

Molecular detection and diversity of xylanase genes in alpine tundra soil

Guozeng Wang · Yaru Wang · Peilong Yang ·
Huiying Luo · Huoqing Huang · Pengjun Shi ·
Kun Meng · Bin Yao

Received: 22 January 2010 / Revised: 7 March 2010 / Accepted: 14 March 2010 / Published online: 15 April 2010
© Springer-Verlag 2010

Abstract Xylan is a major polysaccharide in plant cell walls, and its degradation is mainly conducted by microbial xylanases in nature. To explore the xylanase diversity in the environment, two sets of degenerate primers were designed based on the microbial xylanase sequences in Pfam database of glycosyl hydrolase (GH) family 10 and 11 and were used to amplify objective gene fragments directly from the alpine tundra soil DNA of the Tianshan Mountains, China. Ninety-six distinct GH 10 and 31 GH 11 xylanase gene fragments were retrieved, and most of them have low identities with known sequences in GenBank. Based on phylogenetic analysis, all of the GH 10 xylanase sequences fell into six clusters and were related to xylanases from Actinobacteria, Proteobacteria, Verrucomicrobia, Bacteroidetes, Firmicutes, and Acidobacteria. Three clusters of GH 11 xylanase sequences were established, and two of them were related with enzymes from fungi. These results indicated the diversity of xylanase genes in this cold environment. Four xylanolytic strains were isolated from the soil, and GH 10 xylanase gene fragments were cloned using the same primers. A full-length gene was obtained and expressed in *Escherichia coli*, and the recombinant enzyme showed some cold-related characteristics. Our study provides an efficient molecular approach to study

xylanase in complex environments and casts an insight into the diversity and distribution of xylanases in a cold environment, which is very meaningful to understand their roles in xylan degradation in nature.

Keywords Xylanase · Glycosyl hydrolase · Alpine tundra soil · Gene diversity · Degenerate PCR

Introduction

Hemicellulose, cellulose, and lignin are the major constituents of plant cell walls and represent the largest components of renewable organic resource on earth (Chandrakant and Bisaria 1998). Xylan—the major component of hemicellulose—is the second most abundant polysaccharide (Prade 1996), whose utilization is very important for a bio-based economy. Complete hydrolysis of xylan requires a large variety of cooperatively acting enzymes, including endo-1,4- β -D-xylanase, β -D-xylosidase, α -D-glucuronidase, α -L-arabinofuranosidase, acetyl xylanesterase, and arylesterase (Biely 1985; Thomson 1993; Kulkarni et al. 1999; Collins et al. 2005). Among them, a crucial component is endo-1,4- β -D-xylanase (EC 3.2.1.8) that catalyzes the hydrolysis of xylan to short xylooligosaccharides of varying lengths (Biely 1985). Based on sequence similarities of catalytic domain (Henrissat et al. 1989), xylanases have been classified into glycosyl hydrolase (GH) families (http://www.cazy.org/fam/acc_GH.html; Cantarel et al. 2009) 5, 7, 8, 10, 11, and 43 (Collins et al. 2005); of these, GH 10 and 11 xylanases are the most abundant and are distinct from each other in three-dimensional structures (Biely et al. 1997) and action mechanisms (Jeffries 1996). Xylanases belonging to GH 10 are larger, have an $(\alpha/\beta)_8$ barrel fold structure, and exhibit catalytic versatility

Electronic supplementary material The online version of this article (doi:10.1007/s00253-010-2564-9) contains supplementary material, which is available to authorized users.

G. Wang · Y. Wang · P. Yang · H. Luo · H. Huang · P. Shi ·
K. Meng · B. Yao (✉)

Key Laboratory for Feed Biotechnology of the Ministry of Agriculture, Feed Research Institute,
Chinese Academy of Agricultural Sciences,
No. 12 Zhongguancun South Street,
Beijing 100081, People's Republic of China
e-mail: yaobin@caas-bio.net.cn

(Charnock et al. 1998), while GH 11 xylanases are characterized by lower molecular weight, a compact β -jelly roll fold structure, and substrate specificity to xylan (Collins et al. 2005).

Microbial xylanases have been extensively studied due to their great potential for industrial and agricultural applications (Beg et al. 2001; Subramaniyan and Prema 2002; Polizeli et al. 2005; Collins et al. 2005). To obtain enzyme for use in specific fields, xylanases have been purified and characterized, and numerous xylanase genes have been cloned from various sources (Collins et al. 2005). Generally, more than one xylanase gene is present in the same organism and involved in xylan degradation. For example, Luo et al. (2009a, b, c) have cloned four xylanase genes (one of GH 10, two of GH 11, and one of GH 30) from the acidophilic fungus *Bispora* sp. MEY-1, and all of the genes coded for extracellular xylanases. The whole genome sequencing technique further provides us a more comprehensive understanding of the diversity of xylanase genes and their roles in degradation of plant cell wall polysaccharides (Hatsch et al. 2006; Berg Miller et al. 2009). Both the molecular cloning and genome sequencing data have shown the diversity of xylanase genes in microorganisms. According to the data collected by Pfam (release 24.0, <http://pfam.sanger.ac.uk/>), GH 10 contains more sequences than GH 11 (652 vs. 377), and there are more xylanase sequences from bacteria than from fungi (727 vs. 376).

So far, most information about xylanase is based on cultured microorganisms, which is limited since a vast majority of microorganisms remain uncultured. Metagenomics has become a powerful tool to exploit uncultured microbial communities in the environment (Lorenz and Schleper 2002; Daniel 2005). Using this method, several novel xylanase genes have been cloned directly from the environment (Sunna and Bergquist 2003; Hayashi et al. 2005) or screened from metagenomic library (Brennan et al. 2004; Ferrer et al. 2005; Lee et al. 2006a; Hu et al. 2008). Furthermore, functional gene diversity revealed by metagenomic sequences can provide insights into how microbial communities participate in the biogeochemical cycles in nature (Elifantz et al. 2008), such as chitinase genes (LeClerc et al. 2004; Xiao et al. 2005; Lian et al. 2007; Yasir et al. 2009), fungal GH genes (Jacobsen et al. 2005), and family 5 glycosyl hydrolase genes (Elifantz et al. 2008). Compared to the knowledge of functional genes in the nitrogen cycle, studies of gene diversity involved in organic carbon degradation are limited (Elifantz et al. 2008).

The objective of this study was to explore the diversity of xylanase genes in alpine tundra soil of the Tianshan Mountains, China. The diversity of xylanase genes was examined using clone libraries of amplicons generated with newly designed primers specific for GH 10 and 11 xylanases. Sequence analysis showed that most of the

fragments have low identity to known xylanases, suggesting the existence of a large amount of unidentified xylanase genes in nature. Our results also indicated that even in a very cold environment like alpine tundra soil, xylanases are very diverse with high richness.

Materials and methods

Soil sample collection

In August 2005, alpine tundra soil was collected from the Tianshan Mountains (43°06.1183' N, 86°50.1453' E) in Xinjiang Province, China, at an elevation of 3,525 m and an eastern exposure. Climate in this area is characterized by a long cold winter (from September through next May; -10°C to -19°C) and a short growing season (June to August; 3°C to 5°C). The average annual precipitation of this area is 35.6 mm. The alpine tundra soil was covered mainly by *Kobresia stenocarpa*, *Carex atrofusca*, and *Polygonum viviparum*. Three soil cores (10 cm in depth and 5 cm in diameter) were collected, pooled (~ 800 g in weight), and stored at -70°C until use.

DNA extraction

Soil DNA was extracted using a modified sodium dodecyl sulfate (SDS)-based method (Zhou et al. 1996; Brady 2007). Ten grams of soil was mixed with 100 ml of sterile 0.1 M Tris-EDTA (TE, pH 8.0) buffer with gentle agitation for 30 min. After centrifugation at $16,000\times g$ and 4°C for 10 min, the pellet was washed with 0.1 M TE buffer twice to remove water-soluble materials and incubated in 15 ml of lysozyme solution (10 mg ml^{-1} lysozyme, 25 mM Tris-HCl, 50 mM EDTA, and 0.3 M sucrose) at 37°C for 1.5 h. Then, 20 ml of lysis buffer (0.1 M Tris-HCl, 0.1 M EDTA, 1.5 M NaCl, 2% cetyl trimethyl ammonium bromide (CTAB), and 1% SDS) was added, mixed, and incubated at 70°C for an additional 2 h with gentle shaking every 20 min. After centrifugation at $16,000\times g$ and 4°C for 10 min, genomic DNA was recovered by precipitation with 0.7 volume isopropanol and dissolved in TE buffer. RNase was added to the crude soil DNA to digest RNAs (37°C , 1 h). The presence and size of the crude soil DNA were determined by agarose gel electrophoresis with ethidium bromide staining. Soil DNA was recovered and purified with the Omega Gel Extraction Kit (Norcross, GA, USA). The yield of purified DNA was about $1.8 \mu\text{g DNA g}^{-1}$ soil.

Primer design and testing

Amino acid sequences of 652 GH 10 xylanases and 377 GH 11 xylanases from fungi or bacteria were collected from Pfam

(release 24.0; <http://pfam.sanger.ac.uk/>). Based on the alignment (Fig. 1) and HMM Logos in Pfam (Schuster-Bockler et al. 2004), the conserved regions [W/Y]-D-W-D-V-[V/C/N]-N-E (Sunna and Bergquist 2003) and [D/H]-[G/A/C]-[I/V/L]-G-[M/F/L/I]-Q-[S/G/M/C]-H for GH 10 xylanases (about 85 amino acids between), and S-Y-L[C/S/A]-[V/L]-Y-G-W and T-F-[V/L]-Q-[Y/F]-[W/F]-S-V (Morris et al. 1998) for GH 11 xylanase (about 70 amino acids between) were identified, respectively (Fig. 1). Consensus-degenerate hybrid oligonucleotide primers (Rose et al. 2003) (X₁₀-F and X₁₀-R; X₁₁-F and X₁₁-R) were designed accordingly, in which inosine (I) was used to decrease the degeneracy.

To test the efficiency of these primer sets, a total of 20 representative xylanase-producing bacteria and fungi showing distinct phylogenetic lineages were selected, including strains producing both GH 10 and GH 11 xylanases: *Bispora* sp. MEY-1 (ACS96449, ACN89785), *Penicillium funiculosum* (CAG25554, CAC15487), *As-*

pergillus fumigatus (XP_751237, EAL86316), *Cochliobolus carbonum* (AAT49296, AAA33024), *Magnaporthe grisea* (AAM95237, XP_368051), *Hypocrea jecorina* (BAA89465, CAA49293), *Streptomyces* sp. S27 (ACF57946, ACF57948), *Cellulomonas fimi* (AAZ76373, CAA54145), *Saccharophagus degradans* 2-40 (YP_528105, YP_528530), and *Paenibacillus* sp. JDR-2 (ACS99999, ACT03278); strains only producing GH 10 xylanases: *Bacillus alcalophilus* (AAQ99279), *Pseudoalteromonas atlantica* (YP_662224), *Sphingobacterium* sp. TN19 (ACR61562), *Flavobacterium johnsoniae* UW101 (ABQ05028), and *Thermotoga maritima* (AAP97078); and strains only producing GH 11 xylanases: *Anaerocellum thermophilum* (ACM59249), *Bacillus pumilus* (ACU56779), *Bacillus subtilis* (CAA84276), *Cytophaga hutchinsonii* (YP_678979), and *Nesterenkonia xinjiangensis* (ACY70399). The genomic DNA, extracted using a genome-extracting kit (TIANGEN, Beijing, China) for bacteria or CTAB method for

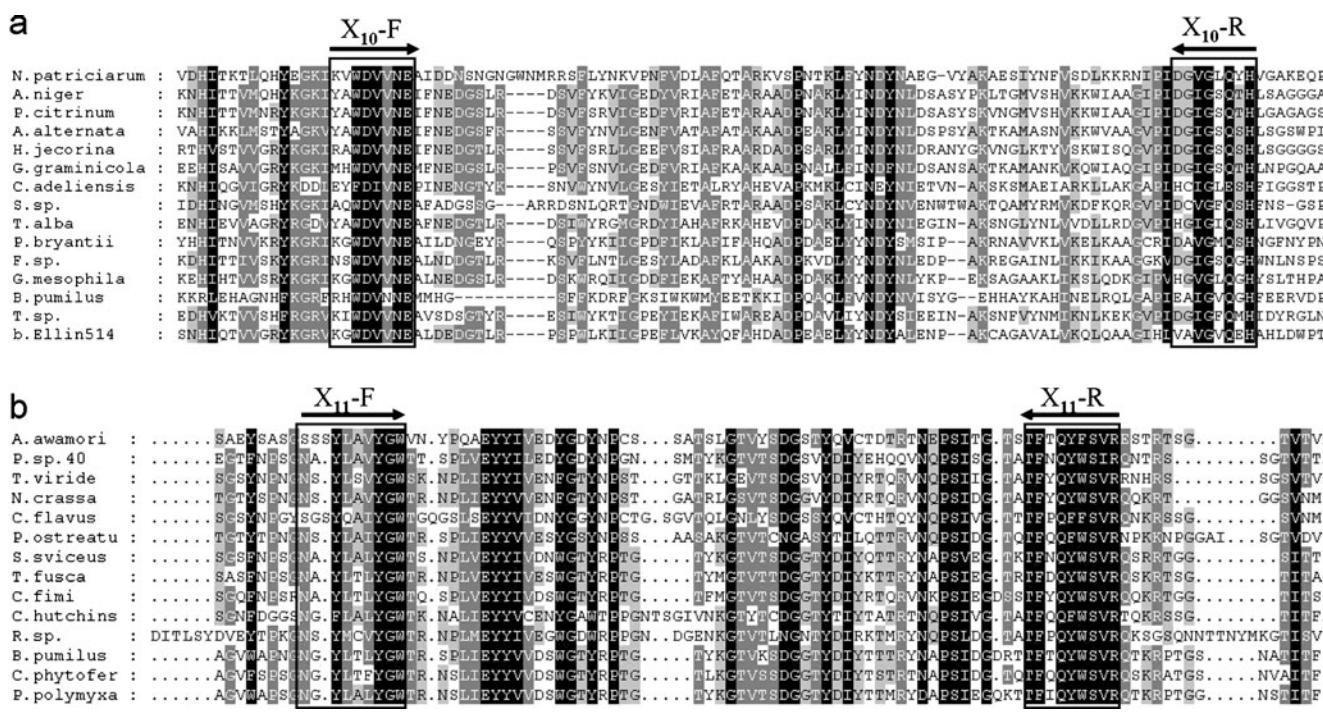


Fig. 1 Primers' design specific for GH 10 and 11 xylanase genes. The amino acid sequences of xylanases from microorganisms representing diverse phylogenetic lineages were aligned. Conserved amino acids are highlighted and consensus regions are boxed. The locations of the two sets of primers and the direction of amplification are indicated by arrows. **a** Sequences of GH 10 xylanases used for primer design. Sequence name, microbial source, and GenBank number were given as follows: N. patriciarum: *Neocallimastix patriciarum* (AAB30669); A.niger: *Aspergillus niger* (XP_001389996); P.citrinum: *Penicillium citrinum* (BAG12101); A.alternata: *Alternaria alternata* (AAF05698); H.jecorina: *Hypocrea jecorina* (BAA89465); G.graminicola: *Glomerella graminicola* (CAQ16209); C.adeliensis: *Cryptococcus adeliensis* (CAA75630); S.sp. S27: *Streptomyces* sp. S27 (CAA75630); T.alba: *Thermobifida alba* (CAB02654); P.bryantii: *Prevotella bryantii* (CAD21011); F.sp. MSY2: *Flavobacterium* sp. MSY2 (AAY98787); G.mesophila: *Glaciecola*

mesophila (ACN76857); B.pumilus SAFR-032: *Bacillus pumilus* SAFR-032 (ABV62242); T.sp.: *Thermotoga* sp. (AAA90913); b. Ellin514: bacterium Ellin514 (EEF60952). **b** Sequences of GH 11 xylanases used for primer design. Sequence name, microbial source, and GenBank number were given as follows: A.awamori: *Aspergillus awamori* (CAA55005); P.sp.40: *Penicillium* sp. 40 (BAA88421); T. viride: *Trichoderma viride* (AAQ67413); N.crassa: *Neurospora crassa* (CAD71059); C.flavus: *Cryptococcus flavus* (ABY50453); P.ostreatu: *Pleurotus ostreatus* (ABY61039); S.sviceus: *Streptomyces sviceus* ATCC 29083 (EDY53512); T.fusca: *Thermobifida fusca* (AAV64879); C.fimi: *Cellulomonas fimi* (CAA54145); C.hutchinsonii: *Cytophaga hutchinsonii* ATCC 33406 (ABG59637); R.sp.: *Ruminococcus* sp. (CAA90271); B. pumilus: *Bacillus pumilus* (AAZ17390); C.phytofermentans: *Clostridium phytofermentans* ISDg (YP_001559210); P.polymyxa: *Paenibacillus polymyxa* (AAZ17384)

fungi, was used as templates and underwent a touchdown PCR. The template DNA (approximately 50 ng) was added to a 50- μ l reaction system containing 1 \times Taq PCR MasterMix (2.5 U of Taq polymerase, 250 μ M of each dNTP, 1.5 mM Mg²⁺, 12.5 mM Tris-HCl (pH8.3), and 100 mM KCl) and 0.6 μ M of each primer. The optimized PCR conditions for both primer sets were: 4 min at 95°C, followed by 12 cycles of 94°C for 30 s, 56°C (for GH 10 xylanases) or 58°C (for GH 11 xylanases; decreasing by 0.5°C after each cycle) for 30 s, and 72°C for 30 s, followed by 28 cycles of 94°C for 30 s, 50°C (for GH 10 xylanases) or 52°C (for GH 11 xylanases) for 30 s, and 72°C for 30 s, and then a final extension at 72°C for 6 min. The presence and size of the amplification products were determined on agarose gels.

Clone library construction and DNA sequencing

The primer sets designed were used to amplify GH 10 and 11 xylanase gene fragments by touchdown PCR (as described above) using the purified soil DNA as templates. PCR products were visualized on an agarose gel, and the objective bands (~260 and 210 bp for GH 10 and GH 11 xylanase gene fragments) were excised and purified using the Qiaquick gel extraction kit (Qiagen, Valencia, CA, USA). To construct the clone library for each xylanase family, the purified PCR products were ligated into vector pGEM-T Easy (Promega, Madison, WI, USA), and electroporated into *Escherichia coli* DH5 α competent cells (TaKaRa, Otsu, Japan) following the procedure recommended by the manufacturer. Cells were grown on Luria-Bertani (LB) agar plates containing 100 μ g ml⁻¹ ampicillin, 80 μ g ml⁻¹ X-Gal, and 0.5 μ M isopropyl- β -D-1-thiogalactopyranoside (IPTG) at 37°C for 15 h. Three hundred positive transformants (white clones) were randomly picked from each library (about 3,000 white clones), amplified with primers M13F (5'-GTAAAACGACGGCCAGT-3') and M13R (5'-GGATAACAATTTTCACACAGGA-3'), and sequenced by Sunbiotech (Beijing, China) for further confirmation.

Phylogenetic analysis

Based on the results of BLASTx, the reading frames were identified for each xylanase gene fragment, and the DNA sequences were translated into amino acid sequences by EMBOSS Transeq (<http://www.ebi.ac.uk/emboss/transeq>) and aligned using ClustalW software. Phylogenetic trees were constructed with MEGA 4.0 (Tamura et al. 2007) using neighbor-joining method (Saitou and Nei 1987). Confidence for tree topologies was estimated by bootstrap values based on 1,000 replicates. Forty-two and 12 related sequences obtained from the GenBank were selected and used as references for GH 10 and 11 xylanase phylogenetic tree constructions, respectively.

Cloning of xylanase genes from xylanolytic strains

Microorganisms were isolated from the alpine tundra soil using an enrichment culture as described by Zhang et al. (2008). Colonies were repeatedly picked up and streaked on LB plates to achieve purity. Xylanolytic strains were screened using the selective medium containing xylan as the only carbon source and Congo red as the indicator of the hydrolysis zone (Morosoli et al. 1986; Wood et al. 1988). Genomic DNA of each strain was extracted using a genome-extracting kit (TIANGEN, Beijing, China) and used as template for amplification of partial 16S rRNA gene sequence. The taxa of these xylan-degrading strains were determined by comparison of their 16S rDNA sequences with those in GenBank.

Xylanase gene fragments of xylanolytic strains were obtained following the same procedure as library construction with genomic DNA of these strains as template. The amplified fragment of the appropriate size was ligated into the pGEM-T Easy vector for sequencing. *Flavobacterium* sp. LW53 showing the highest xylanolytic activity was selected for full-length gene cloning and expression. The 5' and 3' flanking regions of the gene fragment were obtained with the Genome Walking kit (TaKaRa) using thermal asymmetric interlaced (TAIL)-PCR (Liu and Whittier 1995). The xylanase gene (*xyn-fla*) without the signal peptide coding sequence was cloned into plasmid pET-22b(+) and then transformed into *E. coli* BL21 (DE3) competent cells for recombinant expression.

Enzyme expression, purification, and characterization

The positive transformant harboring pET-*xyn-fla* was grown in LB medium containing 100 μ g ml⁻¹ ampicillin at 37°C to an A_{600} of 0.6. Expression was induced by 0.8 mM IPTG at 30°C for 8 h. The recombinant xylanase (Xyn-FLA) was purified to electrophoretic homogeneity by ultrafiltration, ammonium sulfate fraction (40–70% saturation), and ion exchange chromatography through a HiTrap SP Sepharose XL 5-ml column (Amersham Pharmacia Biotech, Uppsala, Sweden). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to determine the purity and apparent molecular mass of recombinant Xyn-FLA. The protein concentration was determined by the Bradford method (1976), using bovine serum albumin as a standard.

Xylanase activity was determined by measuring the release of reducing sugar from oat spelt xylan using the 3,5-dinitrosalicylic acid method (Miller et al. 1960). One unit (U) of xylanase activity was defined as the amount of enzyme that released 1 μ mol of reducing sugar equivalent to xylose per minute under standard conditions (pH6.5, 35°C, 10 min).

The optimal pH for enzyme activity of the purified Xyn-FLA was determined at 30°C in buffers of pH4.0 to 9.0. The enzyme stability at different pH was estimated by measuring the residual enzyme activity after incubating the enzyme solution in buffers at pH2.0–10.0, 37°C for 1 h. The buffers used were McIlvaine buffer (0.2 M Na₂HPO₄/0.1 M citric acid) for pH2.5–7.5, 0.1 M Tris–HCl for pH 7.5–9.0, and 0.1 M glycine–NaOH for pH9.0–10.0. The optimal temperature for Xyn-FLA activity was determined over the range of 10–50°C in McIlvaine buffer (pH6.5). The thermostability of the purified Xyn-FLA was determined after pre-incubation of the enzyme in McIlvaine buffer (pH6.5) at 45°C and 50°C without substrate for various periods.

The K_m , V_{max} , and k_{cat} values for the purified Xyn-FLA were determined using 1–10 mg ml⁻¹ oat spelt xylan as the substrate and measured in McIlvaine buffer (pH6.5) at 35°C. The data were plotted according to the Lineweaver–Burk method.

Nucleotide sequence accession numbers

The nucleotide sequences of GH 10 and 11 xylanase gene fragments were deposited into the GenBank database under accession numbers FJ527976–FJ528073 and FJ623403–FJ623433, respectively. Accession numbers FJ614243, FJ614245, FJ614246, and FJ614250 were assigned to the 16S rDNA sequences of isolated xylanolytic strains. The nucleotide sequence of the full-length xylanase gene (*xyn-fla*) from *Flavobacterium* sp. LW53 was given accession number FJ797706.

Results

GH 10 and 11 xylanase-specific primers

Based on the conserved regions of GH 10 and 11 xylanases (Fig. 1), we designed two primer sets to amplify xylanase gene fragments: X₁₀-F (5'-CTACGACTGGGAYGTNIBS AAYGA-3') and X₁₀-R (5'-GTGACTCTGGAWRCC IABNCCRT-3'), and X₁₁-F (5'-AACTGCTACCTGKCN TNTAYGGNTGG-3') and X₁₁-R (5'-CCGCACGGACC AGTAYTGKIRAANGT-3') (Y: C or T; R: A or G; W: A or T; S: C or G; B: C, G, or T; N: A, C, G, or T; and I: inosine; degenerate regions are underlined). Using X₁₀-F and X₁₀-R, we obtained PCR products of the expected size from 15 representative xylan-degrading strains that served as templates. We also obtained PCR products of the expected size from selected strains using degenerate primers X₁₁-F and X₁₁-R. By sequencing and BLAST analysis, all of the fragments were confirmed to be portions of GH 10 or GH 11 xylanase genes.

Sequence analysis

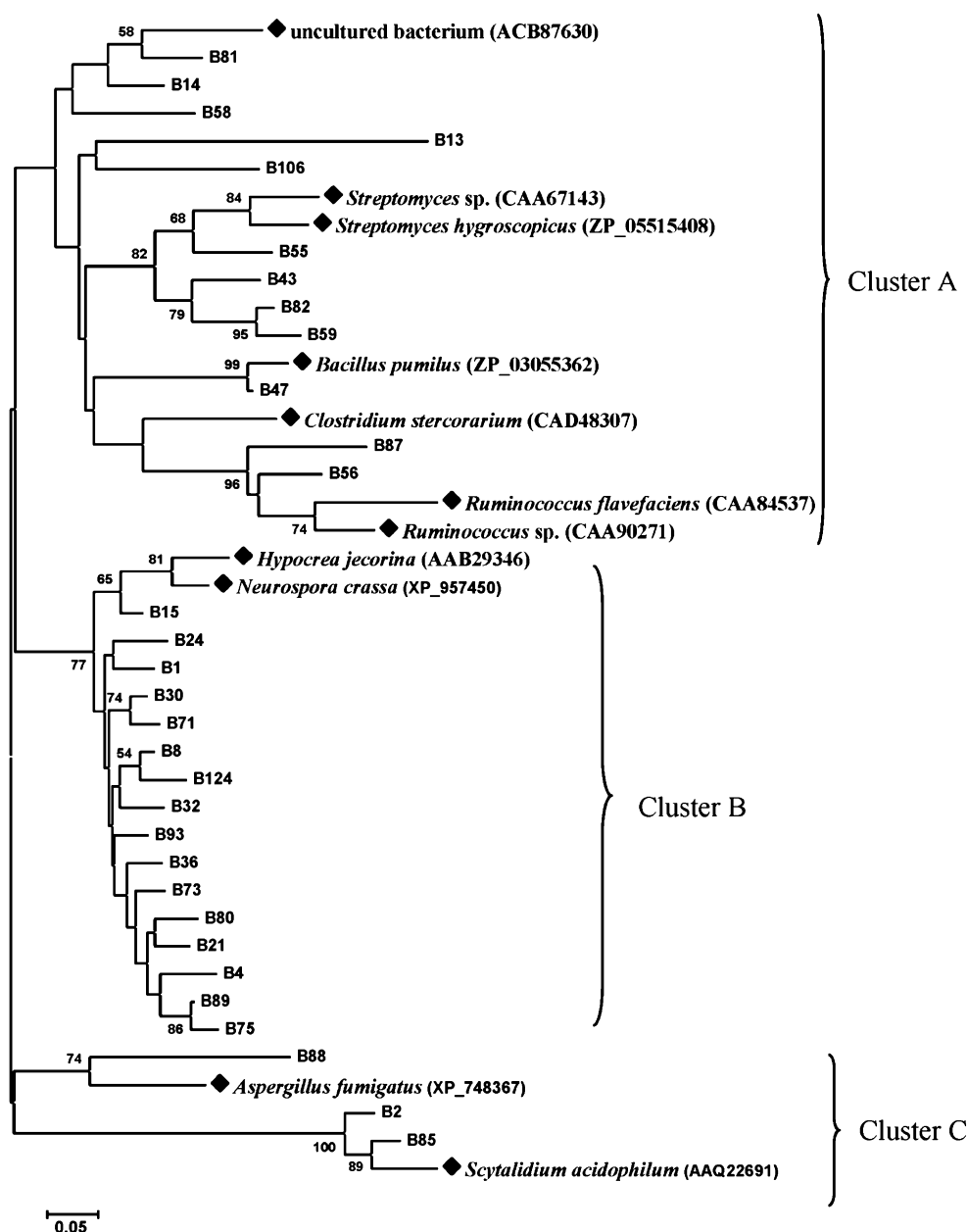
Gene fragments, about 260 bp for GH 10 xylanase and 210 bp for GH 11 xylanase, were amplified directly from the soil DNA using touchdown PCR. Clone libraries for each family (containing about 3,000 white clones) were constructed and 10% clones were selected randomly for further analysis. Of the 300 clones from library GH 10, 216 clones had an insert of the correct size, 147 clones showed 41–94% amino acid identity with known xylanases based on BLAST analysis, and 96 clones showed sequence divergence (sharing <95% identity; Electronic supplementary material—Table 1). Highly conserved residue of GH 10 xylanases (Solomon et al. 2007), Asn, was found at the corresponding position of all protein sequences, which varied from 84 to 102 amino acids in lengths. Of the 300 clones from library GH 11, 197 clones had an insert of the correct size, 132 clones showed 57–95% identity with known xylanases based on BLAST analysis, and 31 clones showed sequence divergence (sharing <95% identity). Highly conserved residue—Glu (catalytic residue) for GH 11 xylanases (Jeffries 1996; Wouters et al. 2001)—was found in all of the protein sequences of 69–72 amino acids in lengths (Electronic supplementary material—Table 2). These results indicated that the clones from both libraries were partial xylanase genes and some of them might be novel.

Phylogenetic analysis

An unrooted protein-level phylogenetic tree for GH 10 xylanases was constructed using the 96 divergent sequences (similarity <95%) from clone library and 42 reference sequences from GenBank. All of the sequences were confined into six clusters, denoted I, II, III, IV, V, and VI, indicating the substantial diversity of GH 10 xylanases in alpine tundra soil (Fig. 2). Many clades formed in the clusters with no reference sequences suggested that these sequences might be novel.

Cluster I contained 32 sequences from clone library and 13 reference sequences of different genera of Actinobacteria, such as *Catenulispora*, *Acidothermus*, *Micromonospora*, *Jonesia*, *Cellulomonas*, and *Streptomyces*. This result indicated the high diversity of xylanolytic Actinobacteria in alpine tundra soil. There were 15 sequences in cluster II, and two sequences (B33 and B69) were closely related with the xylanase from extremely halophilic. A total of 17 sequences from clusters III, IV, and VI were related to xylanases from *Teredinibacter turnerae*, *S. degradans*, *Sorangium cellulosum*, and marine gamma proteobacterium, suggesting that proteobacteria were another predominant group of xylanolytic bacteria in this environment. In cluster IV, 18 sequences closely related to the reference sequence of *Verrucomicrobiae bacterium* were found. Other sequences are related to xylanases from

Fig. 3 Phylogenetic analysis based on the partial amino acid sequences of GH 11 xylanase genes detected in the soil DNA and their relationship with the reference sequences retrieved from GenBank. This tree was constructed using the neighbor-joining method (MEGA 4.0). The lengths of the branches indicate the relative divergence among the amino acid sequences. The reference sequences are marked with a closed diamond with source strains and xylanase GenBank accession numbers in parentheses. The numbers at the nodes indicate bootstrap values based on 1,000 bootstrap replications and bootstrap values (>50) are displayed. The scale bar represents 0.05 amino acid substitutions per position



residues with a typical signal peptide (residues 1–22). The mature protein has a theoretical molecular weight of 39.5 kDa. Sequence homology searches showed that the xylanase shared the highest identity (82%) with a GH 10 xylanase from *Flavobacterium* sp. MSY2 (Lee et al. 2006b). After expression in *E. coli*, the purified recombinant enzyme had a pH optimum of 6.5 (Fig. 4a) and was stable at pH 2.5 to 9.5 (Fig. 4b). The enzyme showed maximal activity at 35°C (Fig. 4c), retaining 16% of the maximal activity even at 10°C, but lost all activity after incubation at 50°C for 4 min (Fig. 4d). Xyn-FLA exhibited substrate specificity to oat spelt xylan (100%), beechwood xylan (86%), and birchwood xylan (81%), respectively. Based on the Lineweaver–Burk plot, the

K_m , V_{max} , and k_{cat} values were 2.75 mg ml⁻¹, 625.3 μmol min⁻¹mg⁻¹, and 411 s⁻¹, respectively, using oat spelt xylan as the substrate at 35°C. These properties indicated that this enzyme had some cold-related enzyme characteristics (Siddiqui and Cavicchioli 2006).

Discussion

To explore xylanase diversity and distribution in nature, we selected a cold environment in Tianshan Mountains. The alpine tundra soil is characterized by unusual environmental conditions such as long periods of low temperature,

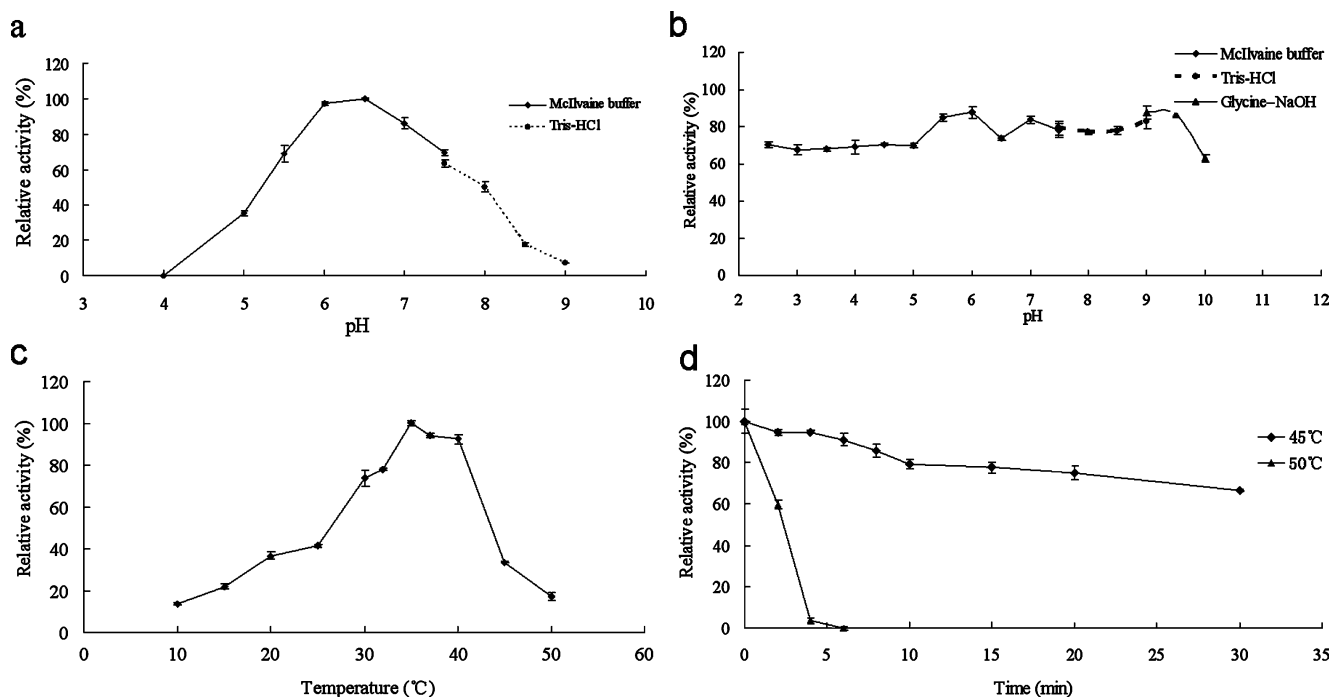


Fig. 4 Properties of Xyn-FLA. **a** Effect of pH on Xyn-FLA activity. Activities at various pH were assayed at 30°C. **b** pH stability of Xyn-FLA. Residual activities after incubation at various pH were assayed at pH6.5 and 35°C. **c** Effect of temperature on Xyn-FLA activity in

McIlvaine buffer (pH6.5). **d** Thermostability of Xyn-FLA. Residual activity was assayed at pH6.5, 35°C after pre-incubation at 45°C and 50°C for different periods of time. The error bars represent mean \pm SD ($n=3$)

frequent freeze-thaw, wet-dry cycling, and transient precipitation (Lipson and Schmidt 2004). Previous studies revealed that the microbial biomass in tundra soil was very rich and active (Brooks et al. 1998; Schadt et al. 2003; Schmidt and Lipson 2004; Nemergut et al. 2005; Wallenstein et al. 2007). Using culture-based methods, more than 200 strains have been isolated from the alpine tundra soil from Tianshan Mountains and about 20% of them displayed diverse glycosyl hydrolase activities by utilizing different carbon sources at low temperature (Zhang et al. 2008). Since a large number of microorganisms are hard to be cultured in laboratory due to the extreme harsh conditions of tundra soils (Lipson and Schmidt 2004; Nemergut et al. 2005), in this study, culture-independent molecular approaches are developed to explore the xylanase gene diversity in the alpine tundra soil.

Two sets of degenerate primers specific for GH 10 and GH 11 xylanases were designed and used to amplify xylanase gene fragments from the soil DNA. Sequence analysis based on BLAST and conserved residues indicated that all of the fragments represented partial GH xylanases. A total of 129 distinct xylanase gene fragments were obtained (Figs. 2 and 3), and most of them are novel, showing low identities of <80% with known xylanases in GenBank. Moreover, similarities among these amplified partial sequences are low and distantly related based on phylogenetic analysis. These results revealed that the

degenerate primers developed in this study are efficient to amplify xylanase gene fragments from the environment than the traditional methods (Sunna and Bergquist 2003; Hayashi et al. 2005).

Microorganisms, mainly bacteria and fungi, are the key agents involved in xylan degradation (Sunna and Antranikian 1997). In this study, phylogenetic analysis of GH 11 partial xylanase genes retrieved directly from soil DNA (Fig. 3) showed that 61% (19/31) of GH 11 xylanase sequences were related to those from Ascomycota, the fungi dominant in tundra soils (Schadt et al. 2003). To our surprise, all of the 96 fragment sequences of library GH 10 are closely related to those from bacteria (Fig. 2). Using the primers X₁₀-F and X₁₀-R, we successfully cloned GH 10 xylanase gene fragments from fungi including *Magnaporthe* sp., *Neosartorya* sp. (with intron), *Penicillium* sp. (with intron), *Phialophora* sp. (with intron), and *Gibberella* sp. (data not shown). The possible reason why we failed to clone fungal GH 10 xylanase gene fragments from the soil is that GH 10 xylanases from bacteria are predominant in this environment and sequence from fungi was omitted from the library. As a fact, there are far more bacterial GH 10 xylanase genes submitted to the GenBank database than those from fungi.

Actinobacteria, Proteobacteria, Verrucomicrobia, CFB group and Firmicutes are the main xylanolytic bacteria to produce xylanases in the alpine tundra soil of Tianshan

Mountains based on the phylogenetic analysis (Fig. 2). It is consistent with the conclusion of Lipson and Schmidt (2004) that such bacteria are the main constituents of microbial community in tundra soils. Soil actinomycetes are one of the important degraders of organic matter in most terrestrial habitats (Ball and McCarthy 1989). In this study, more than 32% (32/98) of GH 10 xylanase gene fragments were closely related to the xylanases from different genera of Actinobacteria (Fig. 2), further confirming that Actinobacteria played important roles in the xylan degradation in cold environment. Many clades without close relatives suggested their novelty, which might be ascribed to the large portion of unidentified microorganisms in this environment (Nemergut et al. 2005; Costello and Schmidt 2006; Babalola et al. 2009). Two sequences (B33 and B69) in cluster II (Fig. 2) are closely related to the xylanase from extremely halophilic archaeon *Halorhabdus utahensis*, suggesting the possibility of xylanase-producing Archaea existing in this environment (Cavicchioli 2006).

Several cold-active xylanases from psychrotrophic or psychrophilic microorganisms have been characterized (Petrescu et al. 2000; Collins et al. 2002; Lee et al. 2006b; Guo et al. 2009). In this study, four xylanolytic strains were isolated from the alpine tundra soil and a full-length GH 10 xylanase gene was cloned from *Flavobacterium* sp. LW53. The recombinant enzyme Xyn-FLA has a temperature optimum at 35°C, 5°C higher than those of the cold-active GH 10 xylanases Xyn10 from *Flavobacterium* sp. MSY2 (Lee et al. 2006b) and XynA from marine *Glaciecola mesophila* KMM 241 (Guo et al. 2009) but lower than most of mesophilic xylanases (50–60°C), and was thermolabile at mesophilic temperatures. Furthermore, the recombinant enzyme Xyn-FLA showed a higher $k_{\text{cat}}/K_{\text{m}}$ value. Xyn-FLA has an apparent K_{m} of 2.75 mg ml⁻¹, V_{max} of 625.3 μmol min⁻¹mg⁻¹, and k_{cat} of 411 s⁻¹ at 35°C toward oat spelt xylan while Xyn10 has an apparent K_{m} of 1.8 mg ml⁻¹, V_{max} of 142 μmol min⁻¹mg⁻¹, and k_{cat} of 100 s⁻¹ at 20°C toward beechwood xylan. Xyn A from *Glaciecola mesophila* has a $k_{\text{cat}}/K_{\text{m}}$ of 10.79 ml mg⁻¹s⁻¹ at 30°C while Xyn-FLA has a $k_{\text{cat}}/K_{\text{m}}$ of 149.45 ml mg⁻¹s⁻¹ at 35°C toward oat spelt xylan. These results indicated that microorganisms in this cold environment did produce xylanases with low-temperature properties. Further studies on other xylanase genes will be conducted.

In summary, a culture-independent molecular method is developed in this study to explore the diversity and distribution of xylanase genes in alpine tundra soil. This method is efficient to amplify gene fragments of distantly related xylanases from a complex environment and might be used in screening xylanase genes from clone library or cloning xylanase genes from environment samples directly. Our results indicated that even in a very cold environment like alpine tundra soil, xylanases are very diverse with high

richness, and implied that the xylanolytic microorganisms in situ is far more complex than we imagined. Our study also drew an insight into the diversity and distribution of xylanases in a cold environment, which is very meaningful to understand their roles in xylan degradation in nature.

Acknowledgments This work was supported by the Agricultural Science and Technology Conversion Funds (Grant no. 2008GB23260388), the Key Program of Transgenic Plant Breeding (2008ZX08011-005), and the Earmarked Fund for Modern Agro-industry Technology Research System (NYCYTX-42-G2-05).

References

- Babalola OO, Kirby BM, Le Roes-Hill M, Cook AE, Cary SC, Burton SG, Cowan DA (2009) Phylogenetic analysis of actinobacterial populations associated with Antarctic Dry Valley mineral soils. *Environ Microbiol* 11:566–576
- Ball AS, McCarthy AJ (1989) Production and properties of xylanases from actinomycetes. *J Appl Microbiol* 66:439–444
- Beg QK, Kapoor M, Mahajan L, Hoondal GS (2001) Microbial xylanases and their industrial applications: a review. *Appl Microbiol Biotechnol* 56:326–338
- Berg Miller ME, Antonopoulos DA, Rincon MT, Band M, Bari A, Akraiko T, Hernandez A, Thimmapuram J, Henrissat B, Coutinho PM, Borovok I, Jindou S, Lamed R, Flint HJ, Bayer EA, White BA (2009) Diversity and strain specificity of plant cell wall degrading enzymes revealed by the draft genome of *Ruminococcus flavefaciens* FD-1. *PLoS ONE* 4: e6650
- Biely P (1985) Microbial xylanolytic systems. *Trends Biotechnol* 11:286–290
- 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
- Brady SF (2007) Construction of soil environmental DNA cosmid libraries and screening for clones that produce biologically active small molecules. *Nat Protoc* 2:1297–1305
- Brennan Y, Callen WN, Christoffersen L, Dupree P, Goubet F, Healey S, Hernández M, Keller M, Li K, Palackal N, Sittenfeld A, Tamayo G, Wells S, Hazlewood GP, Mathur EJ, Short JM, Robertson DE, Steer BA (2004) Unusual microbial xylanases from insect guts. *Appl Environ Microbiol* 70:3609–3617
- Brooks PD, Williams MW, Schmidt SK (1998) Inorganic nitrogen and microbial biomass dynamics before and during spring snowmelt. *Biogeochemistry* 43:1–15
- Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B (2009) The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics. *Nucl Acids Res* 37:D233–D238
- Cavicchioli R (2006) Cold-adapted archaea. *Nat Rev Micro* 4: 331–343
- Chandrakant P, Bisaria VS (1998) Simultaneous bioconversion of cellulose and hemicellulose to ethanol. *Crit Rev Biotechnol* 18:295–331
- Charnock SJ, Spurway TD, Xie H, Beylot MH, Virden R, Warren RA, Hazlewood GP, Gilbert HJ (1998) The topology of the substrate binding clefts of glycosyl hydrolase family 10 xylanases are not conserved. *J Biol Chem* 273:32187–32199

- Collins T, Meuwis M-A, Stals I, Claeysens M, Feller G, Gerday C (2002) A novel family 8 xylanase: functional and physico-chemical characterization. *J Biol Chem* 277:35133–35139
- Collins T, Gerday C, Feller G (2005) Xylanases, xylanase families and extremophilic xylanases. *FEMS Microbiol Rev* 29:3–23
- Costello EK, Schmidt SK (2006) Microbial diversity in alpine tundra wet meadow soil: novel Chloroflexi from a cold, water-saturated environment. *Environ Microbiol* 8:1471–1486
- Daniel R (2005) The metagenomics of soil. *Nat Rev Micro* 3:470–478
- Elifantz H, Waidner LA, Michelou VK, Cottrell MT, Kirchman DL (2008) Diversity and abundance of glycosyl hydrolase family 5 in the North Atlantic Ocean. *FEMS Microbiol Ecol* 63:316–327
- Ferrer M, Golyshina OV, Chernikova TN, Khachane AN, Reyes-Duarte D, Santos VA, Strompl C, Elborough K, Jarvis G, Neef A, Yakimov MM, Timmis KN, Golyshin PN (2005) Novel hydrolase diversity retrieved from a metagenome library of bovine rumen microflora. *Environ Microbiol* 7:1996–2010
- Guo B, Chen X, Sun C, Zhou B, Zhang Y (2009) Gene cloning, expression and characterization of a new cold-active and salt-tolerant endo- β -1, 4-xylanase from marine *Glaciecola mesophila* KMM 241. *Appl Microbiol Biotechnol* 84:1107–1115
- Hatsch D, Phalip V, Petkovski E, Jeltsch J (2006) *Fusarium graminearum* on plant cell wall: no fewer than 30 xylanase genes transcribed. *Biochem Biophys Res Commun* 345:959–966
- Hayashi H, Abe T, Sakamoto M, Ohara H, Ikemura T, Sakka K, Benno Y (2005) Direct cloning of genes encoding novel xylanases from the human gut. *Can J Microbiol* 51:251–259
- Henrissat B, Claeysens M, Tomme P, Lemesle L, Mornon JP (1989) Cellulase families revealed by hydrophobic cluster analysis. *Gene* 81:83–95
- Hu Y, Zhang G, Li A, Chen J, Ma L (2008) Cloning and enzymatic characterization of a xylanase gene from a soil-derived metagenomic library with an efficient approach. *Appl Microbiol Biotechnol* 80:823–830
- Jacobsen J, Lydolph M, Lange L (2005) Culture independent PCR: an alternative enzyme discovery strategy. *J Microbiol Methods* 60:63–71
- Jeffries TW (1996) Biochemistry and genetics of microbial xylanases. *Curr Opin Biotechnol* 7:337–342
- Kulkarni N, Shendye A, Rao M (1999) Molecular and biotechnological aspects of xylanases. *FEMS Microbiol Rev* 23:411–456
- LeClerc GR, Buchan A, Hollibaugh JT (2004) Chitinase gene sequences retrieved from diverse aquatic habitats reveal environment-specific distributions. *Appl Environ Microbiol* 70:6977–6983
- Lee CC, Kibblewhite-Accinelli RE, Wagschal K, Robertson GH, Wong DWS (2006a) Cloning and characterization of a cold-active xylanase enzyme from an environmental DNA library. *Extremophiles* 10:295–300
- Lee CC, Smith M, Kibblewhite-Accinelli RE, Williams TG, Wagschal K, Robertson GH, Wong DWS (2006b) Isolation and characterization of a cold-active xylanase enzyme from *Flavobacterium* sp. *Curr Microbiol* 52:112–116
- Lian M, Lin S, Zeng R (2007) Chitinase gene diversity at a deep sea station of the east Pacific nodule province. *Extremophiles* 11:463–467
- Lipson DA, Schmidt SK (2004) Seasonal changes in an alpine soil bacterial community in the Colorado Rocky Mountains. *Appl Environ Microbiol* 70:2867–2879
- Liu YG, Whittier RF (1995) Thermal asymmetric interlaced PCR: automatable amplification and sequencing of insert end fragments from PI and YAC clones for chromosome walking. *Genomics* 25:674–681
- Lorenz P, Schleper C (2002) Metagenome—a challenging source of enzyme discovery. *J Mol Catal, B Enzym* 19–20:13–19
- Luo H, Wang Y, Li J, Wang H, Yang J, Yang Y, Huang H, Fan Y, Yao B (2009a) Cloning, expression and characterization of a novel acidic xylanase, XYL11B, from the acidophilic fungus *Bispora* sp. MEY-1. *Enzyme Microb Technol* 45:126–133
- Luo H, Li J, Yang J, Wang H, Yang Y, Huang H, Shi P, Yuan T, Fan Y, Yao B (2009b) A thermophilic and acid stable family-10 xylanase from the acidophilic fungus *Bispora* sp. MEY-1. *Extremophiles* 13:849–854
- Luo H, Yang J, Li J, Shi P, Huang H, Bai Y, Fan Y, Yao B (2009c) Molecular cloning and characterization of the novel acidic xylanase XYLD from *Bispora* sp. MEY-1 that is homologous to family 30 glycosyl hydrolases. *Appl Microbiol Biotechnol*. doi:10.1007/s00253-009-2410-0
- Miller GL, Blum R, Glennon WE, Burton AL (1960) Measurement of carboxymethylcellulase activity. *Anal Biochem* 1:127–132
- Morosoli R, Bertrand JL, Mondou F, Shareck F, Kluepfel D (1986) Purification and properties of a xylanase from *Streptomyces lividans*. *Biochem J* 239:587–592
- Morris DD, Gibbs MD, Chin CW, Koh M, Wong KKY, Allison RW, Nelson PJ, Bergquist PL (1998) Cloning of the *xynB* gene from *Dictyoglomus thermophilum* Rt46B.1 and action of the gene product on kraft pulp. *Appl Environ Microbiol* 64:1759–1765
- Nemergut DR, Costello EK, Meyer AF, Pescador MP, Weintraub MN, Schmidt SK (2005) Structure and function of alpine and arctic soil microbial communities. *Res Microbiol* 156:775–784
- Petrescu I, Lamotte-Brasseur J, Chessa JP, Ntarima P, Claeysens M, Devreese B, Marino G, Gerday C (2000) Xylanase from the psychrophilic yeast *Cryptococcus adeliae*. *Extremophiles* 4:137–144
- Polizeli MLTM, Rizzatti ACS, Monti R, Terenzi HF, Jorge JA, Amorim DS (2005) Xylanases from fungi: properties and industrial applications. *Appl Microbiol Biotechnol* 67:577–591
- Prade RA (1996) Xylanases: from biology to biotechnology. *Biotechnol Genet Eng Rev* 13:101–131
- Rose T, Henikoff J, Henikoff S (2003) CODEHOP (consensus-degenerate hybrid oligonucleotide primer) PCR primer design. *Nucl Acids Res* 31:3763–3766
- Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425
- Schadt CW, Martin AP, Lipson DA, Schmidt SK (2003) Seasonal dynamics of previously unknown fungal lineages in tundra soils. *Science* 301:1359–1361
- Schmidt SK, Lipson DA (2004) Microbial growth under the snow: implications for nutrient and allelochemical availability in temperate soils. *Plant Soil* 259:1–7
- Schuster-Bockler B, Schultz J, Rahmann S (2004) HMM logos for visualization of protein families. *BMC Bioinformatics* 5:7
- Siddiqui KS, Cavicchioli R (2006) Cold-adapted enzymes. *Annu Rev Biochem* 75:403–433
- Solomon V, Teplitsky A, Shulami S, Zolotnitsky G, Shoham Y, Shoham G (2007) Structure-specificity relationships of an intracellular xylanase from *Geobacillus stearothermophilus*. *Acta Crystallogr Sect D* 63:845–859
- Subramanian S, Prema P (2002) Biotechnology of microbial xylanases: enzymology, molecular biology, and application. *Crit Rev Biotechnol* 22:33–64
- Sunna A, Antranikian G (1997) Xylanolytic enzymes from fungi and bacteria. *Crit Rev Biotechnol* 17:39–67
- Sunna A, Bergquist P (2003) A gene encoding a novel extremely thermostable 1, 4- β -xylanase isolated directly from an environmental DNA sample. *Extremophiles* 7:63–70
- Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* 24:1596–1599

- Thomson JA (1993) Molecular biology of xylan degradation. *FEMS Microbiol Rev* 10:65–82
- Wallenstein MD, McMahon S, Schimel J (2007) Bacterial and fungal community structure in Arctic tundra tussock and shrub soils. *FEMS Microbiol Ecol* 59:428–435
- Wood PJ, Erfle J, Teather R (1988) Use of complex formation between Congo Red and polysaccharides in detection and assay of polysaccharide hydrolases. *Methods Enzymol* 160:59–74
- Wouters J, Georis J, Engher D, Vandenhoute J, Dusart J, Frere JM, Depiereux E, Charlier P (2001) Crystallographic analysis of family 11 endo- β -1, 4-xylanase Xyl1 from *Streptomyces* sp. S38. *Acta Crystallogr Sect D* 57:1813–1819
- Xiao X, Yin X, Lin J, Sun L, You Z, Wang P, Wang F (2005) Chitinase genes in lake sediments of Ardley Island, Antarctica. *Appl Environ Microbiol* 71:7904–7909
- Yasir M, Aslam Z, Kim SW, Lee SW, Jeon CO, Chung YR (2009) Bacterial community composition and chitinase gene diversity of vermicompost with antifungal activity. *Bioresour Technol* 100:4396–4403
- Zhang GH, Luo HY, Chen JC, Yao B (2008) Pilot studies on enzyme-producing microflora in frozen soil at the root of *Saussureae involucratae* Kar. et Kir. et Maxim from Tianshan, Xinjiang Autonomous Region. *J Agri Sci Technol* 10:82–87
- Zhou J, Bruns M, Tiedje J (1996) DNA recovery from soils of diverse composition. *Appl Environ Microbiol* 62:316–322