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ARTICLE

High-level expression and characterization of *Aspergillus niger* ATCC 1015 xylanase B in *Komagataella phaffii*

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Abstract Owing to the safety issues in food and feed industry, the GH11 xylanase B gene from *Aspergillus niger* ATCC 1015 was cloned and expressed in *Komagataella phaffii*. The highest xylanase B activity of 1827.19 U/ml was obtained after optimization of temperature, pH, and methanol addition through flask cultivation. The optimal temperature and pH were 55 °C and 5.0, respectively, and the highest relative activity of xylanase B reached 133.20% with the addition of 10 mmol/l cupric ions. Thus, the high-level recombinant xylanase B obtained in this study could have potential applications in food and feed industry.

Keywords Xylanase · *Aspergillus niger* · Food safety · Gene expression · *Komagataella phaffii*

Introduction

Xylanase hydrolyzes the β-1,4-xylan bond of hemicellulose, which contributes to 20–50% of the lignocellulosic materials, to produce xylose [1]. This enzyme is widely used in the food industry [2], textile industry [3], and bioenergy field to avoid potential inhibitors production during pretreatment of lignocellulosic materials [4]. In the food industry, xylanase enhances the rheological properties of wheat doughs [5, 6] and improves bioactives extraction from plant cell through hydrolysis of mixed enzymes. Furthermore, xylanase acts under mild temperature and does not damage heat-labile compounds [7]. Enzymatic hydrolysis of plant cells is a green approach for the extraction of new bioactive compounds, such as dietary fiber [8] and antioxidants [9]. Xylooligosaccharides, which are healthy food ingredients with positive effects on gastrointestinal flora, are obtained from xylanase-catalyzed hydrolysis [10]. Similarly, xylitol is increasingly produced from hemicellulose hydrolysis by xylanase owing to advantages such as less toxicity and low generation of inhibitors [11, 12].

Aspergillus niger is a widespread filamentous fungal species with many applications in food industry. Xylanase from *A. niger* is Generally Recognized As Safe (GRAS) according to GRN No. 589 by US Food and Drug Administration (FDA). Based on their hydrolysis characteristics, xylanases have been classified into three glycoside hydrolase families (GH10, GH11, and GH30) [13]. The GH11 xylanases are considered as true xylanases because of their substrate specificity. In addition, GH11 xylanases have advantages of small molecular size, high catalytic efficiency, and high activity at a wide pH and temperature range [14]. One of the most important challenges of xylanase application in food industry is the low production level of xylanase. For example, only 138 U/ml commercial xylanase could be produced from *Thermomyces lanuginosus* [15]. Although several researchers have developed novel xylanase-producing microbial strains to achieve high xylanase production, most of the xylanases obtained from these isolates require long procedure to be accepted as GRAS by the US FDA for food and feed applications (FDA GRN No. 675). Heterologous expression of xylanase is a general, but powerful method to obtain high amount of xylanase from various microbial species [14]. *Pichia pastoris* is a well-known microbial factory for heterologous

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gene expression, and many *Pichia*-based proteins have been approved by the US FDA [16]. High level of heterologous protein is expressed under alcohol oxidase I promoter because of its strong induced by methanol. It must be noted that the *P. pastoris* strains have been reclassified as *Komagataella phaffii* and *Komagataella pastoris* [17, 18], and currently, there are more than 70 *Pichia*-based products in market or in late-stage development [19]. For example, lipase from *P. pastoris* has been confirmed to be safe for application in edible oil degumming [20]. *Aspergillus niger* ATCC 1015 is one of the most common and safe strain in food industry (21 CFR 184.1033) [21]. Xylanase B produced by *A. niger* ATCC 1015 is a GH11 endo-β-1,4-xylanase, which is considered as a GRAS food ingredient. In the present study, *A. niger* ATCC 1015 *xynB* gene encoding xylanase B was cloned, expressed, and characterized into *K. phaffii*, resulting in high-level expression of the enzyme.

Materials and methods

Strains, vectors, and media

Aspergillus niger ATCC 1015 was purchased from American Type Culture Collection (Manassas, VA, USA). *Escherichia coli* Top 10 competent cells were purchased from Takara Biotechnology Co. Ltd (Dalian, China). *Komagataella phaffii* (formerly *P. pastoris* GS115), pPICZA, pPICZaA, and zeocin were purchased from Life Technologies (Carlsbad, CA, USA). All the restriction enzymes, DNA markers, and protein markers used were purchased from Takara Biotechnology Co., Ltd (Dalian, China). All the primers were synthesized by Sangon Biotech Inc (Shanghai, China), and all the chemicals were purchased from Sino Chemical Co. (Shanghai, China).

YPD medium contained the following (per L): yeast extract, 10 g; peptone, 20 g; glucose, 20 g; and agar powder (if necessary), 15 g. BMGY medium comprised the following (per L): yeast extract, 10 g; peptone, 20 g; PBS buffer (pH 7.0), 100 mmol; glycerol, 20 g; YNB, 13.4 g; and biotin, 4×10^{-4} g. BMMY medium was composed of the following (per L): yeast extract, 10 g; peptone, 20 g; PBS buffer (pH 7.0), 100 mmol; methanol, 5 ml; $(NH_4)_2SO_4$, 20 g; and biotin, 4×10^{-4} g. MD medium contained the following (per L): YNB, 13.4 g; biotin, 4×10^{-4} g; and glucose, 20 g. YPDS medium comprised the following (per L): yeast extract, 10 g; peptone, 20 g; glucose, 20 g; sorbitol, 1 mol; and agar powder (if necessary), 15 g.

RNA extraction, gene acquisition, and recombinant *K. phaffii* construction

Aspergillus niger RNA was extracted using RNA kit (Takara Biotechnology Co., Ltd, Dalian, China), and *xynB* cDNA was synthesized by using PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara Biotechnology Co., Ltd, Dalian, China). The primers for *xynB* amplification were designed according to the *xynB* gene [22]. *xynB* fused with α-factor signal was obtained through PCR amplification using primers: P1: 5′-ATTCTCGAGAAAAGATCGACCCCGAGCTCGACC-3′ (*Xho*I) and P2: 5′-ATTGCGGCCGCTTACTGAACAGTGATGGACG-3′ (*Not*I). The PCR was performed using Prime Thermal Cyclers PrimeG (Techne, Staffordshire, UK) as follows: 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 45 s, and 72 °C for 10 min. Subsequently, pPICZαA and the PCR product were simultaneously digested with *Xho*I and *Not*I and then mixed with T4 ligase and incubated overnight at 16 °C to obtain the vector pPICZαA-*xynB*. The constructed vector pPICZαA-*xynB* was transformed into *E. coli* Top 10 competent cells, and the recombinant cells were screened on LB agar plate with 25 μg/ml zeocin and confirmed using PCR and *xynB* gene sequencing. Then, the vector pPICZαA-*xynB* (4–5 μg) was digested using restriction endonuclease, *Dra*I, for linearization, and electro transformed into *K. phaffii* competent cells (80 μl) using Gene Pulser (Bio-Rad, Hercules, CA, USA) at 1.1 kV, 200 Ω, and 25 mF. The recombinant *K. phaffii* cells were screened on YPD plate with 100 μg/ml zeocin (and 1 ml of 1 mol/l sorbitol solution added after 3 h of cultivation).

Screening of recombinant *K. phaffii* for high xylanase B activity

A preliminary screening of recombinant *K. phaffii* cells for high xylanase B activity was conducted by staining with Congo red dye. In brief, the recombinant *K. phaffii* cells were cultivated on YPD agar plate containing 10.0 g/l xylan (Sigma, Munich, Germany) at 30 °C for 3 days. To visualize the hydrolysis zone, the agar plate was flooded with Congo red solution (1% w/v) for 15 min. The plate was further treated by flooding with 1 mol/l NaCl for 15 min after removing Congo red solution. Colonies with large diameters of clear zone were considered to exhibit high xylanase B activity. Furthermore, the activity of xylanase B was determined for conformation after liquid cultivation. Briefly, the recombinant *K. phaffii* cells were inoculated into 15-ml glass tube containing 3 ml of BMGY medium at 30 °C and 180 rpm for 24 h. Subsequently, the *K. phaffii* cells were collected by centrifugation and inoculated into 3 ml of BMMY medium to which 0.5%

methanol was added every day to induce *xynB* expression. The activity of xylanase B was measured after 72 h of induction.

Optimization of recombinant *K. phaffii* cultivation for high xylanase B activity

The recombinant *K. phaffii* cells were cultivated in 250-ml flask containing 50 ml of YPD medium at 30 °C and 200 rpm for 18–24 h up to OD_{600} of about 2.0–8.0. Subsequently, the *K. phaffii* cells were transferred into 100 ml of BMGY medium at an inoculation ratio of 4% and incubated at 200 rpm for 24 h. Then, the culture was centrifuged and the medium was replaced with 100 ml of BMMY medium, and 1.0% methanol was added every 24 h to induce *xynB* expression. The default culture conditions were pH 5.0, 30 °C, 1.0% methanol, and 4% inoculum ratio. The temperatures examined were 26, 28, 30, and 32 °C; the pH values investigated were 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5; and methanol concentrations analyzed were 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0%.

Kinetics parameters of xylanase B activity

The activity of xylanase B was determined at 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0% xylan, respectively. The K_m and V_{max} were calculated using Michaelis–Menten equation.

Effect of temperature on xylanase B activity and thermostability

The xylanase B solution (500 µl) was mixed with 500 µl of 0.2% (w/v) xylan solution in a water bath at 40, 45, 50, 55, 60, 65, and 70 °C for 15 min, respectively, to determine the effect of temperature on its activity. To ascertain the thermostability of xylanase B, the xylanase B solution was heated in a water bath at 50, 55, and 60 °C, respectively, for different time periods. Subsequently, the activity of xylanase B was determined to clarify its thermostability.

Effect of pH on xylanase B activity and stability

The xylanase B solution (500 µl) was mixed with 500 µl of 0.2% (w/v) xylan solution and 50 mmol/l Na_2HPO_4-citric acid buffer at pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0, respectively, in a water bath at 37 °C for 15 min to determine the effect of pH on its activity. To ascertain the stability of xylanase at different pH, the xylanase B solution was pretreated in 50 mmol/l Na_2HPO_4-citric acid buffer at pH 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0, respectively, for different time periods, and the activity of xylanase B was measured.

Effect of ions and chemicals on xylanase B activity

To determine the effect of ions and chemicals (KCl, $CoCl_2$, $ZnCl_2$, $CaCl_2$, NaCl, $MgSO_4$, $CuSO_4$, $LiCl_2$, $AlCl_3$, $FeSO_4$, and EDTA) on the activity of xylanase B, the xylanase B solution was pre-incubated with 5.0, 10.0, and 15.0 mmol/l of each ion and chemical or 0.5, 1.0, and 1% Tween 80, respectively, for 1 h. Subsequently, the xylanase B activity was determined, and reaction mixture without added metal ions or chemical reagents was used as control.

Determination of recombinant *K. phaffii* cell density

The cell density of recombinant *K. phaffii* cells was measured using a spectrophotometer (U-1100, Hitachi Ltd., Tokyo, Japan) against distilled water at 600 nm after appropriate dilution.

Determination of xylanase B activity

The crude enzyme solution was obtained after centrifugation for 10 min at 12,000×*g* 4 °C. The enzyme activity was measured using the crude enzyme solution. The Xylanase B activity was determined according to the Miller method [23] with modification. The xylanase B solution (500 µl) was mixed with 500 µl of 0.2% (w/v) xylan and 1.0 ml of alkaline 3′,5′-dinitrosalicylic acid solution (18.1992 g of sodium potassium tartrate tetrahydrate, 0.6313 g of 3′,5′-dinitrosalicylic acid, 0.5128 g of phenol, 0.5064 g of anhydrous sodium sulfite, and 2.096 g of sodium hydroxide in 100 ml) at 50 °C for 15 min in 50 mmol/l Na_2HPO_4-citric acid buffer (pH 6.0). The hydrolysis reaction was stopped by heating at 100 °C for 10 min. One unit of xylanase B activity was defined as the amount of xylanase B that produced reducing sugars equivalent to 1 µmol of xylose per minute.

PNGase F and Endo H treatment

The PNGase F and Endo H treatment was performed using the PNGase F and Endo H kit (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions. The recombinant xylanase (1–20 µg) was mixed with 1 µl of glycoprotein denaturing buffer (0.5% SDS, 40 mmol/l dithiothreitol) and water (if necessary) to a volume of 10 µl and subjected to denaturation at 100 °C for 10 min. Subsequently, 2 µl of 10 × GlycoBuffer II, 2 µl of 10% NP-40, 2 µl of PNGase F, 2 µl Endo H, and water (if necessary) were added to the mixture to a final volume of 20 µl and incubated at 37 °C for different time.

Statistical analysis

Statistical analysis of the obtained data was performed using Microsoft Excel 2016 (Seattle, WA, USA). One-way *t* test was used for the analysis of xylanase B activity for each dataset. The results were based on a confidence level of 95%, and *p* values lower than 0.05 were considered as significant.

Results

Cloning and expression of *xynB* in *K. phaffii* cells

After amplification, agarose gel electrophoresis and gene sequencing were employed to confirm *xynB* gene. The size of *xynB* gene without native signal sequence was about 550 bp. Sequencing of the *xynB* product revealed that the gene comprised 566 bp, which is similar to that of AY536639 in NCBI. The size of *xynB* and α-factor was about 1100 bp, and the α-factor-*xynB* was sequenced for confirmation. After linearization, transformation, and screening, 18 recombinant *K. phaffii* strains were selected for xylanase activity determination after cultivation in liquid medium. The highest xylanase B activity obtained was 143.94 U/ml (Fig. 1).

Effect of temperature on recombinant *K. phaffii* growth and xylanase B activity

During the induction phase at different temperatures (26–32 °C), the cell density of recombinant *K. phaffii* cells increased from about 6 to about 15 (OD_{600}) after 96 h of induction. Furthermore, the cell densities at different induction temperatures did not show significant difference, indicating that the effect of temperature on recombinant *K. phaffii* cells was not significant (Fig. 2A). However, the effect of temperature on the activity of xylanase B presented significant difference. The activities of xylanase B sharply increased within 48 h of induction and slowly increased during 48–96 h of induction. Moreover, the activities of xylanase B at 30 and 32 °C were higher than those at 26 and 28 °C, with highest xylanase B activity of 1209.60 U/ml noted at 30 °C (Fig. 2B).

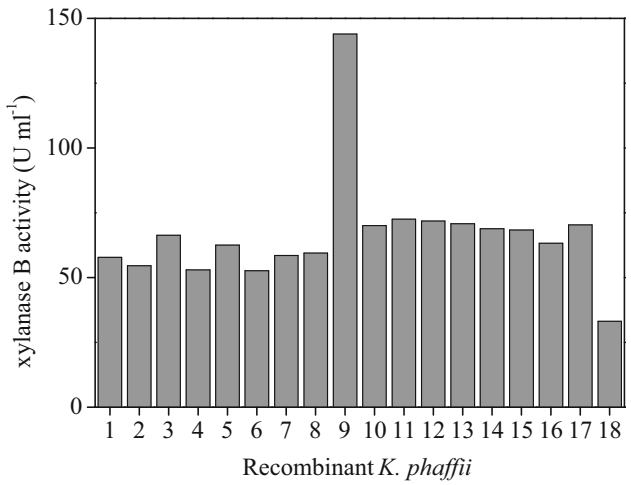

Fig. 1 Screening of recombinant *K. phaffii* cells for high xylanase B activity

Effect of pH on recombinant *K. phaffii* growth and xylanase B activity

The effect of pH on recombinant *K. phaffii* cell density was not significant because the cell densities under all the examined pH values increased from 7.0 to about 15.0 OD_{600} without significant difference (Fig. 2C). Furthermore, at all the examined pH values, the activity of xylanase B sharply increased within 48 h of induction and then slowly increased, reaching 1273.92 U/ml after 72 h of induction. The highest activity of xylanase B (1300.46 U/ml) was obtained at pH 6.5 after 96 h of induction, which was not significantly different from that achieved at 72 h of induction (Fig. 2D).

Effect of methanol addition on recombinant *K. phaffii* growth and xylanase B activity

The cell density of recombinant *K. phaffii* cells increased with the increasing concentration of methanol. The highest cell density was obtained with the addition of 2.0% methanol, and the final density was 17.96 OD_{600} (Fig. 2E). The activity of xylanase B increased within 72 h of induction and exhibited different values and subsequently showed varied trends. With the addition of 0.5 and 1.0% methanol, the activity of xylanase B increased until 96 h, reaching 1143.83 and 1441.16 U/ml, respectively. In contrast, with the addition of methanol at concentration higher than 1.0%, the activity of xylanase B decreased after 72 h of induction, and the highest xylanase B activity of 1827.19 U/ml was observed with the addition of 1.5% methanol after 72 h of induction (Fig. 2F).

Enzyme kinetics parameters

According to Michaelis–Menten equation, the V_{max} and K_m values of xylanase B were 1×10^8 mmol/(L min) and 1.89×10^{-9} mmol/l, respectively. The specific activity of xylanase B was 14432.5 U/mg using ammonium sulfate precipitation process.

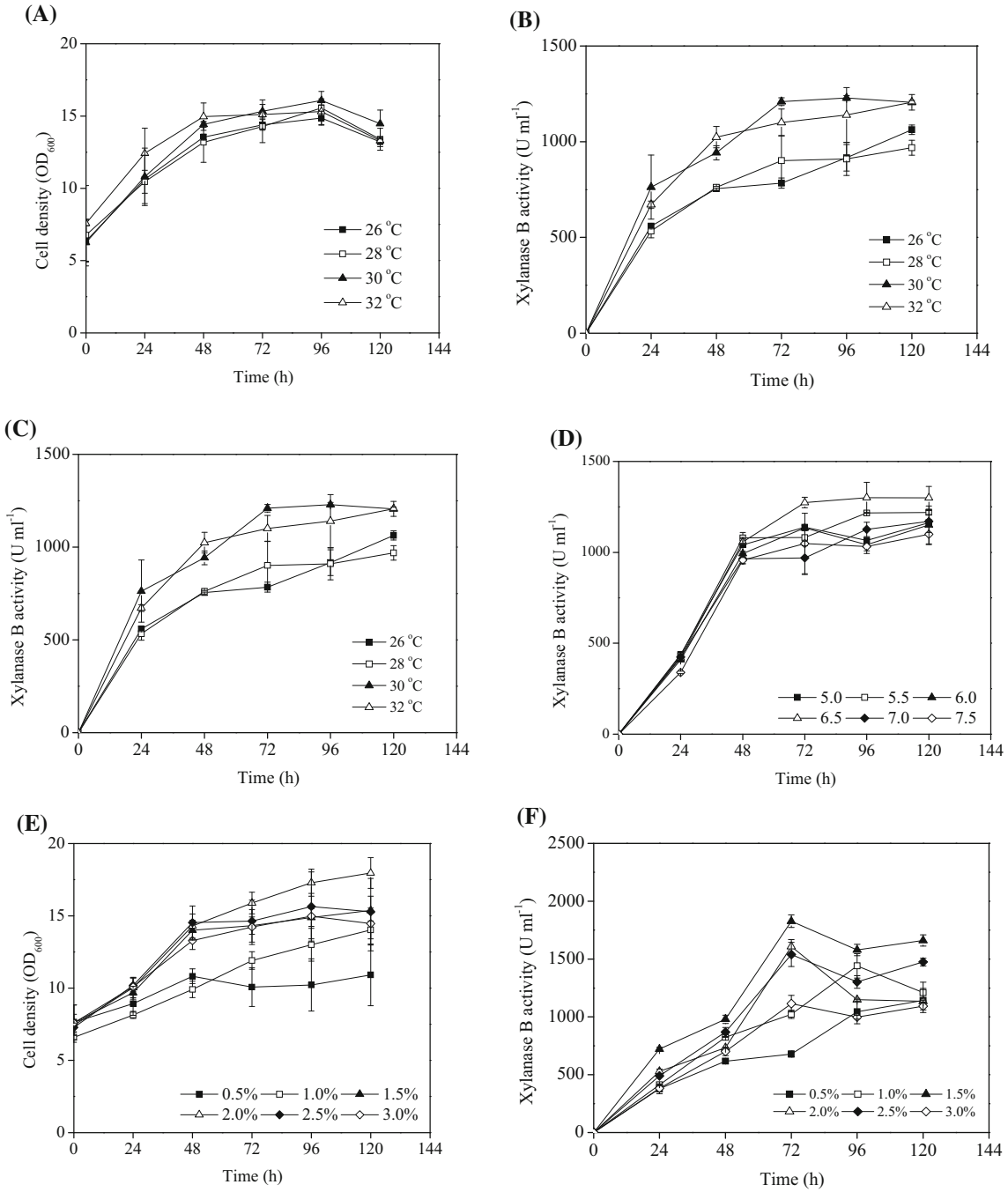

Fig. 2 Effects of temperature, pH, methanol addition on recombinant *K. phaffii* growth and xylanase B activity. (**A**) Effect of temperature on cell density, (**B**) effect of temperature on xylanase B activity, (**C**) effect of pH on cell density, (**D**) effect of pH on xylanase B activity, (**E**) effect of methanol addition on cell density, (**F**) effect of methanol addition on xylanase B activity

Effect of temperature on xylanase B activity and thermostability

The effect of temperature on the activity of xylanase B showed a bell-shaped curve. The xylanase B activity increased with the increasing temperature until 55 °C, and the highest relative activity of 149.52% was achieved at 55 °C, indicating that the optimal temperature for xylanase B activity was 55 °C (Fig. 3A). The thermostability of xylanase B was examined at 50, 55, and 60 °C. With the increasing time period, the activity of xylanase B sharply decreased (Fig. 3B). At 50 °C, the activity of xylanase B decreased to 87.19% after 10 min and then significantly decreased until 120 min, with the final relative activity reaching 0.89%. At 55 and 60 °C, the xylanase relative activity sharply decreased to 42.20 and 2.22% after 10 min.

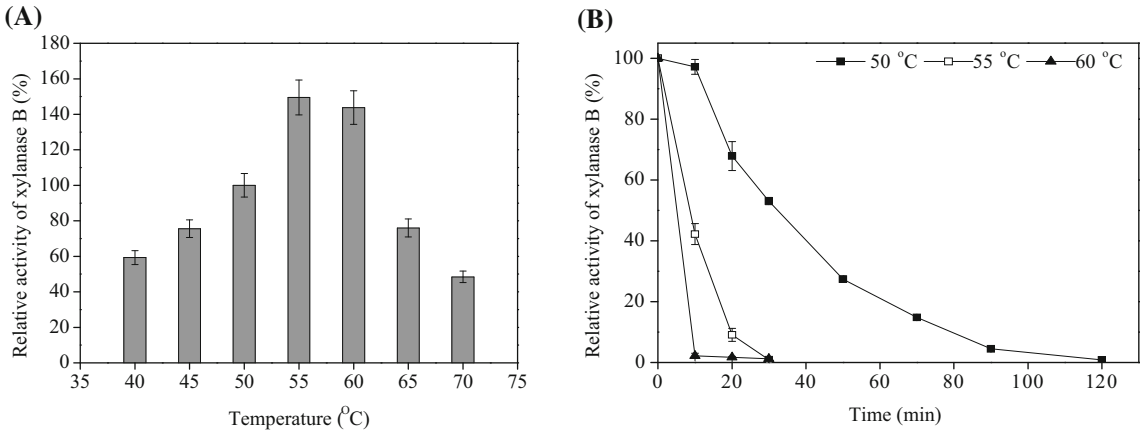

Fig. 3 Effects of temperature on xylanase B activity and thermostability. (**A**) Effects of temperature on relative activity of xylanase B, (**B**) thermostability of xylanase B activity

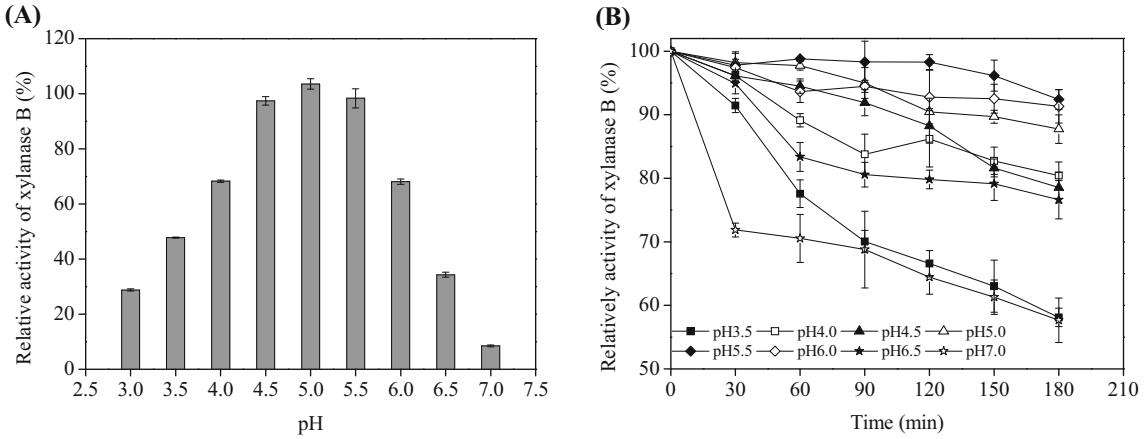

Fig. 4 Effects of pH on xylanase B activity and stability. (**A**) Effects of pH on relative activity of xylanase B, (**B**) pH stability of xylanase B activity

Furthermore, at 55 °C, the xylanase relative activity continuously decreased to 9.11 and 0.93% in 20 and 30 min, respectively (Fig. 3B).

Effect of pH on xylanase B activity and stability

The effect of pH on the activity of xylanase B also showed a bell-shaped curve, with the highest relative activity of 103.56% achieved at pH 5.0 (Fig. 4A). Furthermore, at pH 3.0 and 7.0, the xylanase B activity decreased to 28.78 and 8.52%, respectively. The pH stability of xylanase B was examined at a pH range of 3.0–7.0 at 37 °C. At all the examined pH values, the relative activity of xylanase B continuously decreased with the increasing time period. At pH 5.5, xylanase B exhibited maximum stability with 92.41% relative activity after 180 min. However, at pH higher or lower than 5.5, the activity of xylanase B rapidly decreased (Fig. 4B). At pH 5.0, the activity of xylanase B

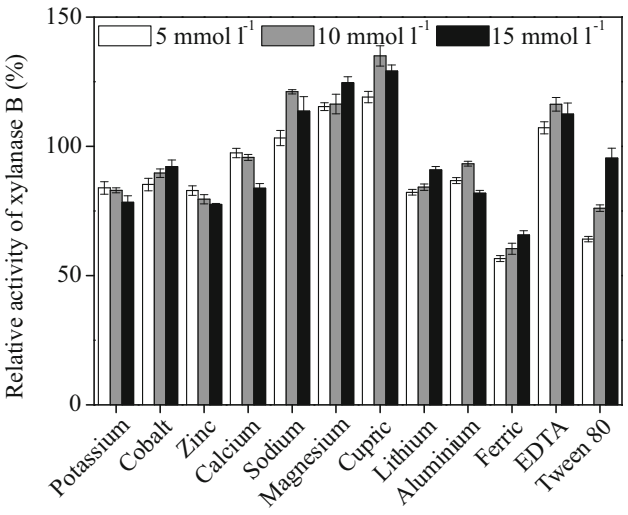

Fig. 5 Effects of ions addition on xylanase B activity (for Tween 80, column with sparse line represents addition of 0.5% Tween 80; column with dense line represents addition of 1.0% Tween 80; and gray column represents addition of 1.5% Tween 80)

Table 1 Heterologous expression of xylanase B from *Aspergillus* strains

	Optimal temperature (°C)	Optimal pH	Activity (U ml^{-1})	References
Aspergillus usamii E001	50	4.6	49.6 U mg^{-1}	[26]
Aspergillus sulphureus	50	5.0	105	[28]
Aspergillus niger CGMCC1067	50	5.0	62	[29]
Aspergillus niger IA-001	50	5.0	15158.23	[30]
Aspergillus niger SCTCC 400264	55	5.0	nd	[25]
Aspergillus niger BRFM281	50	5.0	3492.9	[31]
Aspergillus niger IF0 4066	nd	5.0	nd	[32]
Aspergillus niger ATCC 1015	55	5.0	1827.19	This study

nd not detected

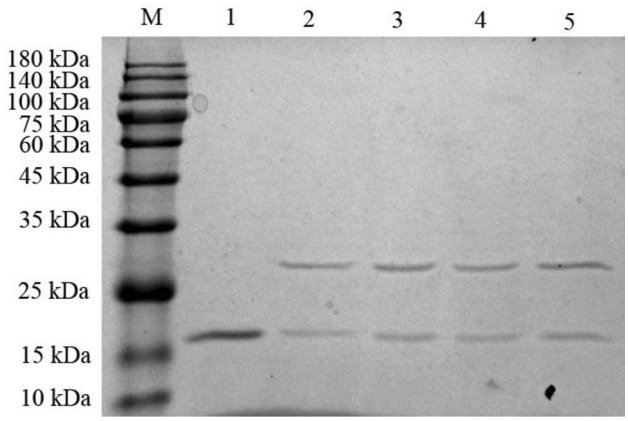

Fig. 6 SDS-PAGE of denatured *K. phaffii* xylanase B after PNGase F and Endo H treatment (M: Marker, 1: xylanase B, 2: 2 h digestion, 3: 4 h digestion, 4: 6 h digestion, 5: 8 h digestion.)

decreased to 87.75%. Thus, xylanase B showed the best stability at pH 5.5 and highest activity at pH 5.0.

Effect of ions and chemicals addition on xylanase B activity

The effect of ions and chemicals addition on the activity of xylanase B is shown in Fig. 5. Based on their effect on the activity of xylanase B, the ions and chemicals were divided into two groups. Sodium, magnesium, cupric, and EDTA ions presented positive effect on xylanase B activity at the concentrations of 5.0, 10.0, and 15.0 mmol/l. In contrast, potassium, cobalt, zinc, calcium, lithium, aluminum, ferric, and Tween 80 showed negative effect on the activity of xylanase B. The highest relative activity of xylanase B (133.20%) was observed with the addition of 10 mmol/l cupric ions. It has been reported that the highest relative activity of xylanase from *A. niger* IA-001, *Aspergillus sulphureus*, and *A. niger* SCTCC 400264 reached 185.2, 140.4, and about 118% with the addition of 0.5% Tween, 10 mmol/l zinc, and 1.0 mmol/l potassium, respectively.

Discussion

Xylanase B is in high demand owing to its wide application in the food industry [24]. In the present study, *xynB* of *A. niger* ATCC 1015 was cloned and expressed in *K. phaffii* cells and exhibited high activity. With the addition of methanol, the cell concentration increases; also because methanol is the inducer of the *AOX1* promoter, the yield of xylanase production will increase. The xylanase yield after 72 h of induction was 1827.19 U/ml. But methanol is toxic at high levels. As the normal cell density of *K. phaffii* cells in a bioreactor is 300 OD_{600}, they are promising host for obtaining high yield of products in large-scale fermentation. In food industry, xylanase is used to improve the specific volume and rheological properties of doughs at

Table 2 Effect of ions and chemicals addition on xylanase B activity

Sodium	Magnesium	Cupric	EDTA	Tween 80	Aluminum	Potassium	Cobalt	Zinc	Calcium	Lithium	Ferric	References
+	+	−	+	nd	nd	+	+	+	+	nd	−	[28]
+	−	+	+	+	+	+	+	+	+	+	−	[30]
nd	+	−	nd	nd	nd	+	nd	nd	nd	nd	nd	[25]
+	+	+	+	+	+	−	−	−	−	−	−	This study

+, positive effect; −, negative effect; nd, not detected

60 °C [5], which is the ideal temperature for xylanase. In the present study, the optimal temperature of xylanase B was 55 °C, which is higher than that of most of the xylanases from other *Aspergillus* strains, such as *Aspergillus usamii* E001 xynI, *A. sulphureus* xylanase B, *A. niger* CGMCC1067 xynB, and *A. niger* IA-001 (Table 1). Only *A. niger* SCTCC 400264 xylanase showed the same optimal temperature and pH as those of xylanase B obtained in the present study [25]. The activity of xylanase B at 60 °C was only 0.04% lower than that at 55 °C. It must be noted that the sequence of *xynI* of *A. usamii* E001 was similar to that of the GH11 family xynB [26], whereas the sequence of xynII of *A. usamii* E001 was not similar to that of GH11 family xynB [27] (Table 1). And the xylanase B was also analyzed by PNGase F and Endo H (Fig. 6) to clarify the influence of glycosylation on recombinant xylanase B, which showed consistent molecular weight before and after digestion. These results indicated the optimal pH and temperature of this xylanase B was attributed to its amino acid sequence.

Table 2 summarizes the effects of ions and chemicals addition on xylanase B activity. It can be noted that none of the ions and chemicals exerted a constant effect on the activity of xylanase B. Among the six positive ions and chemicals, only ferric ions had a constant negative effect on the activity of xylanase B, whereas the other ions or chemicals exhibited varying effect on the activity of xylanase B.

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Author's contributions TL and JZ conceived the project, carried out all experiments and drafted the manuscript. JZ improved the manuscript. Both authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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