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Cloning of a New Xylanase Gene from *Streptomyces* sp. TN119 Using a Modified Thermal Asymmetric Interlaced-PCR Specific for GC-Rich Genes and Biochemical Characterization

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Abstract A bacterial strain, *Streptomyces* sp. TN119, was isolated from the gut of *Batocera horsfieldi* larvae and showed xylanolytic activity. A degenerate primer set was designed based on the base usage of G and C in *Actinobacteria* xylanase-coding sequences belonging to the glycosyl hydrolases family 10 (GH 10), and used to clone the partial xylanase gene from *Streptomyces* sp. TN119. A modified thermal asymmetric interlaced (TAIL)-PCR specific for high-GC genes, named GC TAIL-PCR, was developed to obtain the full-length xylanase gene (*xynA119*; 1089 bp). Rich in GC content (67.8%), *xynA119* encodes a new GH 10 xylanase (XynA119), which shares highest identity (48.8%) with an endo-1,4-β-xylanase from *Cellulosimicrobium* sp. HY-12. Recombinant XynA119 was expressed in *Escherichia coli* BL21 (DE3) and purified to electrophoretic homogeneity. The enzyme showed maximal activity at pH 6.5 and 60 °C, was stable at pH 4.0 to 10.0 and 50 °C, was resistant to most chemicals (except for Cu^{2+}, Mn^{2+}, Ag^{+}, Hg^{2+} and SDS) and trypsin, and produced simple products. The specific activity, K_m, V_{max}, and k_{cat} using oat-spelt xylan as substrate were 57.9 U mg^{-1}, 1.0 mg ml^{-1}, 74.8 μmol min^{-1} mg^{-1}, and 49.2 s^{-1}, respectively.

Keywords Xylanase · *Batocera horsfieldi* · Gut · *Streptomyces* sp. · TN119 · Thermal asymmetric interlaced (TAIL)-PCR · High-GC genes

Introduction

Xylan is the most common hemicellulosic polysaccharide in cell walls of land plants, representing up to 30–35% of the total dry weight [1]. It is a heteropolysaccharide containing substitute groups of acetyl, 4-*O*-methyl-D-glucuronosyl and α-arabinofuranosyl

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residues linked to the backbone of β-1,4-linked xylopyranose units and has binding properties mediated by covalent and noncovalent interactions with lignin, cellulose, and other polymers [2]. Endoxylanases (EC 3.2.1.8) are glycosidases which catalyze the endohydrolysis of β-1,4-D-xylosidic linkages in xylan, and have been extensively applied in many fields, such as bioconversion, prebleaching of kraft pulps and improving the quality of food and feed [3]. Many microorganisms, including bacteria, fungi and yeasts, have been reported to produce xylanases [3]. Based on amino acid sequence similarities, the majority of endoxylanases fall into families 10 and 11 of the glycosyl hydrolase (GH) [4].

Family *Cerambycidae* is particularly noteworthy because the larvae develop deep within the sapwood, a tissue characterized by very low nitrogen content and high lignification [5]. The complex of biota in the digestive tracts of such insects is involved in various metabolic functions including food digestion [6]. Nevertheless, reports about glycosidases from these symbiotic microorganisms in the gut of longicorn beetles, such as *Batocera*, are extremely limited [7].

In this study, a symbiotic strain, *Streptomyces* sp. TN119, in the gut of *Batocera horsfieldi* larvae was isolated and showed xylanolytic activity. Genome-walking techniques based on PCR are successful for gene cloning as they are fast and less labor-intensive [8]; however, it is very hard to obtain the full-length xylanase gene from *Streptomyces* sp. TN119 using the regular techniques due to its high content of GC. Thermal asymmetric interlaced (TAIL)-PCR is an improved PCR method walking in the uncloned genomic DNA to obtain the full-length genes [9]. This method utilizes three nested specific primers in successive reactions together with a shorter arbitrary degenerate (AD) primer of lower melting temperature to improve the relative amplification efficiencies of specific and nonspecific products. In this study, a modified TAIL-PCR specific for high-GC genes consisting of three-step PCR procedure, 12 short arbitrary degenerate (AD) primers, and some commercial reagents was developed to clone the objective gene. The xylanase gene was further expressed in *Escherichia coli*, and the purified recombinant enzyme was characterized.

Experimental

Vectors, Reagents, and Medium

The plasmid pEASY-T3 and *E. coli* Top10 (TransGen, China) were used for gene cloning. The vector pET-22b(+) (Novagen, USA) and *E. coli* BL21 (TransGen) were used for xylanase expression. The His-tagged protein was purified using Nickel-NTA Agarose (Qiagen, USA). Oat-spelt xylan and carboxymethyl cellulose sodium were purchased from Sigma (USA). Xylose, xylobiose, xylotriose, xylotetraose, and xylopentaose were purchased from Wako Pure Chemical Industries (Japan). The genomic DNA isolation, DNA purification, and plasmid isolation kits were purchased from TIANGEN (China). The restriction endonucleases, T4 DNA ligase, DNA polymerase (*TaKaRa rTaq*™, *LA Taq*™, and *Pyrobest*™), dNTP and GC buffer (I and II) were purchased from TaKaRa (Japan). Other reagents were of analytical grade and commercially available. Xylanase screening medium [10], which contained 1% oat-spelt xylan, 0.5% peptone, 0.5% NaCl, and 0.02% congo red, was used to isolate xylanase-producing bacteria.

Microorganism Isolation

The larvae of *B. horsfieldi* (*Coleoptera*, *Cerambycidae*) were collected from oak trees in March 2008 in Jiangxi province, China, and identified by Professor Youqing Luo of

Department of Forestry and Environmental Resources, Beijing Forestry University, China. Dissections were performed under sterile conditions as Schloss et al. [11] described, and the gut luminal contents from 20 larvae were pooled, suspended in 0.7% (*w*/*v*) sterile NaCl, and then spread onto xylanase screening agar plates. Pure cultures, grown in Luria-Bertani (LB) medium at 26 °C, were obtained through repeated streaking. One strain, designated TN119, was selected for further study. The taxon of strain TN119 was identified by comparison of the 16S ribosomal deoxyribonucleic acid (rDNA) sequence with that in GenBank [12].

Primer Design

The guanine+cytosine (GC) content of *Actinobacteria* are high, ranging from 51% to more than 70% [13]. The average GC content in the GH 10 xylanase-coding sequences (CDSs) of *Actinobacteria* is 68.4%, much higher than that of *Proteobacteria* and *Firmicutes* xylanase CDSs (Table 1). Furthermore, the third position of the triplet codon in these *Actinobacteria* xylanase CDSs is almost exclusively G or C, and the first position was designed to be G as much as possible due to the high average between the G and C percentages (G/C%) at this position (173.6).

Based on alignment of the GH 10 xylanases in pfam database (http://pfam.sanger.ac.uk/family?acc=PF00331), two conserved motifs, WDVVNE and FTELDV, were identified, and a degenerate primer set (AXyn10F and AXyn10R; Table 2) was designed to amplify the xylanase gene fragment from strain TN119. Considering the high GC content of *Actinobacteria* and the diversity of residues at the conserved motif of FTELDV, the primer AXyn10R was designed not by strict amino acids back translation but designed rich in S (G or C), especially at the third position of codon triplets. To obtain the full-length xylanase gene, 12 GCAD primers with relatively high-GC (50–85%) and six nested insertion-specific primers were also designed (Table 2). All primers were designed and analyzed using Primer Premier 5.0 (PREMIER Biosoft International, USA) and Oligo 6.0 (Molecular Biology Insights, USA) softwares, and synthesized by Invitrogen (China). Sequence assembly and codon usage analysis were performed using programs of DNASTAR 5.0 (DNASTAR, USA). GC content was calculated by DNAMAN 5.2 (Lynnon, Canada).

Gene Cloning

Genomic DNA of strain TN119 was extracted with the genomic DNA isolation kit following the manufacturer's instructions and used as PCR template. A touchdown PCR using the degenerate primer set (AXyn10F and AXyn10R; Table 2) was performed as follows: 94 °C for 5 min, 11 touchdown cycles of 94 °C for 30 s, 55 °C for 30 s (decreasing 1 °C each cycle), and 72 °C for 40 s, followed by 25 cycles of 94 °C for 30 s, 44 °C for 30 s, and 72 °C for 40 s, and a final extension step at 72 °C for 7 min. The PCR product was gel-purified, ligated into pEASY-T3 vector, and sequenced by Sangon (China).

To obtain the full-length xylanase gene (*xynA119*), a modified TAIL-PCR, named GC TAIL-PCR, using the GCAD and nested insertion-specific primers (Table 2), was performed with an annealing temperature (T_m) of 70 °C (Table 3). All PCR products were electrophoresed in 2% agarose gels, the secondary and tertiary products showing expected sizes were chosen, purified, and cloned into pEASY-T3 vector for sequencing. One more TAIL-PCR was performed using primers u2SP1–3 to clone the upstream sequences presumed as transcription regulator and further test this method. As a control, the regular TAIL-PCR with 11 AD primers was also conducted [9, 14–17].

Table 1 The base usage of G and C in xylanase-coding sequences of *Actinobacteria* belonged to GH 10.

Taxon	Xylanase CDS	% of given G+C%[a]		G+C% of codons				G/C% of codons[c]			
		50–80%	<50%	Overall	Pos.1[b]	Pos.2[b]	Pos.3[b]	Overall	Pos.1	Pos.2	Pos.3
Actinobacteria (High G+C)	*S. ambofaciens* |AM238664|	82.8	2.9	71.2	61.9	55.6	96.2	85.9	245.8	74.3	47.8
	S. avermitilis |AF121865|	83.1	5.1	68.9	61.4	49.8	95.6	87.2	175.3	93.8	52.7
	S. chattanoogensis |AF121864|	83.5	5.9	68.5	57.2	54.0	94.5	77.9	168.5	67.2	52.2
	S. coelicolor |AL939106|	80.5	4.4	70.8	60.4	55.5	96.5	85.8	217.9	77.9	50.5
	S. fradiae |EF429086|	83.2	3.1	70.6	61.7	53.2	97.1	82.0	171.8	83.4	49.6
	S. halstedii |U41627|	84.6	5.6	68.1	61.4	54.0	89.1	96.3	230.1	73.6	63.5
	S. lividans |M64551|	88.0	5.5	68.2	58.7	50.6	95.4	85.3	175.6	101.6	49.1
	S. pristinaespiralis |NZ_ABJI01000137|	84.2	3.0	70.9	68.2	49.5	94.9	88.6	177.2	81.3	55.8
	S. sviceus |NW_002063254|	87.9	5.0	66.8	64.2	41.8	94.6	88.2	137.8	81.7	66.8
	S. tendae |AY190297|	87.5	6.0	67.9	56.3	50.1	97.4	78.7	185.8	106.2	39.1
	S. thermocyaneoviolaceus |AF194024|	86.9	5.5	67.5	59.2	51.7	91.7	87.0	183.3	104.3	47.7
	S. thermoviolaceus |AB110643|	87.1	5.3	67.6	59.4	51.7	91.7	87.3	184.2	104.3	47.7
	A. cellulolyticus |NC_008578|	84.4	12.1	62.9	63.9	42.8	82.0	90.6	152.6	59.1	74.8
	Cellulosimicrobium sp. |EU179736|	87.2	4.6	69.2	64.2	47.6	95.7	76.5	140.4	57.6	57.9
	K. radiotolerans |NC_009664|	81.0	3.6	71.6	69.7	46.5	98.7	83.6	138.7	78.8	59.5
	T. alba |Z81013|	90.7	6.1	65.6	60.9	47.1	88.9	83.2	141.7	93.0	53.8
	T. fusca |NC_007333|	90.9	5.4	65.7	65.8	41.4	89.8	88.3	123.8	78.4	72.7
	Means	85.5	5.3	68.4	62.0	49.6	93.5	85.4	173.6	83.3	55.4
	Streptomyces sp. TN119 | FJ436403|	88.4	4.9	67.8	63.1	43.5	96.7	86.8	154.4	71.9	64.7
Proteobacteria	*C. japonicus* |AF168359|	54.4	44.7	51.3	56.7	38.3	59.1	91.4	124.1	81.5	73.8
Firmicutes (Low G+C)	*C. cellulolyticum* |NZ_AAVC01000026|	20.3	79.7	40.3	46.6	38.8	35.3	107.7	214.9	61.7	82.9

[a] The percentage of sequences with given G+C% (50–80% or <50%) to total sequences constituted by 15 sequential bases in a xylanase CDS

[b] Pos. 1, 2 and 3 represent the first, second and third position of a triplet codon, respectively

[c] G/C%, the percentage between the G and C content

Table 2 Primers used in this study.

Primers	Primer sequences (5′–3′)[a]	T_m (°C)[b]	GC%	Degeneracy
AXyn10F	TGGGAYGTNGTNAAYGAR	52.0–64.6	33.3–61.1	128
AXyn10R	SACSTCSASYTCSGTSAA	40.7–63.4	55.6–61.1	128
XynA119F	CGGAATTCGGCTCCGGCGGACGCGTACCACC	76.1	77.3	1
XynA119R	CCCAAGCTTGTTGAAGCGGCCCTCGGCCAGC	75.4	72.7	1
GCAD1	NYCGASCKTSGWGCT	45.5–61.2	53.3–73.3	128
GCAD2	GTSGRCWGRSMCGSAT	41.2–69.9	56.3–75.0	128
GCAD3	TGYGSAGYASCRSMGA	35.0–73.9	50.0–75.0	128
GCAD4	TGCGNSGWMSCRSAG	43.9–70.4	60.0–80.0	256
GCAD5	AGWGISGSMNCSWGG	40.9–59.9	60.0–80.0	1024
GCAD6	CAWCGSCNGWSRSGT	41.4–66.5	60.0–73.3	256
GCAD7	TCSGICGNACISKSGA	38.7–65.4	56.2–81.2	1024
GCAD8	GTTSIKCSWGCWNSGC	43.1–56.4	56.3–68.8	1024
GCAD9	TCRGSYGWCIGSNSTG	37.0–64.7	56.3–81.3	1024
GCAD10	TCTYICGSRCSWNGGA	45.9–64.4	50.0–75.0	512
GCAD11	TGSWGNGCIRSWCG	44.0–60.0	57.1–78.6	512
GCAD12	GASYGWCSRGWGNSTC	31.2–57.6	56.3–75.0	512
AD1	NTCGASTWTSGWGTT	42.9–40.3	40–46.7	64
AD2	NGTCGASWGANAWGAA	41.1–43.3	31.3–50.0	128
AD3	WGTGNAGWANCANAGA	30.8–48.1	31.3–50.0	256
AD4	TGWGNAGWANCASAGA	30.8–49.1	37.5–50.0	128
AD5	AGWGNAGWANCAWAGG	33.6–50.8	37.5–50.0	128
AD6	CAWCGICNGAIASGAA	42.3–57.0	37.5–62.5	256
AD7	TCSTICGNACITWGGA	46.2–49.8	43.8–62.5	256
AD8	STTGNTASTNCTNTGC	44.1–48.2	37.5–56.3	256
AD9	WCAGNTGWTNGTNCTG	42.3–51.3	37.5–50.0	256
AD10	TCTTICGNACITNGGA	46.2–53.9	37.5–62.5	256
AD11	TTGIAGNACIANAGG	27.6–46.5	26.7–60.0	256
u1SP1	GGCGGTCGACTTCGCGTTGACG	73.4	68.2	1
u1SP2	TGACGTACAGCTTCGCGTGCGGG	73.8	65.2	1
u1SP3	TGTAGGACGGGCCGAGCTGCTGGT	74.6	66.7	1
u2SP1	CGGCGAGGGCGTCCATGTTGA	72.6	66.7	1
u2SP2	GGGCCTTCAGCGGCGCACCCT	75.9	76.2	1
u2SP3	TGACGGCTGCGGCAGCACGGA	76.4	71.4	1
d1SP1	CAGCAGCTCGGCCCGTCCTACA	71.2	68.2	1
d1SP2	GGCCGACCCGCACGCGAAGC	76.3	80.0	1
d1SP3	CTGGTCAAAGAGCTGCGCTCCCA	70.5	60.9	1

[a] IUPAC/IUB symbols are used, and restriction sites are shown in italics

[b] The annealing temperature (T_m) of specific primers is preferred at 70–80 °C

Sequences Analysis

Alignments of DNA and protein sequences were conducted with blastn and blastp programs, respectively (http://www.ncbi.nlm.nih.gov/BLAST/). The signal peptide was predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP/). Key functional residues

Table 3 Settings for GC TAIL-PCR.

Steps	Cycles	Thermal programs	Reaction system	
			Materials	Volume (μl)
Primary	1	94 °C, 5 min	ddH_2O	13.5
	5	94 °C, 30 s; 70 °C, 30 s; 72 °C, 2 min[a]	2×GC Buffer I or II	25.0
	1	94 °C, 30 s; 30 °C, 3 min; ramping to 72 °C, 0.3 °C/s; 72 °C, 2 min	dNTP (2.5 μM)	5.0
	15	94 °C, 30 s; 70 °C, 30 s; 72 °C, 2 min[a]	GCADX or ADX primer (100 μM)[c]	2.5
		94 °C, 30 s; 70 °C, 30 s; 72 °C, 2 min[a]	SP1 primer (10 μM)	1.0
		94 °C, 30 s; 50 °C (44 °C)[b], 1 min; 72 °C, 2 min	*rTaq*™ or *LA Taq*™ (2.5 U μl^{-1})	0.5
	1	72 °C, 7 min	Template (10–20 ng/μl)	2.5
Secondary	1	94 °C, 5 min	The same as the primary PCR reaction except for:	
	15	94 °C, 30 s; 70 °C, 30 s; 72 °C, 2 min[a]		
		94 °C, 30 s; 70 °C, 30 s; 72 °C, 2 min[a]	SP2 primer (10 μM)	1.0
		94 °C, 30 s; 50 °C (44 °C)[b], 1 min; 72 °C, 2 min	Template (50-fold diluted primary PCR product)	2.5
	1	72 °C, 7 min		
Tertiary	The same as the secondary thermal program		The same as the primary PCR reaction except for:	
			SP3 primer (10 μM)	1.0
			Template (50-fold diluted primary or secondary PCR product)[d]	2.5

[a] The annealing temperature (T_m) in this program is set alterably based on the T_m of SP primers, preferred at 70 °C and no lower than 65 °C. This program can be simple and convenient as 94 °C, 30 s; 72 °C, 2 min 30s if the T_m of SP primers are higher than 72 °C

[b] The T_m of GC TAIL-PCR and regular TAIL-PCR in this program are shown in bold and parentheses, respectively

[c] GCADX and ADX represent one of the 12 GCAD and 11 AD primers in Table 1, respectively

[d] To save time and reduce cost, primary PCR product is prior to be used so as to perform the secondary and tertiary steps at the same time

were predicted at the website http://pfam.sanger.ac.uk/search. And the promoter and terminator were predicted by the online tools of Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html) and Sofeberry FindTem (http://linux1.softberry.com/berry.phtml), respectively.

Multiple alignments of sequences were performed with Clustal X [18] and then manually adjusted. Distance matrices were calculated according to the Kimura two-parameter model [19] for nucleotides and PAM Matrix model [20] for amino acids, respectively. Phylogenetic trees were constructed using neighbor-joining algorithm [21] in MEGA 4.0 [22], with its reliability assessed by 1,000 bootstrap repetitions.

Expression of *xynA119* in *E. coli*

To express *xynA119* in *E. coli*, the mature protein-coding sequence was amplified from the genomic DNA of strain TN119 using *Pyrobest*™ DNA polymerase and primers XynA119F and XynA119R (Table 2). The resulting PCR product was gel-purified, digested with *Eco*RI and *Hind*III, and cloned into the corresponding sites of vector pET-22b(+). The recombinant plasmid, *pET-xynA119*, was transformed into *E. coli* BL21 (DE3) competent cells. Positive transformants were identified by enzymatic-digestion assay and PCR analysis, and confirmed by DNA sequencing.

Purification and Identification of the Recombinant Xylanase

The positive transformant harboring *pET-xynA119* was picked up from a single colony and grown overnight at 37 °C in LB medium containing 100 μg ml^{-1} ampicillin. The culture was then inoculated into fresh LB medium (1:100 dilutions) containing ampicillin and grown aerobically at 37 °C to an A_{600} of ~0.8. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.7 mM for induction. After incubation for an additional 8 h at 27 °C, the cells were removed by centrifugation at 12,000×*g* at 4 °C for 10 min. The supernatant was concentrated using a Hollow Fiber Membrane Module (6 KD MWCO, Tianjin Motian Membrane Eng. & Tech., China) and followed by the ultrafiltration membrane (PES5000, Sartorius Stedim Biotech, Germany). The supernatant was further concentrated with polyethylene glycol (PEG8000), and then dialyzed three times against 3 l ddH_2O. The concentrated solution was loaded onto the Nickel-NTA Agarose for purification with a linear imidazole gradient of 20 to 300 mM in Tris–HCl buffer (20 mM Tris–HCl, 500 mM NaCl, 10% glycerol, pH 7.6). The purified enzyme was stored at −20 °C for further characterization.

Twelve percent running gel of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli [23]. Identification of the purified enzyme using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) was performed by the State Key Laboratory of Biology of Biomembrane and Membrane Technology, Institute of Zoology, Chinese Academy of Science. The protein concentration was determined by the Bradford method [24], using bovine serum albumin as a standard.

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 [25]. The standard reaction contained 0.1 ml of appropriately diluted enzyme and 0.9 ml

of McIlvaine buffer (0.2 M Na_2HPO_4/0.1 M citric acid; pH 6.5) containing 1% (*w*/*v*) oat-spelt xylan. After incubation at 60 °C for 10 min, the reaction was stopped with 1.5 ml DNS reagent. The mixture was then boiled for 5 min and cooled down to room temperature, and the absorption at 540 nm was measured. One unit of xylanase activity was defined as the amount of enzyme that released 1 μmol of reducing sugar equivalent to xylose per minute. The enzyme activity in each case was assayed under the standard conditions unless specially noted. Cellulase activity was also determined by the DNS method under standard conditions with carboxymethyl cellulose sodium instead of oat-spelt xylan.

Biochemical Characterization

The optimal pH of the purified recombinant enzyme was determined at 55 °C in buffers with pH ranging from 4.0 to 10.0. The pH stability was estimated by measuring the residual enzyme activity after pre-incubating the enzyme in different buffers of pH 2.0–12.0 at 37 °C for 1 h. The buffers used were: McIlvaine buffer for pH 2.0–8.0, 0.1 M Tris–HCl for pH 8.0–9.0, and 0.1 M glycine–NaOH for pH 9.0–12.0.

The optimal temperature of the purified recombinant enzyme was determined over the range of 30–80 °C in McIlvaine buffer (pH 6.5). Thermostability of the enzyme was monitored by pre-incubating the enzyme in McIlvaine buffer (pH 6.5) at 50 °C or 60 °C without substrate for various periods.

The K_m, V_{max}, and k_{cat} values for the purified recombinant enzyme were determined using 1–10 mg ml^{-1} oat-spelt xylan as the substrate. The data were plotted according to the Lineweaver–Burk method.

To investigate the effects of different metal ions and chemical reagents on the purified recombinant enzyme activity, 1, 5 or 10 mM of NaCl, KCl, $CaCl_2$, LiCl, $CoCl_2$, $CrCl_3$, $NiSO_4$, $CuSO_4$, $MgSO_4$, $FeCl_3$, $MnSO_4$, $ZnSO_4$, $Pb(CH_3COO)_2$, $AgNO_3$, $HgCl_2$, EDTA, SDS or β-mercaptoethanol (β-Met) was added to the reaction system individually.

To examine resistance to different proteases, the purified recombinant enzyme (100 μg ml^{-1}) was incubated at 37 °C for 1 h with 10 μg ml^{-1} trypsin (from bovine, pH 7.5, 14,700 U mg^{-1}, Sigma) or 10 μg ml^{-1} proteinase K (type VIII from *B. licheniformis*, pH 7.5, 30 U mg^{-1}, Amresco, USA).

Analysis of Hydrolysis Product

The reaction mixture containing 3.5 U purified recombinant enzyme and 100 μg oat-spelt xylan in 100 μl McIlvaine buffer (pH 6.5) was incubated at 37 °C for 12 h. After hydrolysis, the enzyme was removed from the reaction system using the Nanosep Centrifugal 3K Device (Pall, USA). The products were analyzed by high-performance anion-exchange chromatography (HPAEC) with a model 2500 system from Dionex (USA) [26]. Xylose, xylobiose, xylotriose, xylotetraose, and xylopentaose were used as standards.

Nucleotide Sequence Accession Numbers

The nucleotide sequences for the *Streptomyces* sp. TN119 16S rDNA and *xynA119* were deposited in GenBank database under accession numbers FJ436402 and FJ436403, respectively.

Results

Strain Identification

Strain TN119 showed a clear zone on xylanase screening agar plates. To identify strain TN119, the partial 16S rDNA (1,391 bp) was PCR-amplified and sequenced. It showed 99.3% identity with *Streptomyces cinnamocastaneus* NBRC14278 (Accession no. AB184588), 99.1% with *Streptomyces fragilis* NBRC12862 (AB184200), and 98.7% with *Streptomyces flaveolus* NBRC3715 (AB184786). Phylogenetic analyses placed TN119 in the subgroup of *S. cinnamocastaneus* and *S. fragilis* (data not shown). Thus, this strain was designated as *Streptomyces* sp. TN119, and deposited at the Agricultural Culture Collection of China under accession No. ACCC 03947.

Cloning of *xynA119*

A fragment of *xynA119* (347 bp) was amplified by PCR using the degenerate primers AXyn10F and AXyn10R specific for high-GC *Actinobacteria*. The 5′ and 3′ flanking regions of this fragment were successfully obtained by GC TAIL-PCR using primers GCAD 2–12. No expected sequences were obtained by using the regular TAIL-PCR with primers AD 1–11 (Fig. 1). Finally, a 3,224 bp DNA sequence with 70.9% GC content was cloned, containing a 1,089-bp full-length *xynA119* with 67.8% GC content. The GC content at the third position of codons of *xynA119* was 96.7%. The upstream of *xynA119* was an incomplete open reading frame (ORF) and transcribed in the same direction as *xynA119*.

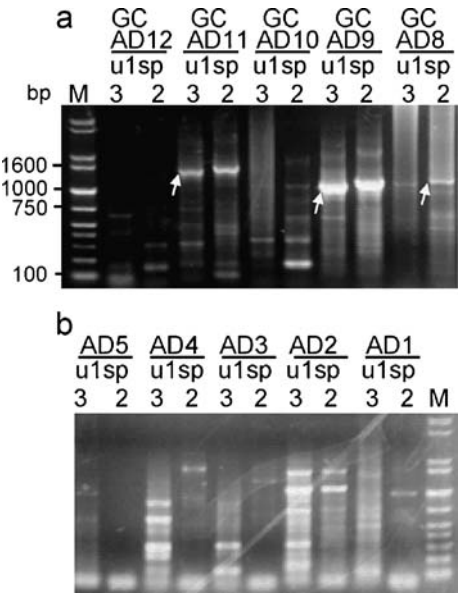

Fig. 1 Gel analysis of the partial results from the GC TAIL-PCR (**a**) and regular TAIL-PCR (**b**) using the same SP primers. The fragments confirmed by sequencing were marked by *white arrows*. *Lanes M* 1 kb Plus DNA ladder, *2* PCR products from the secondary reaction using GCAD or AD primers, *3* PCR products from the tertiary reaction corresponding to GCAD or AD primers

The downstream was a 408-bp gene encoding a hypothetical protein and transcribed in the opposite direction to *xynA119*. The functions of these two hypothetical proteins are unknown so far. The putative promoter, ribosome binding site, and terminator were not detected.

Sequence Analysis

The gene *xynA119* started with the putative codon ATG and ended with TGA, and encoded a 362-residue polypeptide including a putative signal peptide of 27 residues. After processing at cleavage site between Ala27 and Ala28, the mature polypeptide was 335 residues with a calculated molecular mass of 38.0 kDa.

XynA119 contained a complete catalytic domain of GH 10 from Pro40 to Leu356. The partially conserved amino acids of GH 10 in the peptides from XynA119 were underlined as follows: FNSLTAENVMKW, RGHTLLWHNQLPGW, WDVVNE, YVNDYN, GVRVDGFGIQGH, FTEVDV, and WGFADQYSW. Glu171 in WDVVNE and Glu277 in FTEVDV were putative active sites. The amino acid sequence of XynA119 shared highest identity of 61.0% with a putative β-1,4-xylanase from *S. pristinaespiralis* ATCC 25486 (ZP_03187932) and 48.8% with a β-1,4-xylanase from *Cellulosimicrobium* sp. HY-12 (ABX88978). A phylogenetic tree was constructed based on the alignment of *Actinobacteria* xylanases (Fig. 2). High bootstrap values separated these xylanases into two distinct clusters, and XynA119 was closely related to the xylanases of *Streptomyces pristinaespiralis* ATCC 25486, *Kineococcus radiotolerans* (identity of 48.5%), and *Thermobifida fusca* YX (46.3%).

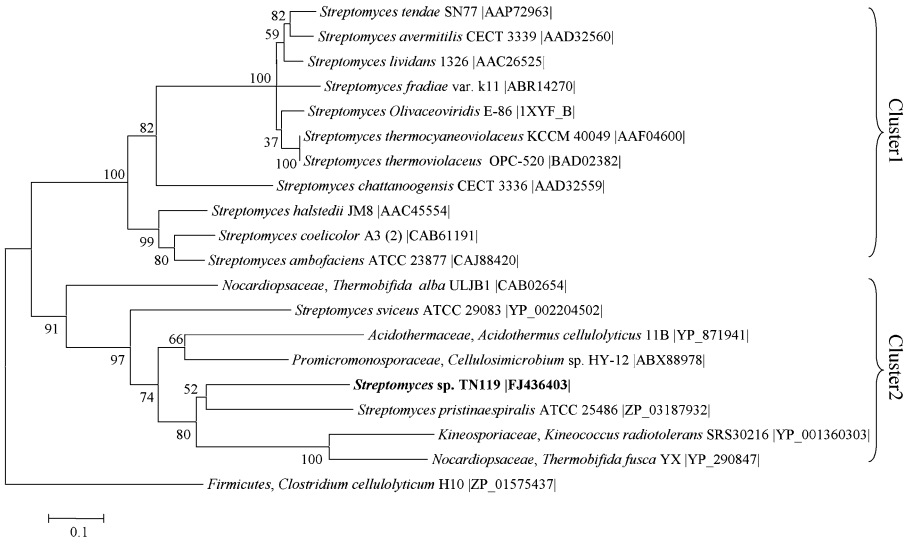

Fig. 2 Phylogenetic dendrogram based on the amino acid sequences of GH 10 xylanases from *Actinobacteria* obtained by neighbor-joining algorithm, showing the position of XynA119 from *Streptomyces* sp. TN119 related to other *Actinobacteria* xylanases. The data was showed with microbial source and GenBank accession no. of xylanases. A GH10 xylanase from *Clostridium cellulolyticum* H10 (ZP_01575437) was used as outgroup. Bootstrap values are expressed as percentages of 1,000 replications. *Bar* 0.1 sequence divergence

Expression, Purification, and Identification of r-XynA119

To produce recombinant xylanase, *xynA119* was expressed in *E. coli*. After induction, the recombinant xylanase, r-XynA119, was secreted to the culture supernatant, showing xylanase activity of 16.4 U mg^{-1}. No xylanase activity was detected in the medium of the uninduced culture or induced transformant harboring the empty plasmid pET-22b(+).

r-XynA119 was purified to electrophoretic homogeneity by Ni^{2+}-NTA metal-chelating affinity chromatography (Fig. 3). The purified enzyme migrated a single band on SDS-PAGE with a molecular mass of about 38.0 kDa, which was identical to the calculated value.

To identify whether the single band of approximately 38.0 kDa was r-XynA119, the band was separated, digested, and analyzed using LC-ESI-MS/MS. The peptide mass fingerprints and tandem MS peptide sequencing obtained from the MS peaks were compared with the amino acid sequence of r-XynA119. Three internal peptides, LYVNDYNVEGVNAK, FADLGVESSFTEVDVR, and GVYDFTQGDALVDFAR, were selected randomly, completely corresponding to the amino acid sequence of XynA119, confirming that the purified protein was r-XynA119 [27]. The specific xylanase activity of the purified r-XynA119 was 57.9 U mg^{-1}, free of cellulase activity.

Enzyme Characterization

The purified r-XynA119 was optimally active at pH 6.5 (at 55 °C), and more than 60% of the maximum activity was retained between pH 5.5 and 7.5 (Fig. 4a). The enzyme was stable over a wide pH range, retaining more than 85% of the initial activity after incubation in buffers ranging from pH 4.0 to 10.0 at 37 °C for 1 h (Fig. 4b).

The optimal temperature for the enzyme activity was 60 °C at pH 6.5. The enzyme retained more than 70% of the maximum activity when assayed at 50–65 °C and more than 45% at 70 °C (Fig. 4c). After incubation at 50 °C for 1 h, the enzyme retained more than 95% of the initial activity. Above 60 °C, the enzyme's stability decreased rapidly (Fig. 4d).

The xylanase activity of purified r-XynA119 in the presence of different metal ions or chemical reagents is shown in Table 4. The activity was absolutely inhibited by Hg^{2+} and strongly inhibited by Ag^{+} and SDS even at a low concentration. Partial inhibition was observed in the presence of some metal ions at 10 mM, and the enzyme inhibition was in the order of $Mn^{2+}>Cu^{2+}>Co^{2+}>Pb^{2+}$. Zn^{2+} and β-mercaptoethanol at 10 mM enhanced the activity of about 1.2- and 1.6-fold, respectively. The addition of other reagents had little or no effect on the activity.

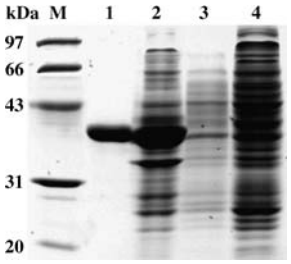

Fig. 3 SDS-PAGE analysis of expression and purification of recombinant XynA119. *Lanes M* low-molecular-weight marker, *1* purified r-XynA119 using Ni^{2+}-NTA metal-chelating affinity chromatography, *2* culture supernatant of the induced transformant harboring *pET-xynA119*, *3* culture supernatant of induced transformant harboring the empty plasmid, *4* culture supernatant of the uninduced transformant harboring *pET-xynA119*

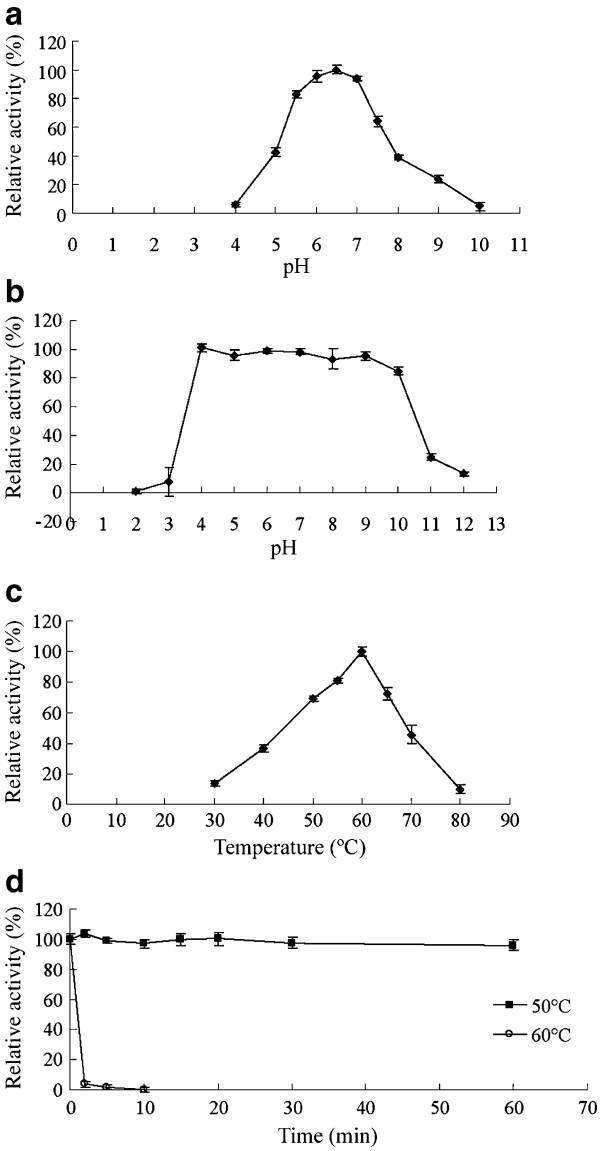

Fig. 4 Characterization of the purified r-XynA119. **a** Effect of pH on r-XynA119. The assay was performed at 55 °C in buffers with pH ranging from 4.0 to 10.0. **b** pH stability of r-XynA119. After pre-incubating the enzyme at 37 °C for 1 h in buffers of pH 2.0–12.0, the activity was measured in McIlvaine buffer (pH 6.5) at 60 °C. **c** Effect of temperature on r-XynA119 measured in McIlvaine buffer (pH 6.5). **d** Thermostability of r-XynA119. The enzyme was pre-incubated at 50 and 60 °C in McIlvaine buffer (pH 6.5) without substrate, and aliquots were removed at specific time points for the measurement of residual activity at 60 °C

The kinetic parameters of purified r-XynA119 with respect to oat-spelt xylan were calculated from a Lineweaver–Burk plot. The K_m, V_{max}, and k_{cat} values were 1.0 mg ml^{-1}, 74.8 μmol min^{-1} mg^{-1} and 49.2 s^{-1}, respectively.

The purified r-XynA119 was resistant to trypsin, retaining about 60% of its activity after treatment, but not resistant proteinase K.

Table 4 Effect of metal ions and chemical reagents on the activity of purified r-XynA119.

Chemicals	Relative activity (%)[a]		
	1 mM	5 mM	10 mM
None	100.0±2.0	100.0±2.0	100.0±2.0
Li^{+}	103.1±6.9	103.3±2.4	93.6±1.8
Fe^{3+}	100.4±2.5	96.8±2.4	94.2±3.2
Ca^{2+}	99.9±6.3	97.0±2.7	94.2±3.5
Na^{+}	99.8±2.0	97.9±2.1	95.4±3.2
K^{+}	99.7±3.6	100.4±2.0	102.6±2.9
Mg^{2+}	97.0±5.5	104.9±7.9	96.4±4.1
Co^{2+}	95.0±1.8	103.0±5.1	80.7±3.0
Cr^{3+}	93.8±2.9	100.7±2.0	93.3±3.8
Zn^{2+}	93.8±2.3	89.8±2.6	120.2±2.9
Mn^{2+}	93.3±2.7	80.8±2.7	39.8±5.2
Pb^{2+}	93.2±1.8	84.3±2.3	81.8±5.2
Ni^{2+}	92.8±2.4	103.8±2.4	99.1±3.9
Cu^{2+}	90.6±2.5	79.3±3.3	66.8±1.6
Ag^{2+}	61.9±5.5	0.0	–
Hg^{2+}	0.0	–	–
β-Met	129.0±5.4	141.9±3.7	156.5±8.8
EDTA	106.0±8.0	90.5±2.1	96.0±4.9
SDS	58.3±5.3	26.0±3.2	0.0

[a] Values represent the means of triplicates relative to the untreated control samples

Analysis of Hydrolysis Products

The hydrolysis products of oat-spelt xylan by purified r-XynA119 were analyzed by HPAEC. The composition of the hydrolysis products was 23.1% xylose and 76.9% xylobiose (*w*/*w*).

Discussion

The digestive enzymes from symbiotic microorganisms harbored in the gut of insects contribute to the digestion of wood constituents such as cellulose, hemicellulose (xylan), lignin, and pectin [28]. To our knowledge, only a GH 11 xylanase of *Paenibacillus* sp. HY-8 from the gut of *Moechotypa diphysis* and a lipase of *Burkholderia* sp. HY-10 from *Prionus insularis* have been isolated [7, 29]. In this study, *Streptomyces* sp. TN119 was isolated from the larvae gut of *B. horsfieldi* and showed xylanolytic activity. This strain is closely related to *S. cinnamocastaneus* and *S. fragilis*. *Actinobacteria* might be important to longhorn beetles since their existence in digestive tract have been verified by cultured or uncultured-based methods [11, 30]. Some xylanases have been cloned from *Actinobacteria*, such as a protease-resistant xylanase from *Streptomyces fradiae* var. k11 [31], a xylanase with broad temperature adaptability from *Streptomyces* sp. S9 [32], and a thermostable xylanase of *Thermomonospora alba* ULJB1 [33]; however, no xylanase has been reported to be produced by *S. cinnamocastaneus* or *S. fragilis* so far.

In this study, the base usage of G and C in the GH 10 xylanases from *Actinobacteria* was analyzed. The first and third positions of triplet codons were almost exclusively G and G or C, respectively. Using this rule, a pair of degenerate primers was designed to amplify the xylanase gene from strain TN119. As a result, a gene fragment containing part of *xynA119* (347 bp), was successfully obtained. Thus, this rule might be useful in primer design for all high-GC genes.

Liu and Whittier [9] developed a TAIL-PCR strategy to successfully isolate flanking regions from known genomic sequences and overcame the shortcomings of existing methods, such as time-consuming and laborious genomic library screening. However, this method is not suitable for high-GC templates, because the keys—AD primers and thermal procedure are inefficient. Usually, AD primers were designed to be of low GC (most <50% and only several <60%) with low T_m for low-stringency PCR [9, 14–17], even the HE TAIL-PCR specific for high-GC plant DNA [34]. However, the GC content of *Actinobacteria* is usually higher than 60%. And the average percentage of sequences with <50% GC content to the total sequences, constituted by 15 sequential bases from 5′–3′ of the tested *Actinobacteria* xylanase CDS, is only 5.3% (Table 1). Under this condition, it is inefficient to anneal the ordinary AD primers with high-GC templates as showed in Fig. 1b. The GC content of GCAD primers we designed are 50–80%. The 15 sequential bases—sequences with 50–80% GC content—account for an average of 85.5% to the total sequences in the tested *Actinobacteria* xylanase CDS (Table 1). Compared to the regular TAIL-PCR procedure, the T_m value in GC TAIL-PCR was increased from 44 °C up to 50 °C in low-stringency cycling (Table 2). Though nonspecific amplifications were not avoided due to the high-GC content of GCAD primers, the expected differential shift between the corresponding secondary and tertiary products were clear and dominant (Fig. 1a). Finally, a 3,224-bp DNA fragment containing the full-length *xynA119* was obtained. Moreover, using this GC TAIL-PCR, we have obtained four high-GC genes, which encoded a pectate lyase, a β-1,3-glucanase, a α-galactosidase and a GH 11 xylanase, respectively (data not shown).

XynA119 shared highest identities with *S. pristinaespiralis* ATCC 25486 and *S. sviceus* ATCC 29083 (YP_002204502). These three xylanases were grouped in cluster 2 (Fig. 2) with those from non-*Streptomyces Actinobacteria*, revealing that GH 10 xylanases from *Streptomyces* might evolve in two or more directions. The special source, the gut of *B. horsfieldi* larvae where strain TN119 was isolated, possibly accounts for the phylogenetic position of XynA19.

Two clusters were separated based on the phylogenetic analysis (Fig. 2). The significant property difference between these clusters is that xylanases in cluster 1 showed higher optimum pH (pH 7.0–7.8) [31, 35, 36] than that in cluster 2 (pH 6.0–6.5) [33, 37]. Most members in cluster 2 are putative xylanases which are analyzed based on conceptual translation of genomes, and characterization of GH 10 xylanases from *Streptomyces* in cluster 2 has not been reported so far. Here, we first reported characterization of a xylanase from *Streptomyces* in cluster 2. The purified r-XynA119 was optimally active at pH 6.5 and 60 °C and stable over a wide pH range, retaining more than 85% of the initial activity at pH 4.0–10.0 (Fig. 4), and was resistant to most metal ions (Table 4). The xylanase $XylA_{CspHY\text{-}12}$ from *Cellulosimicrobium* sp. HY-12 (ABX88978) showed the same optimum pH, optimum temperature, and thermostability with that of r-XynA119, but retained over 80% of its original activity in a narrower pH range of 4.5–7.0 and significantly inactivated at pH over 8.0. $XylA_{CspHY\text{-}12}$ was also sensitive to Ca^{2+}, Cu^{2+}, Co^{2+}, and Fe^{2+} at the concentration of 10 mM [37]. The xylanase XylA from *T. alba* ULJB1 (CAB02654) had similar optimum pH to r-XynA119, but showed worse pH stability and higher optimum temperature (80 °C). Cations at 10 mM, including Zn^{2+}, Ca^{2+}, and Mg^{2+}, inhibited the activity of XylA significantly [33].

Some xylanases often exhibited cellulase activity, thus resulting in cellulose loss, pulp quality degradation and increased effluent treatment cost [3]. Without cellulolytic activity, r-XynA119 would be very attractive to the paper and pulp industry as a biocatalyst in the bleaching process. Generally, xylobiose or a mixture of xylobiose and xylotetraose is the main hydrolysis products of xylan [32, 38]. The hydrolysis products of xylan by r-XynA119 were xylose and xylobiose, which are less complex and more efficient in bioconversion.

In conclusion, a modified PCR technique, GC TAIL-PCR, was developed in this study, and has successfully cloned a new xylanase gene with high GC content from *Streptomyces* sp. TN119. This method might have application potentials in chromosomal walking of all high-GC genes from microorganisms, plants, birds, and mammals. The xylanase, XynA119, exhibiting excellent pH stability and good resistance to some cations and proteases, might be beneficial to basic research and various potential industrial applications.

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