J Ind Microbiol Biotechnol (2010) 37:187–194
DOI 10.1007/s10295-009-0662-4

ORIGINAL PAPER

# A new xylanase from thermoacidophilic *Alicyclobacillus* sp. A4 with broad-range pH activity and pH stability

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Received: 18 August 2009 / Accepted: 24 October 2009 / Published online: 16 November 2009
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**Abstract** We have identified a highly pH-adaptable and stable xylanase (XynA4) from the thermoacidophilic *Alicyclobacillus* sp. A4, a strain that was isolated from a hot spring in Yunnan Province, China. The gene (*xynA4*) that encodes this xylanase was cloned, sequenced, and expressed in *Escherichia coli*. It encodes a 338-residue polypeptide with a calculated molecular mass of 42.5 kDa. The deduced amino acid sequence is most similar to (53% identity) an endo-1,4-$\beta$-xylanase from *Geobacillus stearothermophilus* that belongs to family 10 of the glycoside hydrolases. Purified recombinant XynA4 exhibited maximum activity at 55°C and pH 7.0, had broad pH adaptability (>40% activity at pH 3.8–9.4) and stability (retaining >80% activity after incubation at pH 2.6–12.0 for 1 h at 37°C), and was highly thermostable (retaining >90% activity after incubation at 60°C for 1 h at pH 7.0). These properties make XynA4 promising for application in the paper industry. This is the first report that describes cloning and expression of a xylanase gene from the genus *Alicyclobacillus*.

**Keywords** *Alicyclobacillus* sp. · pH adaptability · pH stability · Thermoacidophilic strain · Xylanase

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## Introduction

Xylanases (EC 3.2.1.8) are ubiquitous in nature and play a crucial role in xylan degradation by catalyzing the endohydrolysis of 1,4-$\beta$-D-xylosidic linkages into short xylooligosaccharides [1]. A variety of xylanases have been reported from fungi, bacteria, and yeast, and some of the corresponding genes have been cloned and expressed [2–4]. Based on the primary structure of the catalytic domain, most xylanases are confined to glycoside hydrolase (GH) families 10 and 11 [5].

Xylanases have attracted particular attention because of their potential for widespread application in the fields of animal feed [6–8], nutrition [9], waste treatment [10], paper production [11], and biofuel development [12]. Many xylanases from microbial sources have optimal activity at mesophilic temperatures (~40–60°C) and neutral pH [5], limiting their potential for industrial applications. Some xylanases from acidophilic and alkaline sources have special properties, such as XyaA from the alkalophilic *Bacillus* sp. strain N137 [13], XylA and XylB from the alkaliphilic *Bacillus* sp. strain AR-009 [14], and XynA from the acidophilic *Penicillum* sp. strain 40 [15], but most of these xylanases are stable and active only under their naturally occurring acidic or alkali conditions.

The objective of this study is to obtain a xylanase with good adaptability and stability over a wide pH range. A thermoacidophilic xylanase-producing *Alicyclobacillus* sp. strain, designated A4, was isolated from a hot spring in Yunnan Province, China. A xylanase gene was cloned and expressed in *Escherichia coli*, and the recombinant protein was subjected to biochemical characterization. The broad pH adaptability, excellent pH stability, and good thermostability make this a potential enzyme for application in many industries.

## Materials and methods

### Microorganism isolation, xylanase activity screening, and growth condition optimization

The outflow of a hot spring (pH 7.23) in Baoshan City, Yunnan Province, China was collected for microorganism isolation. The growth medium contained 0.1% yeast extract and 0.2% peptone and was adjusted to pH 2.0 using 2 N $H_2SO_4$. After incubation at 60°C for 48 h with agitation (100 rpm), cultures were diluted and spread onto agar plates containing 0.1% yeast extract, 0.2% peptone, 0.5% oat spelt xylan, and 3% agar (pH 3.0). Xylanase-producing strains were screened using the Congo red method [16] and identified by 16S rDNA sequences in GenBank. Supernatants and cell lysate of xylanase-producing strains cultured in induced medium (0.1% yeast extract, 0.2% peptone, 0.5% oat spelt xylan, pH 3.0) were subjected to xylanase activity assay. The optimal pH and temperature growth conditions for the strain showing highest xylanolytic activity were determined using liquid medium consisting of 0.1% yeast extract and 0.2% peptone.

### Vectors and materials

Vectors pEasy-T3 (Tiangen, Beijing, China) and pET-22b(+) (Novagen, Madison, WI, USA) were used for gene cloning and recombinant protein expression, respectively. Oat spelt xylan and birchwood xylan were purchased from Sigma (St. Louis, MO, USA). DNA purification kits, restriction endonucleases, T4 DNA ligase, and the Genome Walking kit were purchased from TaKaRa (Otso, Shiga, Japan). The protein purification His-bind kit was purchased from Bio-Rad (Hercules, CA, USA).

### Cloning of the full-length gene *xynA4*

The amino acid sequences of known GH 10 xylanases (http://www.cazy.org/fam/acc_GH.html) were aligned using the ClustalW program [17], and two highly conserved motifs, YDWDV and HGIGM [18], were identified. Based on these conserved sequences, two degenerate oligonucleotide primers, XYN10F and XYN10R (Table 1), were generated to amplify a fragment of the xylanase gene *xynA4*. Genomic DNA purified from *Alicyclobacillus* sp. A4 was subjected to polymerase chain reaction (PCR) amplification in the presence of primers XYN10F and XYN10R as follows: initial denaturation at 94°C for 5 min, 10 touchdown cycles (94°C for 30 s; 53–48°C for 30 s, decreasing by 0.5°C each cycle; 72°C for 30 s), 30 cycles of amplification (94°C for 30 s, 48°C for 30 s, 72°C for 30 s), and a final extension step at 72°C for 10 min. Amplification products were purified by gel electrophoresis and cloned into the pEasy-T3 vector for sequencing. The DNA fragment sequence was analyzed using blastx (http://www.ncbi.nlm.nih.gov/BLAST/).

To obtain the 5′ and 3′ flanking regions of the *xynA4* fragment, thermal asymmetric interlaced (TAIL)-PCR amplification was performed according to Liu and Whittier [19] with modifications described by Zhang et al. [20]. Six nested insertion-specific primers (xynA4F1-3 and xynA4R1-3; Table 1) were designed and synthesized according to the sequence of the *xynA4* gene fragment, and TAIL-PCR was performed using the Genome Walking kit. The upstream and downstream amplification products were purified, sequenced, and assembled with the initial *xynA4* fragment to obtain the full-length gene.

**Table 1** Oligonucleotide primers used for PCR amplification and sequencing of *xynA4*

| Primer | Sequence (5′ → 3′)[a] |
|---|---|
| 27F | AGAGTTTGATCCTGGCTCAG |
| 1492R | GGTTACCTTGTTACGACTT |
| XYN10F | TAYGAYTGGGAYATNGCT |
| XYN10R | YTGCATNCCDATNCCRTG |
| xynA4F | GGGAATTCGATGACTGACACTTATCGGAATATTCC |
| xynA4R | GGGAAGCTTAAATTCAACCACGCTCCAAAACGC |
| xynA4F1 | GCAGTCGTCGATGCGGGTACAGGA |
| xynA4F2 | CAGACGCACTGCTCTTCTACAACGAC |
| xynA4F3 | CGATGAAGGTGTCCCCATCCACGG |
| xynA4R1 | CCGTGGATGGGGACACCTTCATCG |
| xynA4R2 | GTCGTTGTAGAAGAGCAGTGCGTCTG |
| xynA4R3 | TCCTGTACCCGCATCGACGACTGC |
| AD1 | NTCGASTWTSGWGTT |
| AD2 | NGTCGASWGANAWGAA |
| AD3 | WGTGNAGWANCANAGA |
| AD4 | TGWGNAGWANCASAGA |
| AD5 | AGWGNAGWANCAWAGG |
| AD6 | CAWCGICNGAIASGAA |
| AD7 | TCSTICGNACITWGGA |
| AD8 | GTNCGASWCANAWGTT |
| AD9 | NCAGCTWSCTNTSCTT |

[a] N indicates A, G, C or T; M indicates A or C; S indicates C or G; W indicates A or T; D indicates A, G or T; Y indicates C or T

### Sequence analysis

Sequence assembly and prediction of the mature peptide molecular mass was performed using Vector NTI Suite 7.0 software (InforMax, Gaithersburg, MD, USA). Multiple alignments of DNA and protein sequences were carried out using the blastn, blastx, and blastp programs (http://www.ncbi.nlm.nih.gov/BLAST/). The signal peptide of XynA4 was predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP).

Homology modeling was performed using SWISS-MODEL (http://swissmodel.expasy.org//SWISS-MODEL.html) [21].

### Cloning and expression of *xynA4* in *E. coli*

To construct the plasmid for *xynA4* expression in *E. coli*, a gene fragment encoding the mature protein was amplified by PCR using primers XynA4F and XynA4R (Table 1) under the following conditions: 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 90 s. The PCR amplification product was then gel-purified, digested with *Eco*RI and *Hin*dIII, and cloned into the corresponding sites of the pET-22b(+) vector to generate full-length *xynA4* containing a $His_6$ tag at the C terminus for purification and an N-terminal *pelB* signal sequence for periplasmic localization of the xylanase. The recombinant expression plasmid, pET-*xynA4*, was transformed into *E. coli* BL21 (DE3) competent cells, and xylanase-producing recombinants were identified using the Congo red method and were verified by DNA sequencing.

### Purification of recombinant XynA4 and protein analysis

A positive transformant containing pET-*xynA4* was picked from a single colony and grown overnight at 37°C in Luria–Bertani (LB) medium supplemented with 100 μg $ml^{-1}$ ampicillin. The culture was then inoculated into fresh LB medium (1:100 dilution) containing ampicillin and grown aerobically at 37°C to $OD_{600}$ of about 0.6 (2.5–3.0 h). Recombinant xylanase expression was induced by addition of isopropyl-$\beta$-D-1-thiogalactopyranoside (IPTG) to final concentration of 0.8 mM. After incubation at 30°C for 16 h, the cells were centrifuged at 12,000*g* for 10 min at 4°C, and the clear supernatant was concentrated using an ultrafilter membrane (9-kDa; Motianmo, Tianjin, China). The concentrate xylanase was purified by nickel-nitrilotriacetic acid (Ni–NTA) chromatography (Qiagen, Valencia, CA, USA) using a linear gradient of imidazole (20–200 mM) in Tris–HCl buffer [20 mM Tris–HCl, pH 7.6; 500 mM NaCl; 10% (w/v) glycerol]. Fractions exhibiting xylanolytic activity were combined, and the protein concentration was determined using the Bradford method [22].

Purified recombinant xylanase was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis as described by Laemmli [23].

### Enzyme activity assay

Xylanase activity was determined with the 3,5-dinitrosalicylic acid (DNS) method [24]. One unit of xylanase activity was defined as the amount of enzyme required to release 1 μmol of reducing sugar equivalent to xylose from oat spelt xylan or birchwood xylan per minute under the assay conditions (pH 7.0, 55°C, 10 min in McIlvaine buffer).

### Biochemical characterization

The optimal pH for enzyme activity of the purified recombinant xylanase was determined at 55°C for 10 min using oat spelt xylan as substrate in buffers with pH ranging from 2.2 to 12.0. The buffers used were 0.1 M glycine–HCl buffer for pH 2.2–5.4, McIlvaine buffer (0.2 M $Na_2HPO_4$ containing 0.1 M citric acid) for pH 5.4–7.8, 0.1 M Tris–HCl buffer for pH 7.4–8.6, and 0.1 M glycine-NaOH buffer for pH 8.6–12.0. pH stability was estimated by measuring xylanase activity under standard conditions (pH 7.0, 55°C, 10 min) after pre-incubation of the purified recombinant enzyme in buffers of pH 2.2–12.0 at 37°C for 1 h. The optimal temperature for purified enzyme activity was determined by measuring enzyme activity in McIlvaine buffer (pH 7.0) at temperatures ranging from 35°C to 80°C. The thermostability of the enzyme was determined by pre-incubating the enzyme in McIlvaine buffer (pH 7.0) at 60°C, 65°C or 70°C without substrate for various periods and then measuring the residual enzyme activity in each case under standard conditions.

To investigate the effects of different metal ions and chemical reagents on the recombinant enzyme activity, xylanase activity was measured in McIlvaine buffer (pH 7.0) containing 1 or 10 mM NaCl, KCl, $CaCl_2$, LiCl, $CoCl_2$, $CrCl_3$, $NiSO_4$, $CuSO_4$, $MgSO_4$, $FeCl_3$, $MnSO_4$, $ZnSO_4$, $Pb(CH_3COO)_2$, $AgNO_3$, $HgCl_2$, ethylenediamine tetraacetic acid (EDTA), SDS or $\beta$-mercaptoethanol at 55°C. Reactions without added reagents were used as a control.

Substrate specificity of XynA4 was tested against oat spelt xylan, birchwood xylan, barley $\beta$-glucan, CMC-Na, soluble starch, and laminarin using the method described by Liu et al. [25].

$K_m$ and $V_{max}$ values for the purified recombinant enzyme were determined in McIlvaine buffer (pH 7.0) at 55°C using 1–10 mg $ml^{-1}$ oat spelt xylan or birchwood xylan as the substrate. Data were plotted according to the Lineweaver–Burk method [26].

### Analysis of hydrolysis product

Four units of purified recombinant enzyme and 200 μg oat spelt xylan in 400 μl McIlvaine buffer (pH 7.0) were incubated at 37°C for 12 h. Then the solution was treated by Nanosep Centrifugal 3 K Device (Pall, USA). The products were analyzed by high-performance anion-exchange

chromatography (HPAEC) with a model 2500 system from Dionex (USA) [27]. Xylose, xylobiose, xylotriose, and xylotetraose were used as standards. The hydrolytic products were quantified on the basis of their own standard curves.

Nucleotide sequence accession numbers

The nucleotide sequences for the *Alicyclobacillus* sp. A4 16S rDNA and the xylanase gene (*xynA4*) were deposited in the GenBank database under accession numbers GQ240229 and GQ240233, respectively.

## Results

Microorganism identification

Twenty-six strains (A1–A26) showing xylanolytic activity were collected from the hot spring water in Yunnan province, China, and identified based on 16S rDNA sequences. Strain A4 had the highest xylanase activity in the supernatant (0.41 U ml$^{-1}$, pH 4.0) and in the cell lysate (0.17 U ml$^{-1}$, pH 6.0) and was classified into the genus *Alicyclobacillus* because its 16S rDNA sequence exhibited highest nucleotide identity (99%) with *Alicyclobacillus hesperidum* DSM 12766 (AB059679.1). Growth of *Alicyclobacillus* sp. A4 cultures was observed at 45–60°C and pH 2.0–5.0. Optimal growth occurred at 60°C and pH 3.0 (data not shown).

Gene cloning and sequence analysis

A *xynA4* fragment of 259 bp was amplified by PCR using the degenerate primers XYN10F and XYN10R. Nucleotide sequencing revealed that the gene fragment had substantial identity with xylanases from *Bacillus* sp. (CAA84631.1; 61% identity) [13]. Based on the partial sequence, six sequence-specific primers (Table 1) were designed for amplification of the 5′ and 3′ flanking regions of the consensus fragment region using TAIL-PCR. Amplification products of ~700 bp and 495 bp were obtained for the 5′ and 3′ flanking regions, respectively, and were assembled with the consensus region to obtain the full-length gene. The resulting DNA sequence contained one open reading frame of 1,017 bp including one TGA stop codon.

The mature xylanase protein contained 338 residues with a calculated molecular mass of 42.5 kDa. Database searches and alignment of the gene and its deduced amino acid sequence with known xylanases were performed. No signal peptide was predicted using SignalP analysis. XynA4 was classified as a member of the GH 10 family of xylanases based on amino acid sequence comparisons. The deduced amino acid sequence of XynA4 shared highest identity (53%) with an endo-1,4-$\beta$-xylanase (ABI49937.2) from *Bacillus stearothermophilus* [28] and the xylanase (AAZ74783) from *Geobacillus* sp. MT-1 [29]. The tertiary structure of XynA4 was predicted with the SWISS-MODEL server using the xylanase from *Geobacillus stearothermophilus* (protein data bank code, 2Q8X) [30] as the template. The theoretical structure of XynA4 had the typical "bowl" structure of xylanases (Fig. 1), and two putative catalytic residues (Glu137 and Glu244) were identified in the conserved regions [30].

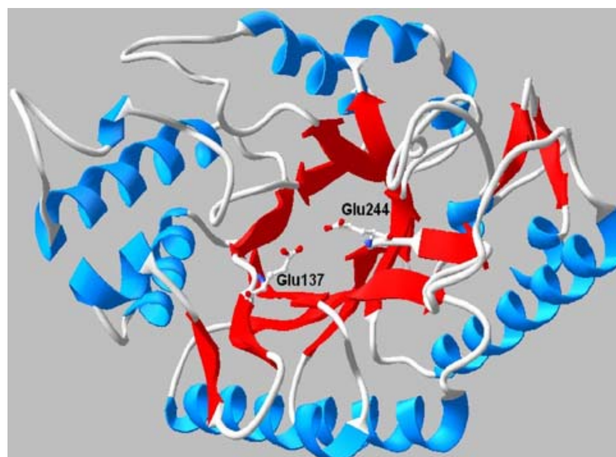


**Fig. 1** The predicted *bowl-like* structure of *Alicyclobacillus* sp. A4 XynA4. The glutamate residues (Glu137 and Glu244) near the active center are predicted to be catalytic and are therefore detailed in ball-and-stick form. Homology modeling was performed using SWISS-MODEL [20] using the xylanase from *Geobacillus stearothermophilus* (protein data bank code, 2Q8X) as the template

Enzyme expression and purification

An *E. coli* BL21 (DE3) transformant that contains pET-*xynA4* was cultivated in LB medium with ampicillin. Induction conditions have a serious effect on recombinant protein expression. In this study, different incubation conditions were examined to optimize recombinant xylanase activity in *E. coli.* After induction with 0.8 mM IPTG at 37°C for 6–12 h, no xylanase activity was detected either in the cell lysate or in the culture supernatant. Using the same concentration of IPTG and incubation at 30°C for 6 h, xylanase activities of 0.15 U ml$^{-1}$ and 0.36 U ml$^{-1}$ were detected in the cell lysate and culture supernatant, respectively. The activities increased to 0.23 U ml$^{-1}$ and 0.81 U ml$^{-1}$, respectively, when cultures were incubated for 16 h. Thus, to induce xylanase expression and increase xylanase yield in the medium, induction with 0.8 mM IPTG at 30°C for 16 h was selected for further production of the recombinant xylanase. After induction with 0.8 mM IPTG at 30°C for 16 h, xylanase activity (0.38 U ml$^{-1}$)

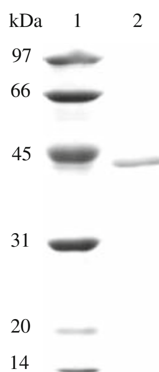


**Fig. 2** SDS–PAGE analysis of purified recombinant XynA4. *Lane 1*, molecular mass markers; *lane 2*, purified recombinant XynA4 from the culture supernatant

was identified in the culture supernatant. No xylanase activity was detected in the supernatants of cultures from the uninduced transformant or from a transformant containing the empty pET-22b(+) vector (data not shown). The recombinant $His_6$-tagged xylanase was purified to apparent homogeneity from the culture supernatant using ultrafiltration followed by Ni–NTA chromatography. The specific activity of the purified recombinant XynA4 was 420.2 U $mg^{-1}$ with a final activity yield of 14.68%. The purified enzyme migrated as a single band with a molecular mass of 43 kDa as determined by SDS–PAGE analysis (Fig. 2), which is similar to the calculated molecular mass of 42.5 kDa.

## Biochemical characterization

Recombinant xylanase activity was assayed at various pH values. The optimum pH for recombinant XynA4 was 7.0, and >40% of the maximum activity was retained at pH 3.8–9.4 (Fig. 3a). XynA4 was pH stable, retaining >80% of its initial activity after pre-incubation at 37°C, pH 2.6–12.0 for 1 h (Fig. 3b). The optimal temperature for enzyme activity was 55°C at pH 7.0, and >50% of the maximum activity was retained at 40–65°C (Fig. 3c). The enzyme was thermostable at 60°C (Fig. 3d), retaining >90% of maximal activity after pre-incubation at 60°C for 1 h. Approximately 30% and 34% of the maximal activity was retained, respectively, after pre-incubation at 65°C for 1 h and at 70°C for 5 min. The half-times of thermal deactivation of XynA4 at 60°C, 65°C, and 70°C were 394, 17, and 3 min, respectively.

The substrate specificity of XynA4 (pH 7.0) was tested against several substrates. The results showed that XynA4 only had ability to catalyze the hydrolysis of oat spelt xylan and birchwood xylan.

XynA4 activity in the presence of different metal ions or chemical reagents was also examined (Table 2). $Ca^{2+}$, $Li^{+}$, $Co^{2+}$, $Cr^{3+}$, $Mg^{2+}$, $Pb^{2+}$ or $\beta$-mercaptoethanol (1 mM each) enhanced the enzyme activity, whereas 1 mM $Zn^{2+}$, $Cu^{2+}$, $Ni^{2+}$, and $Ag^{2+}$ resulted in partial inhibition, and 1 mM $Hg^{2+}$ or SDS substantially inhibited enzyme

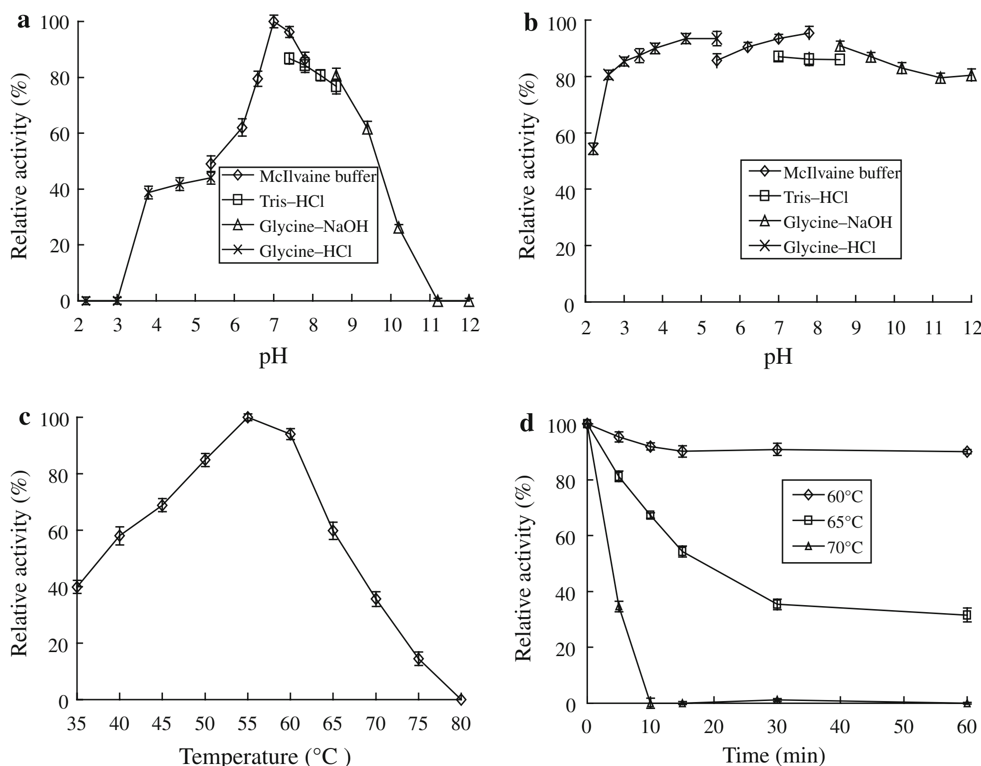


**Fig. 3** Characterization of purified recombinant XynA4 using the DNS method. **a** Effect of pH on xylanase activity. Enzyme activity was determined at 55°C in buffers with pH ranging from 2.2 to 12.0. **b** pH stability of xylanase activity. Xylanase was pre-incubated at 37°C for 1 h in buffers with pH 2.2–12.0, and the residual activity was measured in McIlvaine buffer (pH 7.0) at 55°C. **c** Effect of temperature on xylanase activity. Xylanase activity was measured in McIlvaine buffer (pH 7.0) at the indicated temperatures. **d** Thermostability of recombinant XynA4. The enzyme was pre-incubated at 60°C, 65°C or 70°C in McIlvaine buffer (pH 7.0), aliquots were removed at specific time points, and residual activity was measured in the same buffer at 55°C. Each value in the panels represents the mean of triplicates

**Table 2** Effect of metal ions and chemical reagents on purified recombinant XynA4 activity

| Chemicals | Relative activity (%)[a] 1 mM | 10 mM |
|---|---|---|
| $Ca^{2+}$ | 117.99 ± 2.58 | 96.77 ± 0.50 |
| $Cr^{3+}$ | 115.77 ± 1.57 | 74.47 ± 0.64 |
| $Co^{2+}$ | 111.89 ± 1.10 | 57.87 ± 0.79 |
| $Li^{+}$ | 111.49 ± 3.42 | 74.34 ± 1.70 |
| $Mg^{2+}$ | 109.43 ± 0.18 | 88.24 ± 0.64 |
| $Pb^{2+}$ | 108.07 ± 3.01 | 56.14 ± 0.65 |
| $Na^{+}$ | 102.56 ± 3.28 | 99.36 ± 1.96 |
| $K^{+}$ | 101.52 ± 1.72 | 99.18 ± 1.66 |
| $Fe^{3+}$ | 95.45 ± 1.44 | 81.77 ± 0.68 |
| $Mn^{2+}$ | 90.15 ± 4.06 | ND[b] |
| $Zn^{2+}$ | 76.43 ± 4.18 | 50.43 ± 1.86 |
| $Cu^{2+}$ | 75.26 ± 0.87 | 26.16 ± 1.55 |
| $Ni^{2+}$ | 60.30 ± 0.78 | 28.37 ± 1.98 |
| $Ag^{+}$ | 48.92 ± 2.58 | ND |
| $Hg^{2+}$ | 17.59 ± 1.30 | 0 |
| *β*-Mercaptoethanol | 113.52 ± 0.76 | 132.43 ± 1.27 |
| EDTA | 102.80 ± 0.57 | 100.60 ± 1.51 |
| SDS | 20.02 ± 0.62 | 0 |

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

[b] *ND* not determined

activity. When the concentration of metal ions or chemical reagents was increased to 10 mM, partial or complete inhibition of enzyme activity was observed, with the exception of $K^{+}$, $Na^{+}$, $Ca^{2+}$, and EDTA; 10 mM *β*-mercaptoethanol enhanced activity by 32.43%.

The $K_m$ and $V_{max}$ values for oat spelt xylan as substrate were 1.90 mg $ml^{-1}$ and 417.93 µmol $min^{-1}$ $mg^{-1}$, respectively, and 1.56 mg $ml^{-1}$ and 335.8 µmol $min^{-1}$ $mg^{-1}$, respectively, for birchwood xylan.

The hydrolysis products by purified XynA4 using oat spelt xylanase as substrate were analyzed by HPAEC. The composition of the hydrolysis products was 51.50% xylose, 34.30% xylobiose, 7.53% xylotriose, and 6.65% xylotetraose, indicating that it should be an endoxylanase.

## Discussion

The genus *Alicyclobacillus* was first established by Wisotzkey et al. [31]. The type species, *Thermoplasma acidophila*, was once classified into *Bacillus* [32] and then allocated into *Alicyclobacillus* based on 16S rRNA gene sequence analysis and the presence of unusual *ω*-alicyclic fatty acids in the cell membrane [33]. Seventeen *Alicyclobacillus* spp. have been reported from various sources, such as garden soil, organic compost, orchard soil, fruit processing products, solfataric soil, acidic beverages, and heat-processed foods [34–37]. In this study, we isolated an *Alicyclobacillus* strain from the outflow of a hot spring in Yunnan, China. It showed optimal growth at pH 3.0 and 60°C, similar to that of all known *Alicyclobacillus* spp. Substantial xylanase activity was detected after incubation in the presence of xylan, and the xylanase gene (*xynA4*) was cloned and expressed in *E. coli*. This is the first report that describes cloning and expression of a xylanase gene from *Alicyclobacillus*. The deduced amino acid sequence of *xynA4* is highly homologous to GH 10 endo-1,4-*β*-xylanases and shares the highest identity (53%) with the endo-1,4-*β*-xylanase from *G. stearothermophilus*, suggesting that *xynA4* might be a new xylanase gene.

The purified recombinant XynA4 showed optimal activity at pH 7.0 and retained 40% or more of its maximum activity at pH 3.8–9.4. The enzyme also exhibited stability over a wide pH range, maintaining >80% of its maximal activity after pre-incubation in buffers ranging from pH 2.6 to 12.0 at 37°C for 1 h. Few GH 10 xylanases remain active and stable under such a wide pH range. For example, the recombinant xylanase from *Geobacillus* sp. MT-1, with which XynA4 shares highest identity, has the same pH optimum (pH 7.0) but loses almost all activity below pH 5.0 or above pH 10.0 [29]. XyaA from the alkalophilic *Bacillus* sp., the other highly identical xylanase, has a pH optimum of 8.0, and its activity sharply decreases below pH 5.0, although it is stable between pH 5.0 and 11.0 [13]. XynAS27 from *Streptomyces* sp. is stable over a similar pH range (pH 2.2–12.0) and is active between pH 5.5 and 10.0, but it has almost no activity below pH 5.0 [38]. Some acidic and alkaline xylanases, such as XyaA from *Acidobacterium capsulatum* [39], XylA and XylB from alkaliphilic *Bacillus* [14], xylanase J from alkaliphilic *Bacillus* sp. [40], and xylanase A from *Bacillus* sp. [13], have a broad pH activity profile but lose activity under either acidic (pH <5.0) or alkaline (pH >9.0) conditions and retain their stability only within a narrow pH range. Compared with these enzymes, XynA4 has greater potential for widespread application.

Because XynA4 exhibits substantial thermostability and pH stability and retains its activity over a wide pH range, it is promising for application in many industries. For example, in the paper industry, a multistage bleaching process that incorporates chlorine, chlorine dioxide, and sodium hydrate is required to remove lignin and its associated dark-brown color, and thus the pH varies from acidic to alkaline [41, 42]. Therefore, the xylanase used in this process requires pH stability and activity over a wide pH range. XynA4, as a potential candidate, can reduce the use of chlorine and chlorine

dioxide in the paper-making process [43, 44], thus reducing the whole production cost.

**Acknowledgments** This work was supported by the National High Technology Research and Development Program of China (863 Program; No. 2007AA100601) and the National Key Technology Program of China (No. 2006BAD12B05-03).

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