGCAAATGGCATTCTGACATCCT-3′), which annealed with pPIC9K sequence from both strands using an ABI 3730 automated sequencer by Shanghai Invitrogen Biotechnology Co., Ltd, Shanghai, PR China.

Transformation of P. pastoris KM71

Competent *P. pastoris* KM71 cells (80 μL) were mixed with 5–20 μg of *Sal*I-linearized pPGKX, then transferred into an ice-cold 0.2-cm electroporation cuvette and incubated in an ice bath for 5 min. After electroporation at 1.5 kV and 25 μF, 1 mL of ice-cold 1 M sorbitol was added to the cuvette immediately and 200-μL aliquots were spread on minimal dextrose (MD) medium, containing (in g/L): YNB 13.4, biotin 0.0004, and dextrose 20, and the plates were incubated at 30 °C to screen for His$^+$ transformants according to their capacity to grow in the absence of histidine. The recombinant *P. pastoris* KM71 integrated with pPIC9K was used as control.

PCR analysis of P. pastoris transformants

Isolation of total genomic DNA from yeast was performed according to the procedure described by Querol *et al.* (24). PCR amplifications were then carried out to select positive clones according to Invitrogen's recommendation with 5-μL genomic DNA and primers (5′-*AOX1* promoter primer and 3′-*AOX1* termination primer) complementary to the 5′- and 3′-regions of *AOX1* gene. PCR amplification was performed as follows: initial denaturation at 94 °C for 5 min, followed by 30 amplification cycles (30 s denaturation at 94 °C, 45 s annealing at 55 °C and 1 min elongation at 72 °C) and a final elongation at 72 °C for 10 min.

Expression of recombinant XynII in P. pastoris

The recombinant strains were grown in 100 mL of BMG medium at 30 °C and 250 rpm until the culture reached A_{600}=2.0–6.0. The cells were harvested by centrifugation at 3000 rpm and resuspended at a fivefold concentration in 20 mL of BMM medium to induce protein expression. The cells were incubated for 192 h at 30 °C and 250 rpm, and methanol was added every 24 h to give a final concentration of 2.0 % and to maintain induction. 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.

Purification of XynII

After 144 h of growth, cultures were centrifuged at 10 000 rpm for 10 min and the supernatant was used as the enzyme source. The crude xylanase was precipitated with ammonium sulphate (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 concentrated. No significant loss of activity was seen even after 6 months of storage.

Characterization of XynII

The effect of different temperatures, pH and metal ions on the purified enzyme was studied. The K_m and v_{max} of the purified enzyme were also determined. All the experiments were done in triplicate and the mean values with standard errors were reported.

pH optimum and stability

The effect of pH on xylanase activity was evaluated at 50 °C over a pH range of 3.0–9.0, using appropriate buffers (50 mM): Na_2HPO_4-citric acid buffer (pH=3.0–7.0), KH_2PO_4-NaOH buffer (pH=8.0) and glycine-NaOH buffer (pH=9.0) under xylanase activity assay conditions. Further study on the pH stability of the recombinant xylanase was carried out at 50 °C by preincubation 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 pH=4.0, after which they were subjected to xylanase activity assay.

Temperature optimum and thermostability

The temperature optimum was measured by performing the xylanase activity assay for 15 min at temperatures ranging from 35 to 60 °C under the optimal pH=4.0. The thermostability of xylanase was investigated at temperatures of 40, 45, 50 and 55 °C after incubation of the enzyme solutions in the absence of substrate for 5, 10, 15, 20, 40 and 60 min. Residual activities were determined under xylanase activity assay conditions.

Effect of metal ions

Each metal ion (5 mM) was added to 5 mL of diluted enzyme to obtain a final concentration of 1 mM. The effects of these metals were investigated after 1 h of incubation at 30 °C.

Determination of K_m and v_{max}

For determining the reaction rate, different substrate (birchwood xylan) concentrations were used, ranging from 1.25 to 20.0 mg/mL. The reaction rate *vs.* substrate concentration was plotted to determine whether the enzyme obeys Michaelis-Menten kinetics, and K_m and v_{max} were determined from the Lineweaver-Burk plot.

Analytical methods

Gel electrophoresis was conducted according to the method of Laemmli (*25*) using m/V=15 % acrylamide separating gel and m/V=5.0 % acrylamide stacking gel. Samples were prepared in Tris-glycine buffer at pH=8.8, containing 2.0 % sodium dodecyl sulphate (SDS) and the gel sheets were stained for protein with Coomassie Brilliant Blue R-250. The standard protein markers used were: phosphorylase b (97 400), bovine serum albumin (66 200), ovalbumin (43 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (20 100), and lysozyme (14 400).

Cell dry mass per volume (g/L) was determined by drying a washed cell pellet to a constant mass at 80 °C. Cell concentration was monitored by the A_{600} value measured in triplicate and average values were reported. The linearity of the relationship was invariant at different dilution rates.

Assay of recombinant xylanase activity

The xylanase activity was determined by measuring the release of reducing sugars from birchwood xylan (0.5 %, dry mass per volume) using the dinitrosalicylic acid method (*26*). Reaction mixture containing 2.4 mL of a solution of 0.5 % birchwood xylan in 50 mM citrate buffer, pH=4.0, with 0.1 mL of the diluted enzyme was incubated for 15 min at 50 °C. One unit of xylanase was defined as the amount of enzyme required to release 1 μmol of xylose from xylan in 1 min under the assay conditions.

Results

Construction of expression vector

PCR amplification of the *xynII* cDNA rendered a unique and abundant band of 575 bp (not shown). The cDNA was subcloned into pPIC9K, resulting in the recombinant expression plasmid pPGKX (Fig. 1), which was proved to be corrected by DNA sequencing.
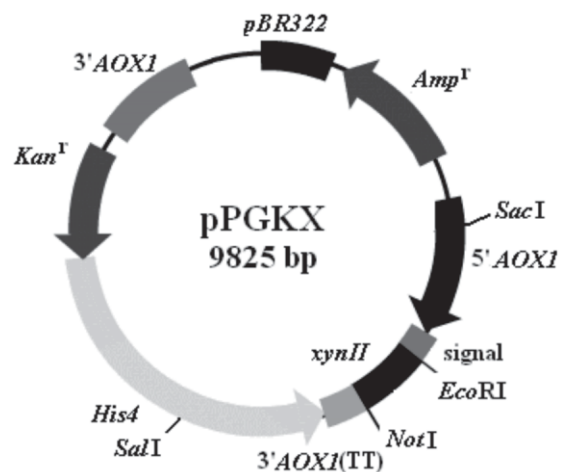

Fig. 1. Gene map of recombinant plasmid pPGKX: *AOX1*: alcohol oxidase gene; *Amp*[r]: ampicillin resistant gene; *pBR322*: *E. coli* replication origin; *Kan*[r]: kanamycin resistant gene; Signal: coding region for the secretion signal from *Saccharomyces cerevisiae* α mating factor peptide; *His4*: histidine coding gene; *xynII*: coding region for XynII

Screening of His⁺Mutˢ transformants and PCR analysis of P. pastoris transformants

Either plasmid pPGKX or pPIC9K was linearized by *Sal*I, and electroporated into the host *P. pastoris* KM71. *XynII* gene was integrated into the *P. pastoris* genome *via* single crossover between the *Sal*I-linearized pPGKX and genome. It is worth mentioning that all *His⁺* transformants in KM71 will be *Mutˢ* because of the disruption of the *AOX1* gene (*aox1::ARG4*). There is no need to test recombinants for Mut phenotype.

PCR amplification results (Fig. 2) showed that plasmid pPIC9K produced a 492-bp product; plasmid pPGKX produced a 1047-bp product containing the *xynII* gene (555 bp); positive recombinants produced the *AOX1* gene (2.2 kb) and a 1047-bp product; negative transformants only produced the *AOX1* gene (2.2 kb) from KM71 genomic DNA; control strains transformed by plasmid pPIC9K produced the *AOX1* gene (2.2 kb) and a 492-bp product.
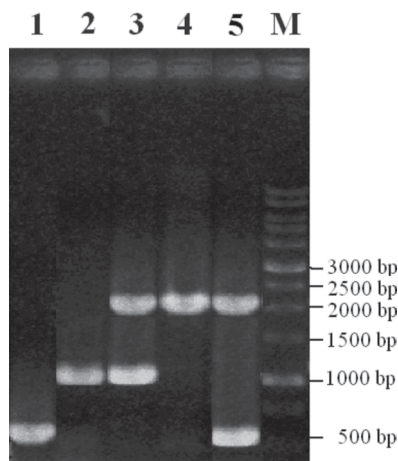
Fig. 2. PCR analysis of *P. pastoris* clones: 10 μL of a 50-μL PCR sample were run on 1 % agarose gel and stained with ethidium bromide. Lane 1: PCR product of pPIC9K; lane 2: PCR product of pPGKX; lane 3: PCR product of positive recombinants; lane 4: PCR product of negative transformants; lane 5: PCR product of control strains; lane M: DNA ladder (DL-10000)

Expression of XynII in P. pastoris

Activity assay was an efficient method to screen the best strain for the production of recombinant XynII. Among 80 positive clones, recombinant *P. pastoris* K3 possessed the highest activity, which was two- or threefold higher than those from other clones. The relationship among induction time, cell density and XynII activity is presented in Fig. 3. The activity increased steadily up to 144 h after the induction to a final value of 1760 U/mL, with the specific activity of 3846.83 U/mg.

Purification of XynII

As seen in Fig. 4, purified XynII displayed a distinctive band, with a relative molecular mass of approx. 21 kDa. Probably due to differences in glycosylation capacity of the expression hosts and (or) the assay conditions, the molecular mass of XynII expressed in *P. pastoris* is
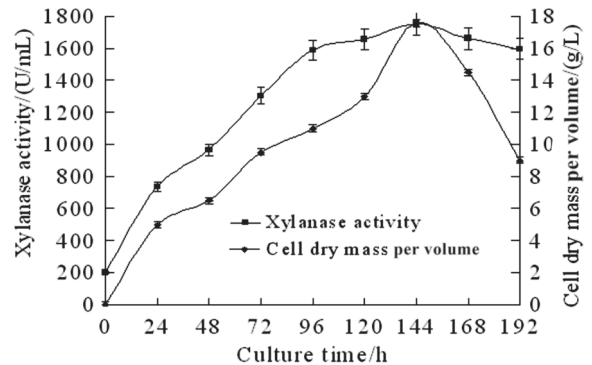
Fig. 3. The relationship between biomass and production of XynII under induction. Time 0 was the starting point for methanol induction. The values represent the means of three independent experiments
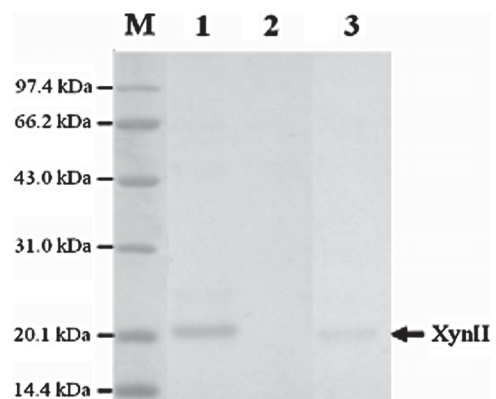
Fig. 4. SDS-PAGE of recombinant XynII secreted by *P. pastoris*. Lane M: protein molecular mass marker; lane 1: culture supernatants of recombinant *P. pastoris* KM71 (pPGKX) after induction for 144 h; lane 2: culture supernatants of *P. pastoris* KM71 transfected with plasmid pPIC9K induced as negative control; lane 3: purified XynII

greater than that of *E. coli* (*15*), but consistent with the native XynII from *A. usamii* (*27*).

Characterization of XynII

Purified xylanase was used to evaluate its biochemical properties.

Effects of pH and temperature on the activity and stability of XynII

In the present study, the pH for optimal activity of XynII was determined to be pH=4.0, with 70 % of maximum activity being retained between pH=3.0 and pH=4.7 (Fig. 5a). At 40 °C, in the absence of substrate, the enzyme was stable for 1 h over a pH range of 3.0 to 5.0. At pH values above 5.0, the enzyme was unstable (Fig. 5b).

In addition, the apparent optimal temperature for enzyme activity at pH=4.0 was 50 °C. With further increase in temperature, xylanase activity was found to decrease (Fig. 6a). Thermal stability studies of this recombinant XynII showed that at pH=4.0, in the absence of substrate, the enzyme was stable at below 50 °C. Temperatures higher than 50 °C inactivated the enzyme

quickly: the residual activity of XynII incubated at 50 °C for 40 min was only 50 % of that at 40 °C (Fig. 6b).

Effect of metal ions and chemical reagents

Several metal ions such as EDTA and Ca^{2+} could activate the enzyme, while the others (*i.e.* Mn^{2+} and Fe^{2+}) could inhibit xylanase activity significantly.

Kinetics of XynII

Substrate (birchwood xylan) concentration varied from 1.25–20.00 mg/mL in the reaction mixture. Initial reaction rates *vs.* substrate concentration (Fig. 7) showed that the enzyme obeys Michealis-Menten kinetics and had K_m of 4.55 mg/mL, v_{max} of 15.15 mM/s and k_{cat} of 455 s^{-1}.
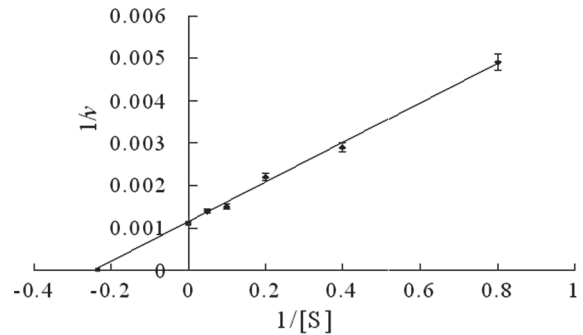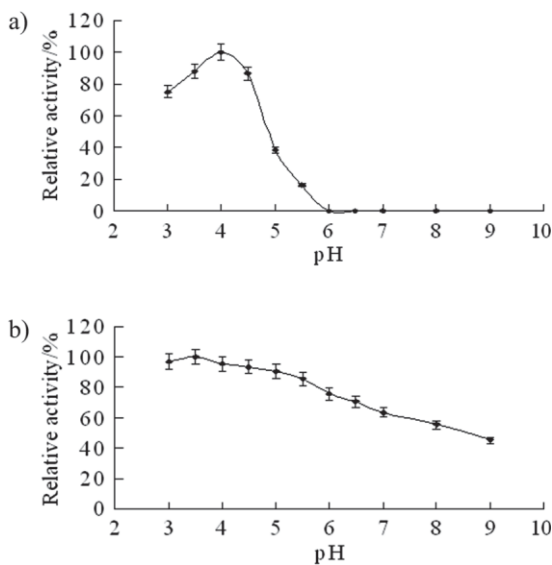

Fig. 7. Lineweaver-Burk plot

Fig. 5. Effect of pH on: (a) recombinant XynII activity, and (b) recombinant XynII stability
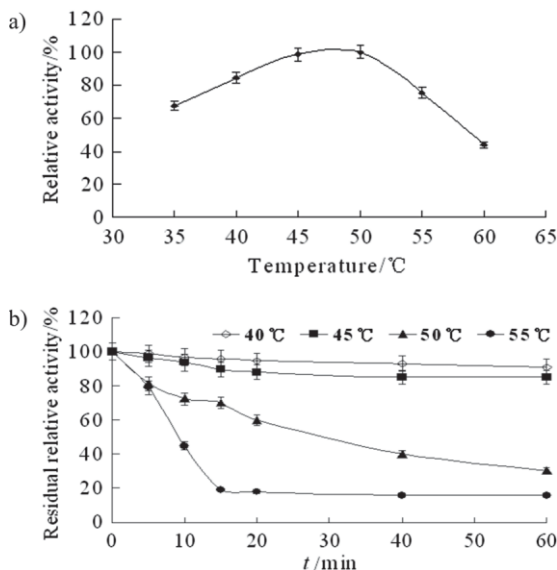

Fig. 6. Effect of temperature on: (a) recombinant XynII activity, and (b) recombinant XynII thermostability

Discussion

Even though various *Aspergillus* xylanases have been cloned and expressed (*28,29*), this is the first report on XynII from *A. usamii* in *P. pastoris*. The recombinant XynII was successfully expressed and secreted in the active form and its enzymatic properties were investigated. Compared to the XynII expressed by *E. coli* (*15*), XynII secreted by *P. pastoris* had a higher biological activity and may hold structural configuration closer to the native form because *P. pastoris* and *A. usamii*, as eukaryotic microorganisms, both have many advantages of higher eukaryotic expression systems such as protein processing, protein folding, and posttranslational modification, while as prokaryotic microorganism, *E. coli* does not have this ability. In addition, it secretes the products with fewer and lower levels of endogenous host proteins into the medium, which greatly favour the purification of target protein. We used *P. pastoris* as XynII expression system and successfully generated a genetically modified strain with maximum activity up to 1760 U/mL, with the specific activity of 3846.83 U/mg, which was higher than other *Pichia* expression systems used for *Aspergillus* xylanases (*30,31*).

In the future, structure-function analysis of the enzyme will be performed to gain a better understanding of this enzyme, which will lead to the modification of the enzyme properties by genetic engineering approach.

Conclusion

XynII gene from *A. usamii* E001 was heterologously expressed in *P. pastoris*. After induction, these strains expressed and secreted biologically active, recombinant XynII. After screening the enzymatic activity, a positive recombinant strain *P. pastoris* K3 with high expression of XynII was obtained. In shake flask culture, its xylanase activity was up to 1760 U/mL, with the specific activity of 3846.83 U/mg. The purified enzyme was a protein of 21 kDa and it was found to give its optimum activity at 50 °C and pH=4.0. The enzyme was active over a wide range of pH values and was stable below 50 °C. All these characteristics are considered favourable for industrial processing, especially in the food processing industry.

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