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A novel thermoacidophilic family 10 xylanase from *Penicillium pinophilum* C1

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ABSTRACT

A novel endo-β-1,4-xylanase gene (*xyn10C1*) was cloned from *Penicillium pinophilum* C1 and overexpressed in *Pichia pastoris*. The 1071-bp full-length gene encodes a 356-residue polypeptide containing the catalytic domain of glycoside hydrolase 10. Deduced XYN10C1 shares highest amino acid sequence identity of 49.3% with a putative xylanase from *Glomeella graminicola* M1.001. Purified recombinant XYN10C1 showed maximal activity at pH 4.0–5.5 and 75 °C, exhibited good adaptability to broad acidic pH and temperature ranges (>69.0% activity at pH 2.5–6.5; and >91.0% activity at 70–80 °C and 22.2% even at 90 °C), and was highly stable at pH 2.0–7.0 for 1 h at 37 °C. The specific activity, K_m and V_{max} values for birchwood xylan and soluble wheat arabinoxylan were 100.7 and 137.4 U/mg, 4.3 and 6.9 mg/ml, and 195.4 and 209.3 μmol/min/mg, respectively. The enzyme was strongly resistant to most metal ions and proteases (pepsin and trypsin). Under simulated mashing conditions, addition of XYN10C1 (80 U) to the brewery mash (12.5 g in 50 ml system) significantly increased the filtration rate by 26.7% and reduced the viscosity by 9.8%, respectively. All these favorable enzymatic properties make XYN10C1 attractive for potential applications in the food and animal feed industries.

1. Introduction

Xylan is one of the major hemicellulose components in plant cell walls of monocots and hard woods, and is the second most abundant polysaccharide after cellulose [1,2]. It is a heteroglycan composed of a backbone of β-1,4-linked xylopyranose residues substituted by a set of glycosidic-ester linkages as the side chains [3]. Due to the heterogeneity and complexity of its structure, complete hydrolysis of xylan requires a large variety of synergistically acting enzymes, including endo-β-1,4-xylanase, β-D-xylosidase, α-L-arabinofuranosidase, α-D-glucuronidase, acetylxylan esterase and feruloyl or coumaroyl esterases [1]. Among them, endo-β-1,4-xylanase is the key enzyme to hydrolyze the main backbone of xylan, yielding different lengths of short xylooligosaccharides [4]. Xylanases are classified into glycosyl hydrolase (GH) families 5, 7, 8, 10, 11, and 43 based on the similarities of the amino acid sequences (http://www.cazy.org/fam/acc_GH.html) [5], and majority of xylanases are confined into GH families 10 and 11 [1]. Compared with family 10 xylanases that have high molecular masses (>30 kDa) and high p*I* values, family 11 xylanases have relatively low molecular weight (19–25 kDa) and lower p*I* values [6].

Various microorganisms have been reported to be xylanase producers, including bacteria, actinomycetes, fungi, protozoa, and yeast. Among them, fungi attract much attention due to their higher-level secretion of xylanolytic enzymes into the medium [3]. Xylanases have been applied in the processes of commercial food production, animal feed, baking, fruit juice clarification, waste treatment, brewing, pulp biobleaching, bioconversion in fuels and chemicals and textile production [7,8]. Each industry has specific requirements, and thus prefers different xylanases with ideal properties. So far, most commercially available xylanases are produced by *Trichoderma* and *Aspergillus* that lack thermostability and are not active at acidic pH, thus limiting their applications in animal feed and brewing industries [1]. Here we report a novel thermoacidophilic xylanase from *Penicillium pinophilum* C1. The recombinant xylanase produced in *Pichia pastoris* exhibited good adaptability to acidic pH and high temperature even above 80 °C, and had superior acid and thermal stability. In addition, the enzyme had strong resistance to most chemicals and proteases. Its application potential in brewing industry was also evaluated.

2. Materials and methods

2.1. Strains, vectors, materials and media

Penicillium pinophilum C1 isolated from the acidic wastewater (pH 3.0) of a tin mine in Yunnan province, China, was obtained from the China General Microbiological Culture Collection Center (CGMCC No. 4432). To induce xylanase production, strain C1 was grown at 30 °C in the wheat bran medium consisting of 5 g/l $(NH_4)_2SO_4$,

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Table 1
Primers used in this study.

Primers	Sequences (5′ → 3′)[a]	Size (bp)
GH10F1	TGGGAYGTNGTNAAYGARGC	20
GH10R1	TAYTCTATRTTRWARTCRTT	20
C1F	CAGCTCAACGAGCTGGCCCAGAAGG	26
C1R	TTACACGTCCAGCGGCTTGCTCCTC	25
GH10PF	GGGGAATTCCAGCTCAACGAGCTGGCCCAGAAGG	34
GH10PR	GGGGCGGCCGCTTACACGTCCAGCGGCTTGCTCCTC	36
GH10 usp1	CTCAACGACAACGGCACGTACCGG	24
GH10 usp2	CGGCACGTACCGGTCCGACATC	22
GH10 usp3	CGACCCGGGCGCGAAGCTG	19
GH10 dsp1	GTACAGCTTCGCGCCCGGGTC	21
GH10 dsp2	CCTCGCTGGCCGCCTGGTAG	20
GH10 dsp3	CGGACCGGTACGTGCCGTTGTC	22

[a] Restriction sites are underlined, and R = A/G, N = A/C/G/T, and Y = C/T.

1 g/l KH_2PO_4, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 0.2 g/l $CaCl_2$, 0.01 g/l $FeSO_4 \cdot 7H_2O$, and 30 g/l wheat bran [9].

Escherichia coli Trans1-T1 (TransGen, Beijing, China) cultivated at 37 °C in Luria–Bertani medium was used for gene cloning and sequencing. *P. pastoris* GS115 from Invitrogen (Carlsbad, CA) was cultivated at 30 °C for gene expression. Plasmids pEASY-T3 (TransGen) and pPIC9 (Invitrogen) were used for gene cloning and expression, respectively. Media for *P. pastoris* growth and induction were prepared according to the instruction of the *Pichia* Expression kit (Invitrogen).

Birchwood xylan, beechwood xylan, soluble wheat arabinoxylan and carboxymethyl cellulose-sodium (CMC-Na) were purchased from Sigma (St. Louis, MO). The DNA purification kit, *LA Taq* DNA polymerase, and restriction endonucleases were supplied by TaKaRa (Tsu, Japan), and T4 DNA ligase and endo-β-*N*-acetylglucosaminidase H (Endo H) were obtained from New England Biolabs (Hitchin, UK). All other chemicals used are of analytical grade and available commercially.

2.2. Cloning of the xylanase gene (xyn10C1)

Genomic DNA of strain C1 was isolated using the fungal DNA Mini kit (Omega Bio-tek, Norcross, GA) and used as a template for PCR amplification. To obtain the core region of the xylanase gene from strain C1, a degenerate primer set (GH10F1 and GH10R1) specific for fungal GH 10 xylanase genes and a touchdown PCR were used as described by Luo et al. [9]. The resulting PCR fragment with appropriate size was purified and ligated into the pEASY-T3 vector for sequencing and subjected to BLAST analysis.

Thermal asymmetric interlaced (TAIL)-PCR was performed using nested insertion-specific primers (Table 1) with an annealing temperature of 60 °C to obtain the 5′ and 3′ flanking fragments of the core region. The resulting amplified fragments with correct size were cloned into pEASY-T3 vector and sequenced, and then assembled with the known fragment sequence to obtain the full-length xylanase gene.

Mycelia of strain C1 were harvested after 4-day growth in wheat bran-inducing medium at 30 °C. Total RNA was extracted and purified using the total RNA isolation system (Promega, Madison, WI) according to the instructions of manufacturer. Reverse transcription (RT)-PCR was carried out to obtain the full-length xylanase cDNA, using primers C1F and C1R (Table 1) with an annealing temperature of 60 °C.

2.3. Sequence analysis

Vector NTI 7.0 software was employed to assemble and analyze the DNA sequence, and to calculate the molecular mass of mature peptide. The signal peptide was predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP/). The exon–intron structure and transcription initiation sites of the full-length gene were predicted using the online software FGENESH (http://linux1.softberry.com/berry.phtml). Sequence homology database searches were conducted using the BLAST server (http://www.ncbi.nlm.nih.gov/BLAST). Multiple alignments of protein sequences were performed using the ClustalW program (http://www.ebi.ac.uk/clustalW). Homology modeling and electrostatic potential analysis was performed using Accelrys Discovery Studio software (DS 2.5, http://www.accelrys.com) with the GH 10 xylanase from *Cellulomonas fimi* (PDB: 3CUF) as the template.

2.4. Expression of xyn10C1 in P. pastoris

The gene fragment coding for the mature protein without the signal peptide was amplified using the expression primers GH10PF and GH10PR (Table 1). The PCR products were digested with *Eco*RI and *Not*I and cloned into pPIC9 under the control of the alcohol oxidase promoter. The recombinant plasmid pPIC9-*xyn10C1* was linearized using *Bgl*II and then transformed into *P. pastoris* GS115 competent cells by electroporation. The fermentation in shake tubes and 1-l shake flasks was carried out following the method of Qiu et al. [10].

2.5. Xylanase activity assay

The xylanase activity was determined using the 3,5-dinitrosalicylic acid (DNS) method by measuring the release of reducing sugar from birchwood xylan [11]. The standard assay mixture was composed of 0.1 ml of appropriately diluted enzyme (2 U) and 0.9 ml 0.1 M citric acid–Na_2HPO_4 (pH 5.0) containing 1% (w/v) birchwood xylan. After incubation at 75 °C for 10 min, the reaction was terminated by adding 1.5 ml DNS reagent, boiled for 5 min and cooled to room temperature. The absorbance was measured at 540 nm. Each assay and its control were done in triplicate. One unit of xylanase activity was defined as the amount of enzyme required to produce 1 μmol of reducing sugar per min under the standard conditions (pH 5.0, 75 °C, 10 min).

2.6. Purification of recombinant XYN10C1

To purify recombinant XYN10C1, the induced culture was centrifuged at 12,000 × *g* for 10 min at 4 °C to remove cell debris and undissolved materials. The cell-free culture supernatant was concentrated by ultrafiltration with Vivaflow 200 membrane of 10-kDa molecular weight cut-off (Vivascience, Hannover, Germany), and loaded onto a FPLC HiTrap Q Sepharose XL 5 ml column (GE Healthcare, Uppsala, Sweden) that was equilibrated with 20 mM citric acid–Na_2HPO_4 (pH 6.5). Proteins were eluted using a linear gradient of NaCl (0–1.0 M) in the same buffer at a flow rate of 3.0 ml/min. Fractions containing the enzyme activity were pooled and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [12]. To determine the protein concentration, a Bradford assay was used with bovine serine albumin as a standard [13]. The purified recombinant XYN10C1 (1 μg) was treated with 250 U of Endo H for 2 h at 37 °C according to the supplier's instructions, and the deglycosylated and untreated purified XYN10C1 were analyzed by SDS–PAGE.

2.7. Biochemical characterization of purified recombinant XYN10C1

The optimal pH of the purified recombinant XYN10C1 for xylanase activity was determined at 50 °C and pH 2.0–8.0 for 10 min in the presence of 1% (w/v) birchwood xylan. The pH stability of XYN10C1 was estimated by measuring the residual activity under standard conditions (pH 5.0, 75 °C, 10 min) after pre-incubation of the enzyme solution in buffers at pH 2.0–7.0, 37 °C for 1 h without substrate. The activity of XYN10C1 without incubation was set as 100%. The buffer used was 0.1 M citric acid–Na_2HPO_4 (pH 2.0–8.0). The optimal temperature for xylanase activity was determined by performing the reaction at temperatures ranging from 25 to 90 °C in 0.1 M citric acid–Na_2HPO_4 (pH 5.0) containing 1% (w/v) birchwood xylan for 10 min. Thermostability of XYN10C1 was determined after pre-incubation of the enzyme in 0.1 M citric acid–Na_2HPO_4 (pH 5.0) at 65, 70 and 75 °C without substrate for different periods. The remaining activity was assessed under standard conditions.

The effects of various metal ions (Na^+, K^+, Ca^{2+}, Li^+, Co^{2+}, Cr^{3+}, Ni^{2+}, Cu^{2+}, Mg^{2+}, Fe^{3+}, Mn^{2+}, Zn^{2+}, Pb^{2+}, and Ag^+) and chemical reagents (EDTA, SDS, and β-mercaptoethanol) on the activity of purified XYN10C1 was determined at the final concentrations of 1 mM and 5 mM, and its resistance to proteolysis by pepsin and trypsin was determined as described before [14].

The kinetic parameters, K_m and V_{max}, for the purified recombinant enzyme were determined in 0.1 M citric acid–Na_2HPO_4 (pH 5.0) containing 0.25–10 mg/ml birchwood xylan or soluble wheat arabinoxylan at 75 °C for 5 min. The resulting data were plotted according to Lineweaver–Burk method [15] using the non-linear regression computer program GraFit (Version 7, Erithacus Software, Surrey, UK). Each experiment was repeated three times and each experiment had three replicates.

2.8. Substrate specificity and analysis of xylan hydrolysis products

To investigate the substrate specificity of recombinant XYN10C1, the enzyme activity was determined in the standard assay system containing 1% (w/v) of birchwood xylan, beechwood xylan, CMC-Na, soluble wheat arabinoxylan, insoluble wheat arabinoxylan or barley β-glucan.

To study the hydrolysis products of birchwood xylan and soluble wheat arabinoxylan by purified recombinant XYN10C1, reactions containing 25 U purified recombinant XYN10C1 and 0.5% (w/v) birchwood xylan or soluble wheat arabinoxylan in 500 μl 0.1 M citric acid–Na_2HPO_4 (pH 5.0) were incubated at 37 °C for 12 h. The excess enzyme was removed from the reaction using the Nanosep Centrifugal 3 K Device (Pall, Chicago, IL). The products were analyzed by high-performance anion-exchange chromatography (HPAEC) with a model 2500 system from Dionex (Sunnyvale, CA) as described previously [14]. Xylose, xylobiose, xylotriose, xylotetraose, and xylopentaose were used as standards.

2.9. Effects of XYN10C1 on the filtration rate and viscosity of mash

Mash was prepared according to the method of Celestino et al. [16] with some modifications. Malt was firstly triturated in a disintegrator followed by filtration through a 0.2-mm sieve, and 12.5 g of malt was dissolved in 50 ml 0.1 M citric acid–Na_2HPO_4 (pH 5.5) containing 40 U or 80 U XYN10C1. The reaction was incubated at 45 °C for 30 min, 50 °C for 30 min, 60 °C for 60 min, and 70 °C for 30 min, and boiled for 5 min. Addition of 0.1 M citric acid–Na_2HPO_4 (pH 5.5) instead of the

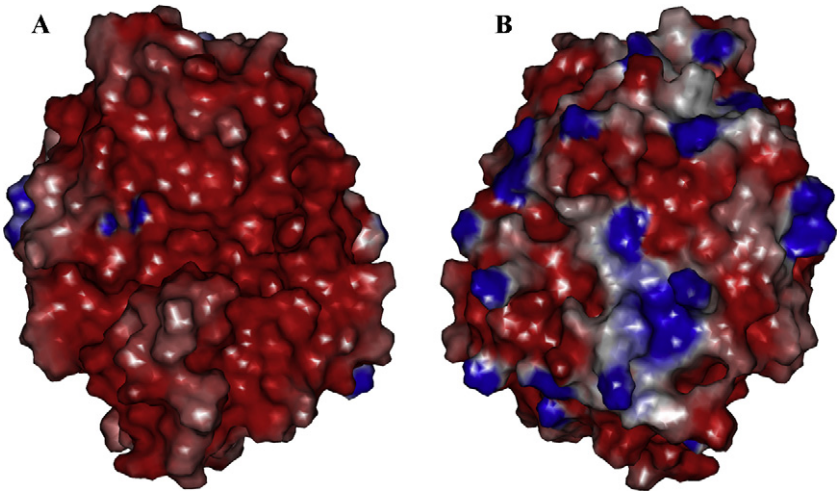

Fig. 1. Charge distribution on the surface of a three-dimensional model of XYN10C1. Negatively and positively charged surfaces are coloured red and blue, respectively. (A) The surface showing the substrate cleft with the active site. (B) The opposite-side surface. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

enzyme solution was treated as a control. Each reaction was terminated by addition of 50 ml cold water and immediately cooled down to room temperature.

The filtration rate was determined by filtering 10 ml of mash after reaction through a 101 filter paper (Xinhua, Beijing, China). Filtration rate in the absence of enzyme was used as a control. The reduction of filtration rate was calculated using the following equation [10,16]:

$$\Delta\psi = \frac{(\psi_{\text{control}} - \psi) \times 100}{\psi_{\text{control}}}$$

where ψ is the total flow time of 10 ml and $\Delta\psi$ is the filtration time reduction.

Viscosimetry was used to measure the mash viscosity with or without enzyme treatment. Mash supernatant (5 ml) after filtration was subjected to viscosity assay at 20 °C. Mash viscosity without enzyme addition was used as the control. The viscosity reduction was calculated using the following equations:

$$\mu = \frac{\mu_{\text{water}} \times t \times \rho}{t_{\text{water}} \times \rho_{\text{water}}}$$

$$\Delta\mu = \frac{(\mu_{\text{control}} - \mu) \times 100}{\mu_{\text{control}}}$$

where μ is the viscosity, t is the total flow time through viscometer, $\Delta\mu$ is the viscosity reduction, and ρ is the density.

2.10. Nucleotide sequence accession number

The nucleotide sequence for the GH 10 xylanase gene (*xyn10C1*) of *P. pinophilum* C1 was deposited into the GenBank database under the accession number HQ668176.

3. Results

3.1. Gene cloning and sequence analysis

A 158-bp xylanase gene fragment was amplified from the genomic DNA of strain C1 with degenerate primers GH10F1 and GH10R1. The fragment showed highest amino acid sequence similarity of 64% with the family 10 xylanase from *Glomerella graminicola* M1.001 (EFQ31642). The 3′ and 5′ flanking regions were obtained by TAIL-PCR, and assembled with the core region to give a 1939-bp fragment. One complete chromosomal gene of 1253 bp was identified by FGENESH.

The full-length cDNA sequence (1071 bp, see supplementary Fig. S1) of the xylanase gene, denoted *xyn10C1*, was obtained by RT-PCR from *P. pinophilum* C1. Two introns (100 bp and 82 bp, respectively) interrupted the coding sequence. A putative 18-residue signal peptide at N-terminus was identified by SignalP analysis. The deduced mature protein of XYN10C1 contained 356 residues with an estimated theoretical molecular weight of 38.0 kDa. Based on the sequence comparison, XYN10C1 had the catalytic domain typical of GH 10 and no cellulose binding domain. The deduced amino acid sequence of XYN10C1 exhibited highest identity of 49.3% with a putative xylanase from *G. graminicola* M1.001, followed by an experimentally verified xylanase (XYND) from *Penicillium funiculosum* (CAG25554; 44.4%). The putative tertiary structure of XYN10C1 is the classical $(\alpha/\beta)_8$ fold, and the two catalytic residues (Glu148 and Glu255) are located on the inner surface of the protein. Analysis of solvent-exposed amino acids revealed a low frequency of positively charged residues on the surface (Fig. 1).

3.2. Expression and purification of recombinant XYN10C1

The gene fragment for mature protein without the signal peptide was cloned into vector pPIC9 and successfully expressed in *P. pastoris*. Positive transformants were screened by measuring the xylanase activity in the culture supernatant. The transformant showing the highest extracellular enzyme activity (2.3 U/ml) was subjected to large-scale expression. After methanol induction for 72 h in 1 l shaker flask, the enzyme activity of 477 U/ml was detected. XYN10C1 in the culture supernatant was purified to electrophoretic homogeneity by a single-step anion-exchange chromatography. The specific activity of the purified recombinant XYN10C1 was 100.7 U/mg towards birchwood xylan. The apparent molecular mass of purified recombinant XYN10C1 on SDS–PAGE gel was found to be 60.0 kDa, which was much higher than its theoretical molecular mass (38.0 kDa). After deglycosylation with Endo H, the purified recombinant XYN10C1 yielded a single band of about ~40.0 kDa (Fig. 2), which was in agreement with the calculated molecular weight.

3.3. Enzyme characterization

Purified recombinant XYN10C1 had a pH optimum of 4.0–5.5 at 50 °C and broad pH adaptability, retaining more than 80% of the maximal activity at pH 3.0–6.5 and 69% activity at pH 2.5 (Fig. 3A). The apparent temperature optimum of XYN10C1 was 75 °C at pH 5.0 (Fig. 3B). The enzyme retained almost all of the activity after incubation for 1 h at pH 2.0–7.0 (Fig. 3C). In the thermostability assay, XYN10C1 was highly stable at 65 °C for 1 h, but lost almost half of its activity at 70 °C for 30 min and 80% activity at 75 °C for 10 min (Fig. 3D).

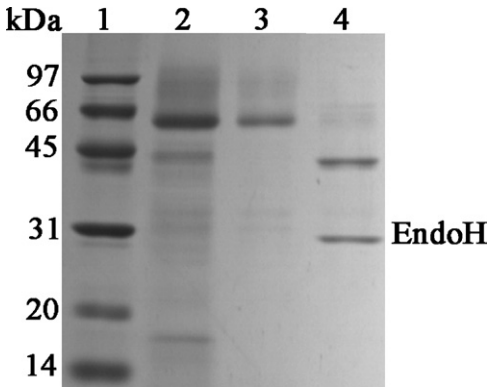

Fig. 2. SDS–PAGE analysis of the purified recombinant XYN10C1. Lane 1, standard protein molecular weight markers; lane 2, the culture supernatant; lane 3, the purified recombinant XYN10C1; lane 4, the purified recombinant XYN10C1 after deglycosylation with Endo H.

The effect of different metal ions or chemical reagents on the activity of purified XYN10C1 was determined at a final concentration of 1 and 5 mM (Table 2). At low concentration, all the chemicals except for SDS enhanced or had no effects on the enzyme activity. At the concentration of 5 mM, Ag^+, Cu^{2+}, and EDTA inhibited the enzyme activity partially. SDS was a strong inhibitor for XYN10C1 activity, resulting in 40% loss of activity at 1 mM and almost complete loss at 5 mM. XYN10C1 was highly resistant to proteolytic digestion, showing 118.6% and 126.0% of its initial activity after incubation for 2 h at 37 °C with pepsin and trypsin, respectively.

Table 2
Effects of metal ions and chemical reagents on purified recombinant XYN10C1 activity.

Chemicals	Relative activity (%)[a]	
	1 mM	5 mM
None	100.0	100.0
Na^+	110.9 ± 1.2	110.0 ± 1.1
K^+	111.3 ± 1.5	107.8 ± 2.0
Ca^{2+}	108.0 ± 0.8	103.7 ± 0.5
Li^+	111.4 ± 1.0	109.6 ± 1.0
Co^{2+}	110.3 ± 1.8	104.3 ± 1.8
Cr^{3+}	109.9 ± 1.4	112.3 ± 0.8
Ni^{2+}	106.8 ± 1.7	104.6 ± 0.9
Cu^{2+}	103.4 ± 2.6	67.0 ± 1.4
Mg^{2+}	104.2 ± 1.7	107.4 ± 1.1
Fe^{3+}	101.7 ± 0.9	102.3 ± 0.8
Mn^{2+}	106.8 ± 2.3	100.8 ± 2.3
Pb^{2+}	103.9 ± 2.0	93.2 ± 1.8
Ag^+	98.4 ± 0.9	57.5 ± 1.1
SDS	60.4 ± 1.2	4.8 ± 1.4
EDTA	97.2 ± 2.0	89.7 ± 0.6
β-Mercaptoethanol	118.6 ± 1.8	126.0 ± 2.7

[a] Values represent the mean ± SD (n = 3) relative to untreated control samples.

3.4. Substrate specificity and kinetic parameters

The enzyme activity of purified recombinant XYN10C1 towards different types of substances was detected. When defined the activity towards birchwood xylan as 100.0%, the enzyme showed highest activity on soluble wheat arabinoxylan (136.4%), followed by beechwood xylan (101.7%). No activity was detected in the presence of barley β-glucan, CMC-Na and insoluble wheat arabinoxylan.

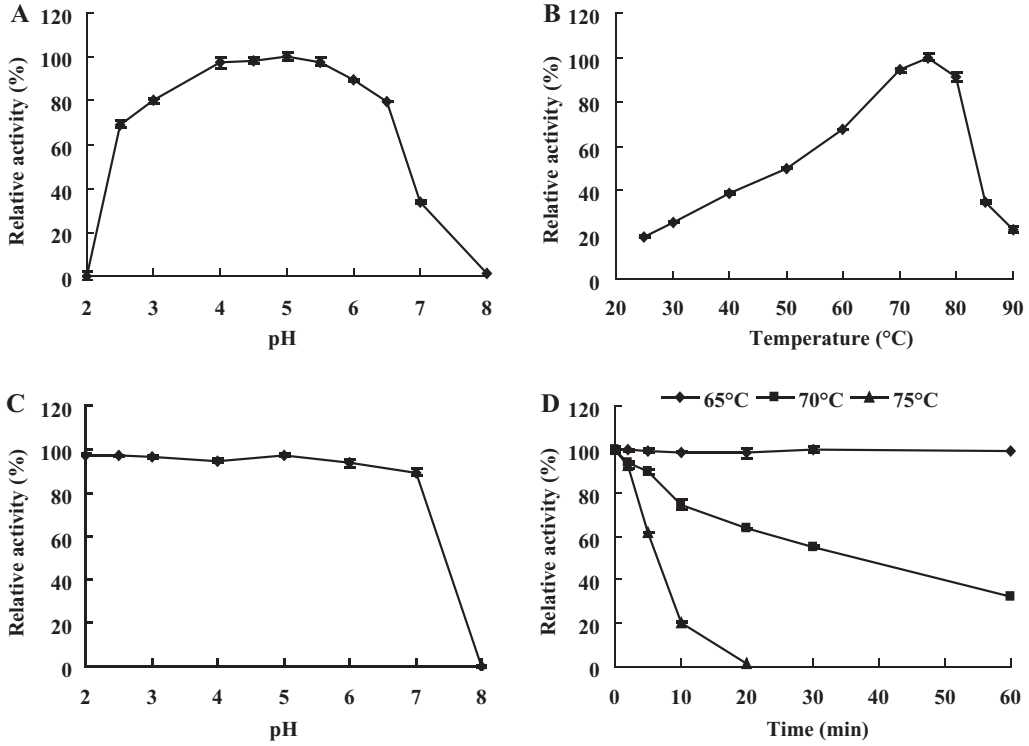

Fig. 3. Characterization of the purified recombinant XYN10C1. (A) The effect of pH on xylanase activity. The activity assay was performed at 50 °C in buffers of pH 2.0–8.0 for 10 min. (B) The effect of temperature on xylanase activity measured in 0.1 M citric acid–Na_2HPO_4 (pH 5.0) for 10 min. (C) pH stability of XYN10C1. After pre-incubating the enzyme at 37 °C for 1 h at pH 2.0–8.0, the residual activity was measured in 0.1 M citric acid–Na_2HPO_4 (pH 5.0, 75 °C). (D) Thermostability of purified XYN10C1. The enzyme was pre-incubated at 65 °C (♦), 70 °C (■), or 75 °C (▲) in 0.1 M citric acid–Na_2HPO_4 (pH 5.0). Aliquots were removed at specific time points for measurement of residual activity in the same buffer at 75 °C. Each value in the panel represents the mean ± SD (n = 3).

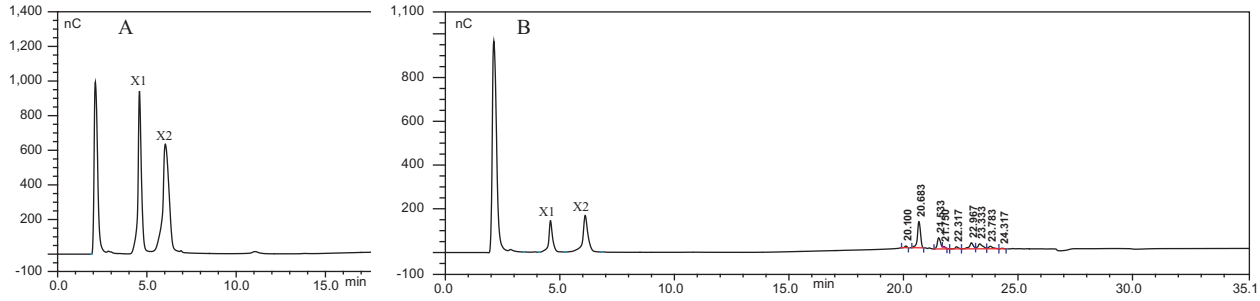

Fig. 4. HPAEC analyses of the hydrolysis products of birchwood xylan (A) and soluble wheat arabinoxylan (B) by XYN10C1. X1 represents xylose, and X2 represents xylobiose.

Kinetic parameters of XYN10C1 on soluble wheat arabinoxylan and birchwood xylan were determined. The K_m and V_{max} values towards soluble wheat arabinoxylan were 6.9 mg/ml and 209.3 μmol/min/mg, respectively. Using birchwood xylan as the substrate, the K_m and V_{max} values were 4.3 mg/ml and 195.4 μmol/min/mg, respectively.

3.5. Analysis of hydrolysis products

The products of birchwood xylan and soluble wheat arabinoxylan by XYN10C1 hydrolysis were analyzed by HPAEC (Fig. 4). The main products were xylose and xylobiose. The composition of hydrolysis products from birchwood xylan were 44.5% xylose and 55.5% xylobiose. The hydrolysis products of soluble wheat arabinoxylan were 38.8% xylose and 61.2% xylobiose.

3.6. Effects of XYN10C1 on the filtration rate and viscosity of mash

The specific filtration rate and viscosity of mash (12.5 g in 50 ml 0.1 M citric acid–Na_2HPO_4, pH 5.5) were reduced by 22.3% and 5.0% with 40 U of purified XYN10C1, respectively. Higher reduction in the filtration rate (26.7%) and viscosity (9.8%) were observed when 80 U XYN10C1 was used.

4. Discussion

The genus *Penicillium* is a promising producer of xylanolytic enzymes suitable for applications in various industries [8]. In this study, a novel xylanase from *P. pinophilum* C1 was identified, cloned, functionally expressed and characterized. XYN10C1 has a pH optimum at 4.0–5.5, similar to most fungal xylanases, such as XYL10C (pH 4.5–5.0) from *Bispora* sp. MEY-1 [9], XynD (pH 4.2–5.2) from *P. funiculosum* [17], and xylanase II (pH 4.5) from *Penicillium sclerotiorum* [18], which are different from the neutral and alkaline pH optima of bacterial and few fungal xylanases [1,19]. Moreover, XYN10C1 is adaptable to a broader range of acidic pH. At pH 2.5, the enzyme still exhibited 69% of the maximal activity, significantly higher than xylanase II from *P. sclerotiorum* and XYN10G5 from *Phialophora* sp. G5 that have little enzyme activity (<30%) at pH 3.0 [18,20]. XYN10C1 has more acidic residues—the ratio of acidic (Asp and Glu) to basic residues (Lys and Arg) is 48:28. It might be the key factor of its acidophilicity and broad pH catalytic adaptation. XYN10C1 was only stable at acidic and neutral conditions (pH 2.0–7.0). This property is similar to reported GH 10 fungal xylanases, such as XYL10C from *Bispora* sp. MEY-1 [9] and XynA from *Acidobacterium capsulatum* [21]. Electrostatic potential analysis indicates that GH 10 fungal xylanases have more negative residues around the catalytic sites and on the surface by comparison with alkaline active xylanases (Fig. 1). The acid stability of these enzymes has been ascribed to the acidic residues on the surface and low p*I*s [22]. The enzyme activities decreased greatly at alkaline pH, and this instablility has been reported to be a repulsion of its excess negative charges and the altered conformation of the enzyme [23]. The enzyme could be denatured when it loses its functional conformation.

The optimal temperature of XYN10C1 for xylanase activity was 75 °C, which was much higher than that of most known GH 10 xylanases of *Penicillia*, such as XynA (60 °C) from *Penicillium purpurogenum*, XynA and XynB (47–48 °C) from *Penicillium capsulatum*, and XYLP from *Penicillium chrysogenum* (40 °C) [8]. Only a few fungal xylanases have the similar or higher optimal temperature (≥75 °C), including the xylanase (75 °C) from thermophilic fungus *Talaromyces thermophilus* [24], and XYL10C (85 °C) from *Bispora* sp. MEY-1 [9]. XYN10C1 was stable at 65 °C, and this thermostable character is an important prerequisite for commercialization and industrial applications [25]. Besides, XYN10C1 showed high activity towards various xylan substrates (soluble wheat arabinoxylan, beechwood xylan and birchwood xylan), but had no ability to degrade CMC-Na. In such a case, XYN10C1 can be applied in a number of biotechnological applications where the existence of cellulose is undesirable [26].

The effects of various metal ions on xylanase activity of XYN10C1 were tested. Of tested ions, Cu^{2+} has been reported to be an inhibitor of most GH 10 xylanases [10,20,27], Co^{2+} and Ni^{2+} strongly inhibited the activity of XYN10G5 from *Phialophora* sp. G5 [20], and Zn^{2+} had a moderate to significant inhibitory effect on xylanase activity [10,19]. XYN10C1 under study shows resistance to Cu^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+} as XYL10C from *Bispora* sp. MEY-1 is [9]. Moreover, the residual activity increased by 1.2–1.3 folds after treatment with pepsin and trypsin. These properties suggest XYL10C has potential for application in the animal feed industry.

During the mashing process, some factors might influence the production of beer, such as producing the extracts at low levels, slowing down the filtration rate, and forming some gelatinous precipitates that leads to the high wort viscosity and increased turbidity [28]. To increase mash efficiency, higher temperature and addition of xylanase are developed to facilitate the degradation of arabinoxylan in barley malts [29]. Under simulated mashing conditions, XYN10C1 at the concentration of 80 U could efficiently increase the filtration rate (26.7%) and decrease the mash viscosity (9.8%), significantly higher than the commercial xylanase (200 U) that increased the filtration rate by 20.2% and reduced the viscosity by 4.8% of the mash [30]. So, XYN10C1 might be used as a possible candidate for application in the brewing industry.

In summary, XYN10C1 was a novel acidic xylanase of family 10, exhibited optimal activity at pH 4.0–5.5 and 75 °C, and had good adaptability and stability to a broad acidic pH range. Moreover, the enzyme showed high hydrolysis efficiency towards wheat arabinoxylan, had simple hydrolysis products, and was highly resistant to most metal cations and proteases (pepsin and trypsin). All these favorable characters make XYN10C1 potentially beneficial in various biotechnological processes, especially the food and feed industries.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.procbio.2011.09.018.

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