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ORIGINAL PAPER

A thermophilic and acid stable family-10 xylanase from the acidophilic fungus *Bispora* sp. MEY-1

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Abstract A complete gene, *xyl10C*, encoding a thermophilic endo-1,4-β-xylanase (XYL10C), was cloned from the acidophilic fungus *Bispora* sp. MEY-1 and expressed in *Pichia pastoris*. XYL10C shares highest nucleotide and amino acid sequence identities of 57.3 and 49.7%, respectively, with a putative xylanase from *Aspergillus fumigatus* Af293 of glycoside hydrolase family 10. A high expression level in *P. pastoris* (73,400 U ml^{-1}) was achieved in a 3.7–l fermenter. The purified recombinant XYL10C was thermophilic, exhibiting maximum activity at 85°C, which is higher than that reported from any fungal xylanase. The enzyme was also highly thermostable, exhibiting $\sim$100% of the initial activity after incubation at 80°C for 60 min and >87% of activity at 90°C for 10 min. The half lives of XYL10C at 80 and 85°C were approximately 45 and 3 h, respectively. It had two activity peaks at pH 3.0 and 4.5–5.0 (maximum), respectively, and was very acid stable, retaining more than 80% activity after incubation at pH 1.5−6.0 for 1 h. The enzyme was resistant to Co^{2+}, Mn^{2+}, Cr^{3+} and Ag^{+}. The specific activity of XYL10C for oat spelt xylan was 18,831 U mg^{-1}. It also had wide substrate specificity and produced simple products (65.1% xylose, 25.0% xylobiose and 9.9% xylan polymer) from oat spelt xylan.

Keywords Thermophilic xylanase · Acidophilic fungus · *Bispora* sp. MEY-1 · *Pichia pastoris*

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Introduction

Hemicellulose is the second most abundant natural polysaccharide after cellulose and it is comprised mainly of xylan (Collins et al. 2005). Because xylans are structurally heterogeneous, the xylan-degrading enzyme system generally includes several types of hydrolytic enzymes. Among them, a crucial enzyme component is endo-β-1, 4-xylanase (EC 3.2.1.8), which catalyzes the endohydrolysis of 1,4-β-D-xylosidic linkages in xylan to short xylooligosaccharides of varying length (Biely 1985). Thus, xylanases have potential applications in various industrial processes such as commercial food production, animal feed, baking, fruit juice clarification, pulp biobleaching and bioconversion (Beg et al. 2005).

Xylanases have been purified and characterized from various microorganisms, including bacteria, fungi and yeasts, and some xylanase genes have been cloned, sequenced and expressed (Kulkarni et al. 1999; Subramaniyan and Prema 2002). Based on the structural similarity among catalytic domains, majority of xylanases fall into glycoside hydrolase (GH) family 10 and 11 (Collins et al. 2005). These two families differ in substrate specificity, enzyme

action, molecular weight, and net-electric charge (Henrissat and Bairoch 1993).

Xylanases with different properties are useful in different applications. For example, alkaline xylanases are used in the pulp industry, whereas acidic xylanases are used in the feed and food industries. The majority of microbial xylanases are maximally active at, or near, mesophilic temperatures ($\sim$40–60°C) and neutral or slightly acidic pH (Collins et al. 2005), thus limiting their potential application in industry. Some fundamental properties of xylanases, such as specific activity, thermal stability, and resistance to cations and chemicals, also are pivotal factors that affect the industrial potential of these enzymes.

Recently, much effort has been devoted to isolating xylanases from extremophilic microorganisms, because these organisms produce enzymes that, by definition, function under extreme conditions. Among others have a thermophilic xylanase from *Thermotoga* sp. (Simpson et al. 1991), an alkaliphilic xylanase from *Bacillus* sp. (Nakamura et al. 1993), and an acidophilic xylanase from *Penicillium* sp. 40 (Kimura et al. 2000) been reported. Here, we report a novel xylanase from *Bispora* sp. MEY-1 CGMCC 2500, an acidophilic fungus isolated from the acidic wastewater of a uranium mine and showed optimal growth at pH 2.5–3.0 and 28–30°C (Luo et al. 2009). This enzyme is active under acidic conditions and at temperatures even above 90°C, has superior acid and thermal stability, is resistant to Co^{2+}, Mn^{2+}, Cr^{3+} and Ag^{+}, and has activity against various xylans, lichenan and carboxymethyl cellulose (CMC). All of these features suggest that the xylanase may have great potential in various biotechnological applications.

Materials and methods

Strains, vectors, materials and media

Bispora sp. MEY-1 CGMCC 2500 from the China General Microbiological Culture Collection Center was cultivated at 30°C in wheat bran medium consisting of 5 g l^{-1} $(NH_4)_2SO_4$, 1 g l^{-1} KH_2PO_4, 0.5 g l^{-1} $MgSO_4{\cdot}7H_2O$, 0.2 g l^{-1} $CaCl_2$, 0.01 g l^{-1} $FeSO_4{\cdot}7H_2O$, and 30 g l^{-1} wheat bran to induce xylanase production. *Escherichia coli* JM109 (TaKaRa, Tsu, Mie, Japan) was cultivated at 37°C in Luria–Bertani medium. *Pichia pastoris* GS115 (Invitrogen, Carlsbad, CA, USA) was cultivated at 30°C in yeast extract peptone dextrose medium. The plasmids pGEM-T Easy (Promega, Madison, WI, USA) and pPIC9 (Invitrogen) were used as cloning and expression vectors, respectively.

Oat spelt xylan, birchwood xylan, CMC sodium (CMCNa), lichenan, laminarin, chemical deglycosylation kit (TFMSA) and enzymatic protein deglycosylation kit (E-DEGLY) were purchased from Sigma (St. Louis, MO, USA). LA *Taq* DNA polymerase was obtained from TaKaRa, and restriction endonucleases, T4 DNA ligase and endo-β-*N*-acetylglucosaminidase H (Endo H) were obtained from New England Biolabs (Hitchin, Herts, UK). All other chemicals were analytical grade and commercially available.

Cloning of GH 10 xylanase gene (*xyl10C*)

Genomic DNA isolated from *Bispora* sp. MEY-1 using the fungal DNA Mini kit (Omega Bio-tek, USA) was used as a template for PCR amplification. The core region of *xyl10C* from *Bispora* sp. MEY-1 was amplified using degenerate primers XCP1 (5′-TGGGAYGTNGTNAAYGARGC-3′) and XCP2 (5′-TAYTCTATRTTRWARTCRTT-3′) (W, Y, N and R representing A/T, C/T, A/C/T/G and A/G, respectively), which were designed based on the conserved amino acid sequences (WDVVNEA and NDY(F)NL(I)EY) of GH 10 xylanases. The touchdown PCR conditions were as follows: 95°C for 3 min, followed by 10 cycles of 95°C for 30 s, 46°C (decreased by 1°C after each cycle) for 30 s, 72°C for 30 s, followed by 27 cycles of 95°C for 30 s, 37°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. The resulting amplified fragment was ligated into pEASY-T_3 vector for sequencing and subjecting to BLAST analysis. The 5′ and 3′ flanking regions of the core region were obtained using thermal asymmetric interlaced (TAIL)-PCR (Liu and Whittier 1995) with the genome walking kit (TaKaRa).

Bispora sp. MEY-1 was grown in wheat bran-inducing medium at 30°C for 3 days. Mycelia were collected and immediately ground to a fine powder in liquid nitrogen. Total RNA extraction and RT-PCR were carried out according to the methods of Luo et al. (2009), using the specific primers XCF (5′-ATGTCTTTCCACTCGCTTCT AATCTCAGGTCTTC-3′) and XCR (5′-TCATGGACTTT CCGCCTTATGTTGCAAAGCC-3′) with an annealing temperature of 60°C.

Sequence analysis

The signal peptide was predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP/). The molecular mass of the mature peptide was predicted using Vector NTI 7.0 software. Homology searches of GenBank were performed using the BLAST server (http://www.ncbi.nlm.nih.gov/BLAST). Multiple alignments of protein sequences were accomplished using the ClustalW program (http://www.ebi.ac.uk/clustalW) and GeneDoc software (Nicholas and Nicholas Jr 1997). Homology modeling was

performed using SWISS-MODEL (http://swissmodel.expasy.org//SWISS-MODEL.html) (Arnold et al. 2006).

Vector construction and expression of *xyl10C* in *P. pastoris*

To construct the expression vector in *P. pastoris*, the gene fragment (*xyl10C*) encoding the mature protein was amplified using primers PxcF (*Eco*RI) (5′-GACGAATTC GTCCCCAAAGAAGCTTGGGGAATTACAGTG-3′) and PxcR (*Not*I) (5′-CAGGCGGCCGCTCATGGACTTTCCG CCTTATGTTGCAAAGCC-3′) (restriction sites are underlined). The PCR product was digested by *Eco*RI and *Not*I and cloned into pPIC9. Recombinant expression and high-cell density fermentation were performed following the method as previously described (Luo et al. 2009).

Enzyme assay

Xylanase activity was determined by measuring the release of reducing sugar from oat spelt xylan using the 3,5-dinitrosalicylic acid (DNS) reagent as described by Miller (1959). The standard reaction contained 0.1 ml of appropriately diluted enzyme and 0.9 ml of citric acid–Na_2HPO_4 buffer (pH 4.5) containing 1% (w/v) oat spelt xylan. Following incubation at 85°C for 10 min, the reaction was terminated with 1.5 ml DNS reagent. The mixture was then boiled for 5 min and cooled to room temperature, and the absorption was measured at 540 nm. One unit of xylanase activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar equivalent to xylose per minute. Each reaction and its control were run in triplicate.

Purification of recombinant xylanase XYL10C

To purify the recombinant XYL10C, the induced culture supernatant (~300 ml) was collected by centrifugation at 12,000*g* for 10 min at 4°C and then concentrated by progressively adding solid ammonium sulfate to 80% saturation. The protein precipitate was harvested by centrifugation, re-suspended in 5 ml 20 mM Tris–HCl (pH 7.0) and dialyzed against the same buffer overnight. After removing the undissolved materials by centrifugation at 14,000*g* for 10 min at 4°C, the clear supernatant was loaded onto an HiTrap Q Sepharose XL 5-ml FPLC column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with 20 mM Tris–HCl buffer (pH 7.0). Proteins were eluted with a step gradient of NaCl (0–1.0 M) in the same buffer and a peak xylanase activity was detected when eluted at 0.38 M NaCl. Fractions having enzyme activity were pooled and concentrated by ultrafiltration at 4,000*g* for 20 min using a PL-10 Amicon ultracentrifugal filter (Millipore, Billerica, MA, USA).

Protein analysis, deglycosylation and mass spectrometry analysis of XYL10C

The purified XYL10C was analyzed by SDS-PAGE as described by Laemmli (1970). The protein concentration was determined using the Bradford (1976) assay with bovine serum albumin as a standard. To estimate the native molecular weight of XYL10C, the purified protein was subjected to non-denaturing gradient (4–12%, w/v) PAGE analysis as described previously (Cao et al. 2007).

Enzymatic deglycosylation of purified recombinant XYL10C was treated with 250 U of Endo H at 37°C for 2 h according to the manufacturer's instructions (New England Biolabs, Ipswich, MA, USA). Deglycosylation under native conditions were conducted according to the instructions of enzymatic protein deglycosylation kit (Sigma). XYL10C (100 μg) was treated with 1 μl each of PNGase F, *O*-glycosidase, α-2(3, 6, 8, 9) neuraminidase solutions, *β*-(1–4) galactosidase and *β*-*N*-acetylglucosaminidase at 37°C for 48 h. Chemical deglycosylation were conducted according to the instructions of chemical deglycosylation kit (TFMSA, Sigma). The deglycosylated and untreated XYL10C were analyzed by SDS-PAGE.

To identify the purified protein, the corresponding band was excised from the gel, digested by trypsin under denaturing conditions, and analyzed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) for peptide fingerprinting at the Institute of Zoology, Chinese Academy of Sciences.

Biochemical characterization of XYL10C

The optimum pH for XYL10C activity was measured at 85°C over 10 min in 0.1 M KCl–HCl buffer (pH 0.5–2.2), 0.1 M citric acid–Na_2HPO_4 buffer (pH 2.2–8.0), 0.1 M Tris–HCl (pH 8.0–9.0), and 0.1 M glycine–NaOH (pH 9.0–10.0), respectively. For the pH stability assay, the enzyme was incubated at 37°C in the buffers described above over the pH range of 1.0–10.0 for 1 h, and residual enzyme activity was then determined under standard conditions (pH 4.5, 85°C, 10 min).

To determine the temperature for maximal activity at pH 4.5, enzyme activity was measured at temperatures between 25 and 100°C in 0.1 M citric acid–Na_2HPO_4 buffer (pH 4.5) for 10 min. Thermal stability of the purified recombinant XYL10C was determined by measuring residual enzyme activity under standard conditions after incubating the enzyme solution in the absence of substrate at either 80 or 90°C for 0, 2, 5, 8, 10, 15, 20, 30, 45 and 60 min. The half lives of XYL10C in the absence of substrate at 80 and 85°C were also determined by measuring the residual activity for different durations (1−48 h).

The effect of chemicals on purified recombinant enzyme activity was determined by adding 1, 5 or 10 mM of different metal salts (NaCl, KCl, $CaCl_2$, LiCl, $CoCl_2$, $CrCl_3$, $NiSO_4$, $CuSO_4$, $MgSO_4$, $FeCl_3$, $MnSO_4$, $ZnSO_4$, $Pb(CH_3COO)_2$, $AgNO_3$ and $HgCl_2$) or other chemical reagents (SDS, EDTA and β-mercaptoethanol) to the assay system. The reaction system without any additive was used as a control.

Substrate specificity and kinetic parameters

The substrate specificity of XYL10C was determined by measuring the enzyme activity after incubating in 0.1 M citric acid–Na_2HPO_4 buffer (pH 4.5) containing 1.0% of each substrate (oat spelt xylan, birchwood xylan, lichenan, laminarin or CMCNa) at pH 4.5 and 85°C for 10 min. The amount of reducing sugars produced was estimated using the DNS method described above.

Kinetic parameters, $K_{0.5}$ and apparent v_{max}, for the purified recombinant XYL10C were determined by assaying in 0.1 M citric acid–Na_2HPO_4 buffer (pH 4.5) at 85°C for 5 min with 0.5–10 mg ml^{-1} oat spelt xylan or birchwood xylan as the substrate. A Michaelis–Menten plot was constructed to determine kinetic parameters. Three independent experiments were averaged, and each experiment included three replicate samples.

Analysis of hydrolysis products

Reactions containing 50 U purified recombinant XYL10C and 100 μg oat spelt xylan in 200 μl 0.1 M citric acid–Na_2HPO_4 buffer (pH 4.5) were incubated at 85°C for 2 h. Samples in which XYL10C was omitted were used as controls. After hydrolysis, the enzyme was removed from the reaction using the Nanosep Centrifugal 3 K Device (Pall, East Hills, NY, USA). The products were analyzed by high-performance anion-exchange chromatography with a model 2500 system from Dionex (Sunnyvale, CA, USA) (Li et al. 2008). Xylose, xylobiose, xylotriose, xylotetraose and xylopentaose were used as standards.

Nucleotide sequence accession numbers

The nucleotide sequence for the *Bispora* sp. MEY-1 xylanase GH 10 gene *xyl10C* was deposited in GenBank under accession number FJ492963.

Results

Gene cloning and sequence analysis

A single 176 bp fragment was amplified from genomic DNA of *Bispora* sp. MEY-1 with the degenerate primers XP1 and XP2. Nucleotide sequencing revealed that the sequence was similar to GH 10 xylanases and shared low amino acid sequence similarity with known sequences. The 5′ and 3′ flanking regions of the consensus fragment region were amplified by TAIL-PCR; products of about 879 and 985 bp were obtained for the 5′ and 3′ flanking regions, respectively, and were assembled with the core region. The resulting DNA sequence was 2,040 bp and contained one complete chromosomal gene of 1,336 bp. The full-length cDNA sequence (1,275 bp) of the xylanase was obtained through RT-PCR using the specific primers XCF and XCR. One intron of 61 bp interrupted the coding sequence.

SignalP analysis identified an N-terminal signal peptide at residues 1–18. The mature protein contained 407 residues with a calculated molecular mass of 44.9 kDa. Database searches and alignment of the gene and its deduced amino acid sequence with known xylanases were performed. XYL10C was classified as a member of the GH 10 xylanase based on the sequence comparison. No carbohydrate-binding domain was predicted in XYL10C. The N-terminal region (residues 19–76) is Ser/Thr-rich and shares no similarity to known sequences. Without this sequence, the deduced amino acid sequence of XYL10C shares highest identity (49.7%) with a putative xylanase from *Aspergillus fumigatus* Af293 (XP754103) and high homology to the xylanases from *Penicillium funiculosum* (46.2% identity, CAG25554), *Phanerochaete chrysosporium* (41.6% identity, AAG44992), *Agaricus bisporus* (40.5% identity, O60206) and *Penicillium simplicissimurn* (32.7% identity, P33559) (Fig. 1). There are six potential *N*-glycosylation sites in the deduced amino acid sequence.

The three-dimensional structure of XYL10C was predicted using the SWISS-MODEL server with the *P. simplicissimurn* xylanase (Protein data bank code, 1B30) as the template. The theoretical structure of XYL10A has the classical $(\alpha/\beta)_8$-fold, and the two catalytic glutamate residues (E219 and E332) are located on the central cavity. Except for the two catalytic residues, XYL10C has another two glutamate residues (E175 and E300) located near the active site, which are different from other GH 10 members (Fig. 2). Residues near the catalytic center, including E219, E332, W172, W372, W380, H168 and H302, were strictly conserved in GH 10 xylanases (Fig. 1). Two disulfide bridges (C167 and C211, C360 and C366) might be formed to stabilize the extensive loops and only one disulfide bridge in *P. simplicissimurn* xylanase.

Expression and high-cell density fermentation of *xyl10C* in *P. pastoris*

The gene encoding the mature XYL10C was cloned into an *Eco*RI and *Not*I-digested pPIC9 expression vector behind the α-factor signal sequence under the control of the

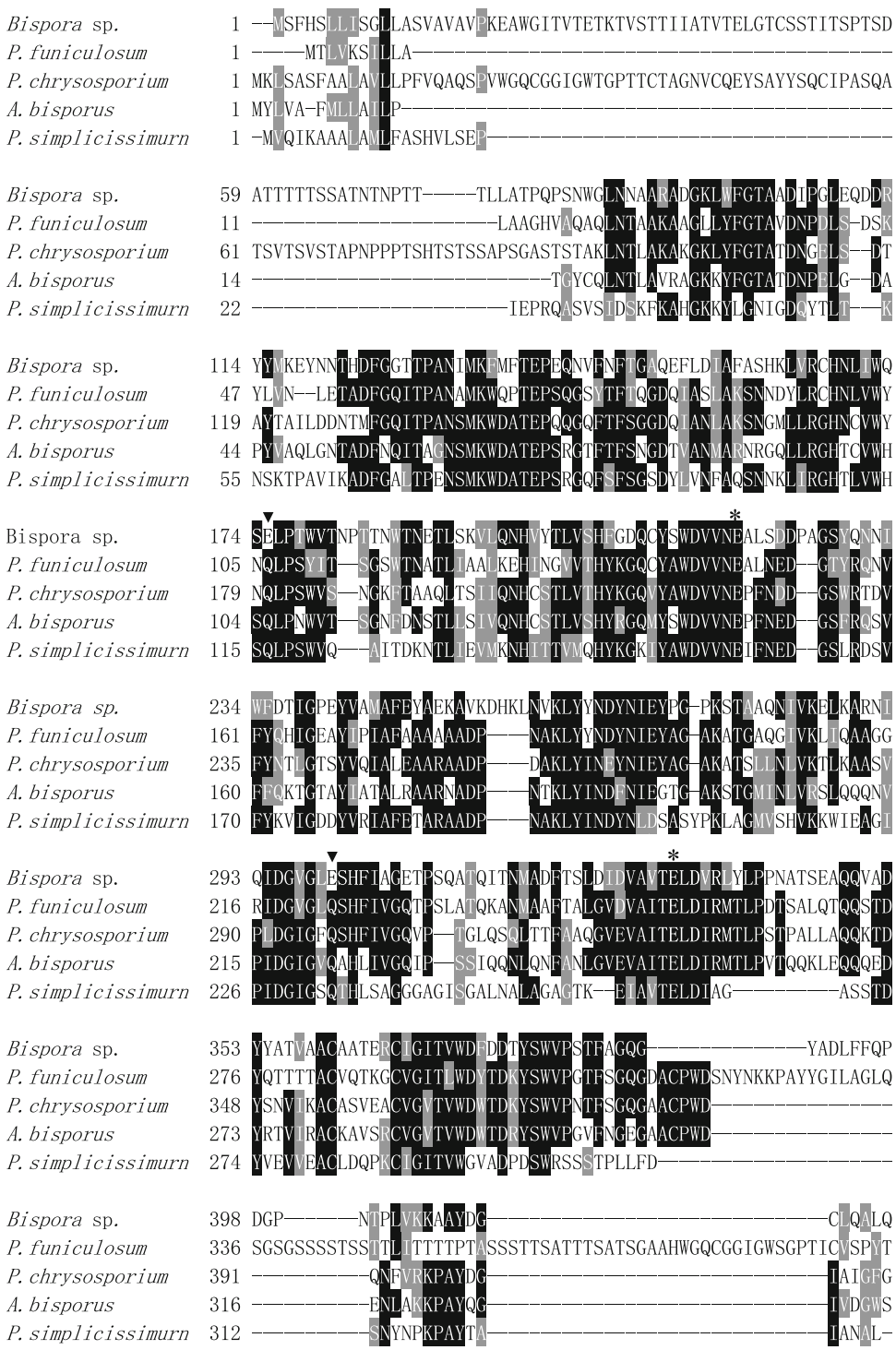

Fig. 1 Multiple alignment of the amino acid sequence of XYL10C with other known GH 10 xylanases using the ClustalW program. Xylanases from the following organisms were included: *Bispora* sp. (accession no. FJ492963), *Penicillium funiculosum* (accession no. CAG25554), *Phanerochaete chrysosporium* (accession no. AAG44992), *Agaricus bisporus* (accession no. O60206) and *Penicillium simplicissimurn* (accession no. P33559). Identical and similar amino acids are indicated by *solid black* and *gray boxes*, respectively. The predicted catalytic glutamate residues (E219 and E332) are indicated by *asterisks* and the other two glutamate residues (E175 and E300) which are different from other xylanases and near the active center are indicated by *inverted triangles*

alcohol oxidase 1 promoter. The recombinant plasmid DNA digested by *Bgl*II was electroporated into host *P. pastoris* cells, and the transformants were screened for xylanase activity. Transformant 92# showing the highest activity reached 587 U ml^{-1} of activity after methanol induction for 48 h in shaker flask culture confirming that *xyl10C* encodes a functional xylanase.

The transformant with the highest xylanase activity in the shaker flask was subjected to high-cell density fermentation. Before the induction phase, no xylanase was detected in the culture supernatant. After 150 h of methanol induction, the xylanase activity in the supernatant increased to its maximum of 73,400 U ml^{-1}. The maximal secreted concentration of XYL10C was 3.9 mg ml^{-1}.

Purification, mass spectrometry analysis and deglycosylation of recombinant XYL10C

Even before purification, the crude enzyme expressed in *P. pastoris* exhibited only one band after SDS-PAGE analysis. The specific activity of XYL10C was 18,831 U mg^{-1} after 1.1-fold purification with anion-exchange chromatography. The apparent molecular weight of the band was ~90 kDa (Fig. 3a), much higher than the calculated value of 44.9 kDa. To determine whether the single band at ~90 kDa was the recombinant XYL10C expressed by *xyl10C*, the band was excised, digested by trypsin, and analyzed using MALDI-TOF/MS. The amino acid sequences obtained from the mass peaks were compared with the XYL10C sequence. All four of the identified peptides LWFGTAADIPGLEQDDR, EYNNTHDFGGT TPANIMK, DHKLNVKLYYNDYNIEYPGPK and KAA YDGCLQALQHK corresponded exactly to the XYL10C sequence confirming that the purified protein was indeed recombinant XYL10C. Native gradient gel electrophoresis of the purified XYL10C demonstrated that the purified protein migrated as two bands of ~360 or 540 kDa suggesting that native XYL10C might be a tetramer or hexamer of identical subunits.

After EndoH treatment, the purified recombinant XYL10C migrated as a single band of ~70 kDa on SDS-PAGE. Deglycosylation by chemical method (TFMSA) resulted in a band of ~55 kDa, still a little higher than the calculated value of 44.9 kDa (Fig. 3b).

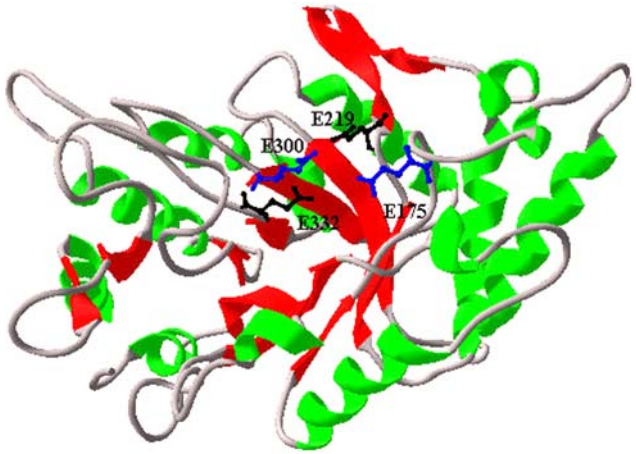

Fig. 2 Predicted tertiary structure of XYL10C from *Bispora* sp. MEY-1. The three-dimensional structural modeling was run on Swiss-Model server with the *P. simplicissimurn* xylanase (Protein data bank code, 1B30) as the template. The predicted catalytic glutamate residues (E219 and E332) and the other two glutamate residues (E175 and E300) near the active center are labeled

Properties of purified XYL10C

Purified recombinant XYL10C exhibited enzymatic activity over a pH range of 2.0–7.0 (measured at 85°C), and the activity had two peaks at pH 3.0 (63%) and 4.5–5.0 (100%), respectively (Fig. 4a). The enzyme was stable from pH 1.5 to 6.0, retaining more than 80% of its initial activity (Fig. 4b). Thus, the xylanase was acidophilic and stable under acid conditions.

Activity of purified recombinant XYL10C was maximal at about 85°C (pH 4.5, 10 min) (Fig. 4c). More than 80% of the maximum activity was retained between 75 and 90°C, and about 20% of activity remained at 100°C. It was also highly thermostable. After incubation at 80°C for 60 min, enzyme activity was 103% of the initial activity; the enzyme retained >87% of activity after incubation at 90°C for 10 min (Fig. 4d). The half lives of XYL10C at 80 and 85°C were approximately 45 and 3 h, respectively. After deglycosylation under native conditions, the thermostability of XYL10C declined retaining about 58% of the initial activity after incubation at 80°C for 60 min and

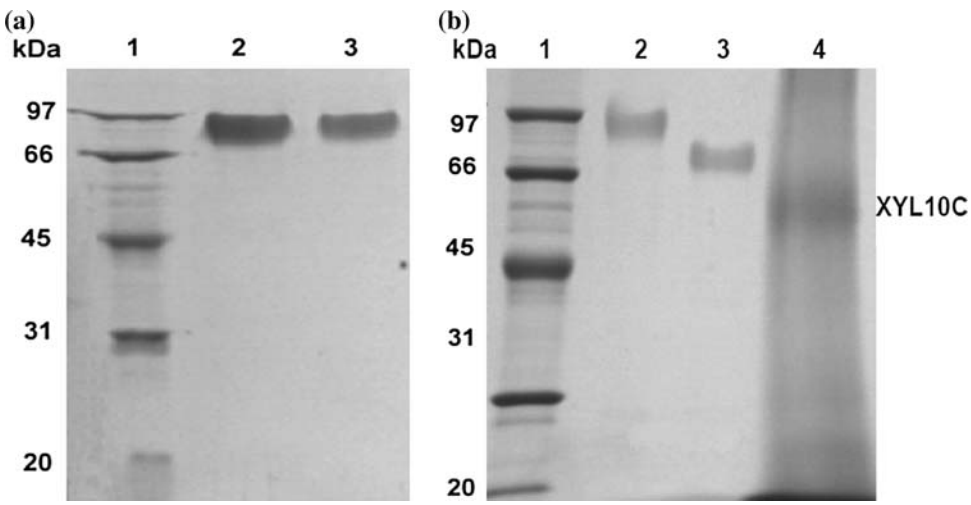

Fig. 3 SDS-PAGE analysis of purification (**a**) and deglycosylation (**b**) of recombinant XYL10C. **a** *Lane 1* molecular mass standard, *lane 2* culture supernatant from the induced transformant, *lane 3* extract following 80% ammonium sulfate precipitation and anion-exchange chromatography. **b** *Lane 1* molecular mass standard, *lane 2* purified recombinant XYL10C, *lane 3*, *4* XYL10C after deglycosylation with EndoH, *lane 5* XYL10C after deglycosylation with TFMSA

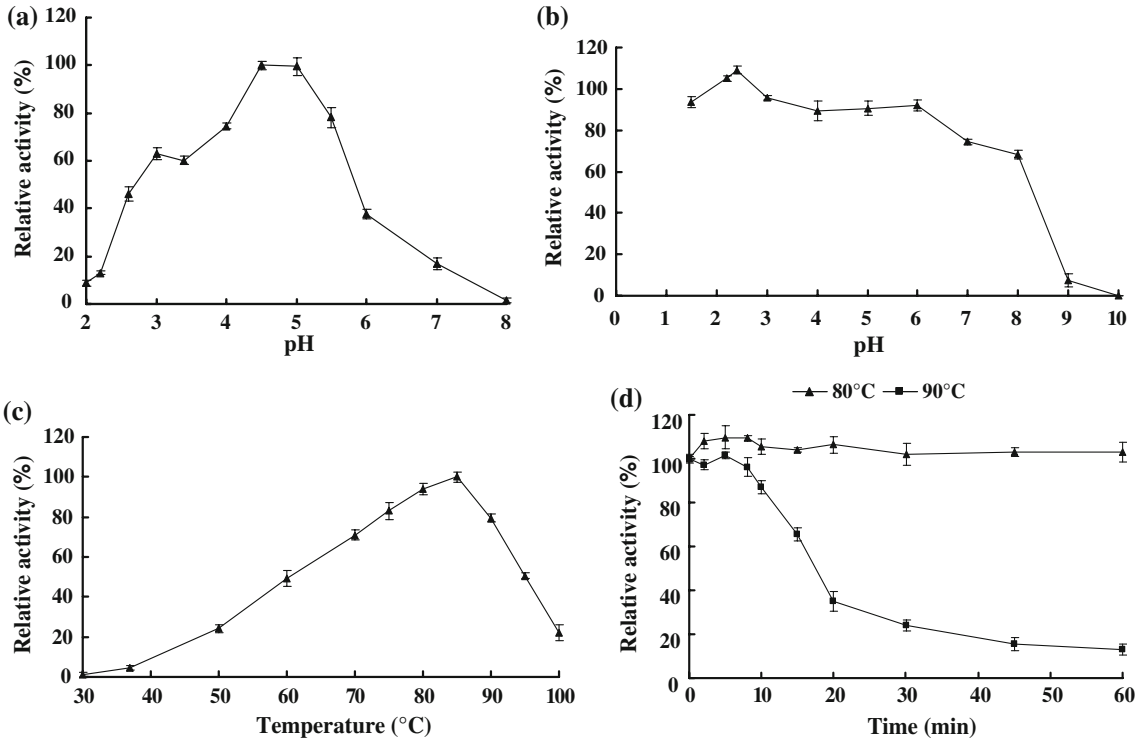

Fig. 4 Characterization of purified recombinant XYL10C. **a** Effect of pH on xylanase activity. The activity of recombinant XYL10C was assayed at 85°C in buffers ranging from pH 0.5 to 9.0. **b** Stability of xylanase activity at different pH values. After incubation of the enzymes at 37°C for 1 h in buffers ranging from pH 1.0 to 10.0, activity was determined in 0.1 M citric acid–Na_2HPO_4 buffer (pH 4.5) at 85°C. **c** The effect of temperature on xylanase activity was measured in 0.1 M citric acid–Na_2HPO_4 buffer (pH 4.5) for 10 min. **d** Thermostability of xylanase activity. Purified XYL10C was pre-incubated at 80°C (*triangles*) or 90°C (*squares*) in 0.1 M citric acid–Na_2HPO_4 buffer (pH 4.5) for 60 min. Aliquots were removed at specific time points to measure residual activity. Each data point represents the mean ± SD ($n = 3$)

almost completely inactive after incubation at 90°C for 10 min.

The enzyme activity was significantly enhanced in the presence of Cr^{3+}, Ag^{+} and β-mercaptoethanol (Table 1). Partial inhibition (<14%) was observed in the presence of 10 mM Ca^{2+}, Zn^{2+} or Mg^{2+}. XYL10C activity was completely inhibited by 5 and 10 mM SDS or Hg^{2+}, whereas Cu^{2+}, Pb^{2+} and Fe^{3+} (each at 10 mM) reduced its activity by 44.5, 35.0 and 33.0%, respectively. Other cations, such as Na^{+}, K^{+}, Li^{+}, Co^{2+}, Ni^{2+} and Mn^{2+}, had little or no effect on the activity at the three concentrations tested.

Substrate specificity, kinetic parameters and hydrolysis products

Purified recombinant XYL10C was highly active against oat spelt xylan (18,831 U mg^{-1}), slightly less active on birchwood xylan (14,236 U mg^{-1}), and weakly active on lichenan (138 U mg^{-1}), and CMCNa (2.1 U mg^{-1}). Kinetic parameters were determined for oat spelt xylan and birchwood xylan. The calculated $K_{0.5}$ and apparent v_{max} values were 0.98 mg ml^{-1} and 23,728 U $min^{-1}mg^{-1}$, respectively, for oat spelt xylan, and 1.27 mg ml^{-1} and 18,744 U $min^{-1}mg^{-1}$, respectively, for birchwood xylan. High-performance anion-exchange chromatography revealed that XYL10C-mediated digestion of oat spelt xylan mainly generated xylose (65.1%), xylobiose (25.0%) and some short xylan polymers (9.9%).

Discussion

The temperatures for maximal activity of most of xylanases are between 40 and 60°C. Thus far, only a few xylanases with optimum temperatures over 80°C have been reported, and most of these xylanases were isolated from thermophilic and hyperthermophilic organisms, including the xylanases from the thermophilic eubacterium *Thermotoga* (Simpson et al. 1991), extreme thermophilic anaerobic bacterium *Thermotoga thermarum* (Sunna and Antranikian 1996), *Dictyoglomus thermophilum* (McCarthy et al. 2000) and thermophilic fungus *Talaromyces thermophilus* (Maalej et al. 2008). The optimum temperature of XYL10C was 85°C, higher than that reported for any xylanases from fungal strains (Ritschkoff et al. 1994), including some thermophilic fungi (Tuohy and Coughlan 1992; Damaso

Table 1 Effect of metal ions and chemical reagents on the xylanase activity of purified recombinant XYL10C

	Relativity xylanase activity (%)[a]		
	1 mM	5 mM	10 mM
None	100.0	100.0	100.0
Ag^{+}	166.5 ± 3.3	ND[b]	ND
Cr^{3+}	107.0 ± 2.1	118.4 ± 4.5	119.2 ± 1.3
Co^{2+}	100.7 ± 1.6	92.1 ± 3.2	94.0 ± 2.4
Mg^{2+}	99.4 ± 0.9	95.7 ± 1.5	90.3 ± 2.3
Fe^{3+}	99.1 ± 2.1	108.0 ± 5.3	67.0 ± 3.1
Na^{+}	98.4 ± 4.3	97.5 ± 2.4	99.5 ± 0.8
Ni^{2+}	97.8 ± 3.6	94.6 ± 0.7	95.5 ± 2.3
Li^{+}	97.4 ± 1.4	98.5 ± 0.8	100.6 ± 3.9
K^{+}	97.4 ± 1.7	98.9 ± 1.9	102.4 ± 4.2
Ca^{2+}	97.0 ± 0.8	91.8 ± 1.5	85.9 ± 3.7
Cu^{2+}	96.1 ± 1.1	78.7 ± 3.4	55.5 ± 2.0
Mn^{2+}	95.7 ± 2.6	ND	ND
Hg^{2+}	91.6 ± 1.3	0	0
Zn^{2+}	90.8 ± 2.8	89.0 ± 1.6	86.6 ± 3.0
Pb^{2+}	81.4 ± 1.9	66.5 ± 3.6	65.0 ± 1.2
β-mercaptoethanol	108.4 ± 3.1	118.7 ± 4.3	115.9 ± 3.4
EDTA	98.2 ± 1.8	97.1 ± 0.9	101.4 ± 1.3
SDS	80.1 ± 3.3	0.0	0.0

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

[b] *ND* not determined

et al. 2003; Maalej et al. 2008). It was also thermostable, retaining all of the initial activity after incubation at 80°C for 60 min and about 80% at 90°C for 10 min. Heavy glycosylation of enzyme might underlie its thermostability as demonstrated by Han et al. (1999). In XYL10C, the existence of six potential *N*-glycosylation sites and a Ser/Thr-rich region might result in its extensive glycosylation. After deglycosylation under native conditions, the thermostability of XYL10C declined.

XYL10C shares high-sequence identity (41.6%) to the *Phanerochaete chrysosporium* xylanase XynA (Decelle et al. 2004), but their properties are distinct. For example, in the case of temperature properties, XYL10C exhibited maximal activity at 85°C and was thermostable at 80°C; XynA was maximal active at 70°C, but lost stability at temperatures above 60°C. Moreover, XYL10C had a broad range of pH adaptation (pH 1.5–7.0), exhibited peak activities at pH 3.0 (60%) and pH 4.5–5.0 (100%), respectively, and was acid stable at pH 1.5–6.0; XynA showed maximum activity at pH 4.5 but no activity blow pH 3.0, and was unstable at pH 1.0–3.0. When compared with XynA and other GH 10 xylanases, XYL10C has more acidic amino acid residues (E and D, 11.3%) and four glutamate residues located at the active site, the function of the additional glutamate residues near the active site need to be further explored.

The size of the deduced mature XYL10C (44.9 kDa) is comparable to that of other endo-β-1,4-xylanases belonging to family 10, which have a relatively high-molecular weight (>30 kDa) (Biely et al. 1997). The apparent molecular weight of XYL10C (~90 kDa) is much greater than that calculated one, even after deglycosylation (~55 kDa), suggesting that the enzyme underwent a complex post-translational modification. Native XYL10C might work as a tetramer or hexamer based on non-denaturing gradient PAGE analysis. The crude enzyme of recombinant XYL10C was a single band after SDS-PAGE analysis, and the activity assay indicated that the purity of XYL10C was enhanced only marginally by the purification process. Because XYL10C was relatively homogeneous even in the absence of purification, as such, crude XYL10C preparations may be useful directly in many industrial processes, thereby reducing overall production costs.

High-specific activity and efficient hydrolysis are necessary for potential applications of an enzyme in industrial processes. XYL10C has high-specific activity (18,831 U mg^{-1}), is expressed at high levels in *P. pastoris* (3.9 mg ml^{-1}), and has high activity during fermentation (73,400 U ml^{-1}); thus, this enzyme potentially may be more cost-effective than any of the xylanases currently used in industry. Furthermore, it may be possible to further increase XYL10C expression by altering the nucleotide sequence according to codon usage bias, using a more favorable signal sequence, or by increasing the gene copy number.

In addition to degrading xylan, recombinant XYL10C also hydrolyzed and CMCNa. Wide substrate specificity is common to all the enzymes in the GH 10 family. XYL10C also exhibited some beta-glucanase activity with lichenan as the substrate, which has seldom been reported for GH 10 xylanases. The major xylan hydrolysis products produced by XYL10C were mainly xylose and xylobiose, which are less complex in structure with the comparison of the products of other xylanases.

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References

Arnold K, Bordoli L, Kopp J, Schwede T (2006) The SWISS-MODEL workspace: a web-based environment for protein structure homology modeling. Bioinformatics 22:195–201

Beg QK, Kapoor M, Mahajan L, Hoondal GS, Terenzi HF, Jorge JA, Amorim DS (2005) Xylanases from fungi: properties and industrial applications. Appl Microbiol Biotechnol 67:577–591

Biely P (1985) Microbial xylanolytic systems. Trends Biotechnol 3:286–290

Biely P, Vrsanska M, Tenkanen M, Kluepfel D (1997) Endo-β-1,4-xylanase families: differences in catalytic properties. J Biotechnol 57:151–166

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Cao Y, Yang P, Shi P, Wang Y, Luo H, Meng K, Zhang Z, Wu N, Yao B, Fan Y (2007) Purification and characterization of a novel protease-resistant α-galactosidase from *Rhizopus* sp. F78 ACCC 30795. Enzyme Microb Tech 41:835–841

Collins T, Gerday C, Feller G (2005) Xylanases, xylanases families and extremophilic xylanases. FEMS Microbiol Rev 29:3–23

Damaso MCT, Almeida MS, Kurtenbach E, Martins OB, Andrade CMMC, Pereira N Jr, Albano RM (2003) Optimized expression of a thermostable xylanase from *Thermomyces lanuginosus* in *Pichia pastoris*. Appl Environ Microbiol 69:6064–6072

Decelle B, Tsang A, Storms RK (2004) Cloning, functional expression and characterization of three *Phanerochaete chrysosporium* endo-1, 4-β-xylanases. Curr Genet 46:166–175

Han Y, Wilson DB, Lei XG (1999) Expression of an *Aspergillus niger* phytase gene (*phyA*) in *Saccharomyces cerevisiae*. Appl Environ Microbiol 65:1915–1918

Henrissat B, Bairoch A (1993) New families in the classification of glycosyl hydrolases on amino acid sequence similarities. Biochem J 293:781–788

Kimura T, Ito J, Kawano A, Makino T, Kondo H, Karita S, Sakka K, Ohmiya K (2000) Purification, characterization, and molecular cloning of acidophilic xylanase from *Penicillium* sp. 40. Biosci Biotechnol Biochem 64:1230–1237

Kulkarni N, Shendye A, Rao M (1999) Molecular and biotechnological aspects of xylanases. FEMS Microbiol Rev 23:411–456

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Li N, Meng K, Wang YR, Shi PJ, Luo HY, Bai YG, Yang PL, Yao B (2008) Cloning, expression, and characterization of a new xylanase with broad temperature adaptability from *Streptomyces* sp. S9. Appl Microbiol Biotechnol 80:231–240

Liu YG, Whittier RF (1995) Thermal asymmetric interlaced PCR: automatable amplification and sequencing of insert end fragments from P1 and YAC clones for chromosome walking. Genomics 25:674–681

Luo HY, Wang YR, Wang H, Yang J, Yang YH, Huang HQ, Yang PL, Bai YG, Shi PJ, Fan YL, Yao B (2009) A novel highly acidic β-mannanase from the acidophilic fungus *Bispora* sp. MEY-1: gene cloning and overexpression in *Pichia pastoris*. Appl Microbiol Biotechnol 82:453–461

Maalej I, Belhaj I, Masmoudi FN, Belghith H (2008) Highly thermostable xylanase of the thermophilic fungus *Talaromyces thermophilus*: purification and characterization. Appl Biochem Biotechnol 158:200–212

McCarthy AA, Morris DD, Bergquist PL, Baker EN (2000) Structure of XynB, a highly thermostable beta-1, 4-xylanase from Dictyoglomus thermophilum Rt46B.1, at 1.8 Å resolution. Acta Crystallogr D Biol Crystallogr 56:1367–1375

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–428

Nakamura S, Wakabayashi K, Nakai R, Aono R, Horikoshi K (1993) Purification and some properties of an alkaline xylanase from alkaliphilic *Bacillus* sp. strain 41M–1. Appl Environ Microbiol 59:2311–2316

Nicholas KB, Nicholas Jr HB (1997) GeneDoc: a tool for editing and annotating multiple sequence alignments. (Distributed by the author. http://www.psc.edu/biomed/genedoc)

Ritschkoff AC, Buchert J, Viikari L (1994) Purification and characterization of a thermophilic xylanase from the brown-rot fungus *Gloeophyllum trabeum*. J Biotechnol 32:67–74

Simpson HD, Haufler UR, Daniel RM (1991) An extremely thermostable xylanase from the thermophilic eubacterium *Thermotoga*. Biochem J 277:413–417

Subramaniyan S, Prema P (2002) Biotechnology of microbial xylanases: enzymology, molecular biology, and application. Crit Rev Biotechnol 22:33–64

Sunna A, Antranikian G (1996) Growth and production of xylanolytic enzymes by the extreme thermophilic anaerobic bacterium *Thermotoga thermarum*. Appl Microbiol Biotechnol 45:671–676

Tuohy MG, Coughlan MP (1992) Production of thermostable xylan-degrading enzymes by *Talaromyces emersonii*. Biores Technol 39:131–137