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BIOCATALYSIS

# A novel xylanase with tolerance to ethanol, salt, protease, SDS, heat, and alkali from actinomycete *Lechevalieria* sp. HJ3

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**Abstract** A xylanase-coding gene (*xynAHJ3*, 1,104 bp) was cloned from *Lechevalieria* sp. HJ3 harbored in a saline soil sampled from Heijing town, aka the "town of salt", on the famous "Silk Route of the South". The gene encodes a 367-residue polypeptide (XynAHJ3) with the highest identity of 74.0 % with the endoxylanase from *Streptomyces thermocarboxydus* HY-15. The coding sequence of the mature protein (without the predicted signal peptide from M1 to S22) of *xynAHJ3* was expressed in *Escherichia coli* BL21 (DE3). The activity of the purified recombinant XynAHJ3 (rXynAHJ3) was apparently optimal at 70 °C and pH 6.0, retained greater than 55 % xylanase activity at a concentration of 0.2–2.0 M $Na^+$ and 26 % at 4.0 M $Na^+$ (pH 7.5 20 °C), and showed 110.2 and 44.2 % xylanase activities in the presence of 100 mM SDS (pH 6.0 37 °C) and 10 % ethanol (pH 5.0 37 °C), respectively. rXynAHJ3 activity was stable at 50 °C and pH 4.0–11.0 for more than 60 min, in trypsin or proteinase K at 20 °C for 24 h (pH 7.5), in 10 % ethanol (v/v) (pH 5.0) at 30 or 37 °C for 72 h, in 80 % ethanol (v/v) for 1 h, and in 0.6 or 3 M NaCl (20 °C, pH 7.5) for 72 h. Compared with the majority of xylanases with tolerance to ethanol, salt, SDS, or protease ($K_m$ values of 1.42–15.1 mg $ml^{-1}$), rXynAHJ3 showed a low $K_m$ value (0.8 mg $ml^{-1}$) and showed only limited amino acid sequence identity with those other xylanases (less than 47 %).

**Keywords** Xylanase · Actinomycete · *Lechevalieria* sp.

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## Introduction

Xylan, composed mainly of $\beta$-1,4-linked xylose residues, represents up to one-third of the plant cell wall content—the most common component of renewable biomass on earth [6]. Endoxylanase (EC 3.2.1.8), which catalyzes the random endohydrolysis of $\beta$-1,4-xylosidic linkages, is the crucial member of xylanases widely isolated from various microorganisms. The majority of endoxylanases fall into the glycosyl hydrolase (GH) families 10 and 11 on the basis of amino acid sequence similarities [8].

The important roles of endoxylanases in basic research, the bioconversion of biomass materials, and various potential industrial applications have been widely reviewed [6]. Specific applications of xylanases usually require one or more significant enzyme characteristics such as thermo-alkali tolerance for the textile industry [7], protease resistance for the feed industry [19], activity at low to moderate temperatures for the food industry [6], ethanol tolerance for

improvement of alcohol yield [29], salt tolerance for processing of marine materials [9], and freedom from cellulase activity for the paper and pulp industry [37]. A xylanase possessing all of the above characteristics would have great value for basic research (e.g., engineering enzyme activity by rational design) and versatile industrial applications but has never been reported.

Xylanases obtained from extremophilic sources attract much attention and interest [6]. As an extremophilic source, the saline environment provides novel genetic resources and enzymes, such as unidentified bacteria and salt-tolerant xylanases in saline sediments and marine samples, respectively [9, 35]. In this study, a novel GH 10 endoxylanase gene was cloned from *Lechevalieria* sp. HJ3 harbored in a saline soil. The gene was expressed in *Escherichia coli*, and the purified recombinant enzyme was characterized and showed tolerance to ethanol, salt, protease, SDS, heat, and alkali.

## Materials and methods

### Vectors and reagents

*Escherichia coli* Trans1-T1 (TransGen, Beijing, China) and the pMD 18-T vector (TaKaRa, Otsu, Japan) were used for gene cloning. *E. coli* BL21 (DE3) (TransGen) and the pET-28a(+) vector (Novagen, San Diego, CA, USA) were used for gene expression. Nickel-NTA agarose (Qiagen, Valencia, CA, USA) was used to purify the $His_6$-tagged protein. Genomic DNA isolation, DNA purification, and plasmid isolation kits were purchased from Tiangen (Beijing, China). Restriction endonucleases, T4 DNA ligase, DNA polymerase (*Taq* and Pyrobest), and dNTPs were purchased from TaKaRa. Substrates birchwood xylan, beechwood xylan, oat spelt xylan, barley $\beta$-glucan, pullulan (from *Aureobasidium pullulans*) and carboxymethyl cellulose sodium salt were purchased from Sigma (St. Louis, MO, USA). Isopropyl-$\beta$-D-1-thiogalactopyranoside (IPTG) was purchased from Amresco (Solon, OH, USA). All other chemicals were of analytic grade.

### Microorganism isolation

The saline soil was sampled from an abandoned salt mine located in Heijing town, aka the "town of salt", in Yunnan province, China, a town on the famous "Silk Route of the South". Two grams of soil was suspended in 0.7 % (w/v) NaCl and spread onto screening agar plates containing 0.5 % (w/v) soybean meal, 0.1 % (w/v) peptone, and 0.1 % (w/v) NaCl. The pure culture of strain HJ3 was obtained through repeated streaking on xylanse-inducing medium containing 0.5 % (w/v) birchwood xylan, 0.1 % (w/v) peptone, and 0.1 % (w/v) NaCl, and showed extracellular xylanase activity at 20–80 °C. The taxon of the strain was identified by 16S rDNA sequence PCR-amplified using primers 27F and 1492R [14].

### Gene cloning and sequence analyses

As a result of the low yield (less than 0.1 U $ml^{-1}$) and difficulty of purification of native xylanase from HJ3, the cloning and heterologous expression of the xylanase gene (*xynAHJ3*) was carried out. Genomic DNA from strain HJ3 was extracted using the Tiangen genomic DNA isolation kit following the manufacturer's instructions. The degenerate primer set (Table 1; Xyn10F and Xyn10R), corresponding to conserved G-H-T-L-[V/I/L]-W-H and W-D-V-V-N-E blocks of GH 10 endoxylanase (Fig. 1) was designed and analyzed using Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, CA, USA) and Oligo 6.0 (Molecular Biology Insights, Cascade, CO, USA) software, and synthesized by Invitrogen (Carlsbad, CA, USA). Touchdown-PCR was performed as follows to amplify a partial xylanase gene: 94 °C for 5 min; then 15 touchdown cycles of 94 °C for 30 s, 60 °C for 30 s (decreasing by 1 °C each cycle), and 72 °C for 30 s; followed by 30 cycles of 94 °C for 30 s, 44 °C for 30 s, and 72 °C for 30 s; and one final extension at 72 °C for 5 min. The PCR product was gel purified, ligated to pMD 18-T vector, transformed into *E. coli* Trans1-T1, and sequenced by Beijing Genomics Institute (Guangzhou, China).

**Table 1** Primers used in this study

| Primer name | Primer sequence (5′ → 3′)[a] | $T_m$ (°C)[b] |
|---|---|---|
| Xyn10F | GGYCAYACBCTNRTNTGGCA | 44–60 |
| Xyn10R | YTCRTTNACNACRTCCCA | |
| xynAHJ3uSP1 | CCCACTGCTGGACCTTGCCCTTGTAC | 65 |
| xynAHJ3uSP2 | CCTGCTCCAGCCACTCCGGGTTCT | |
| xynAHJ3dSP1 | AACCCGGAGTGGCTGGAGCAGG | 65 |
| xynAHJ3dSP2 | CCGGTACAAGGGCAAGGTCCAGC | |
| rxynAHJ3EF | CCGGAATTCGTCTCGGCCCCGCCGGACGTGA | 44 |
| rxynAHJ3XR | CCGCTCGAGGGCTCGCTTCGCCAGCGTGGAC | |

[a] IUPAC/IUB symbols are used; restriction sites are underlined

[b] $T_m$, annealing temperature. The $T_m$ values of specific primers (SP) are used for the high-stringency step in GC TAIL-PCR

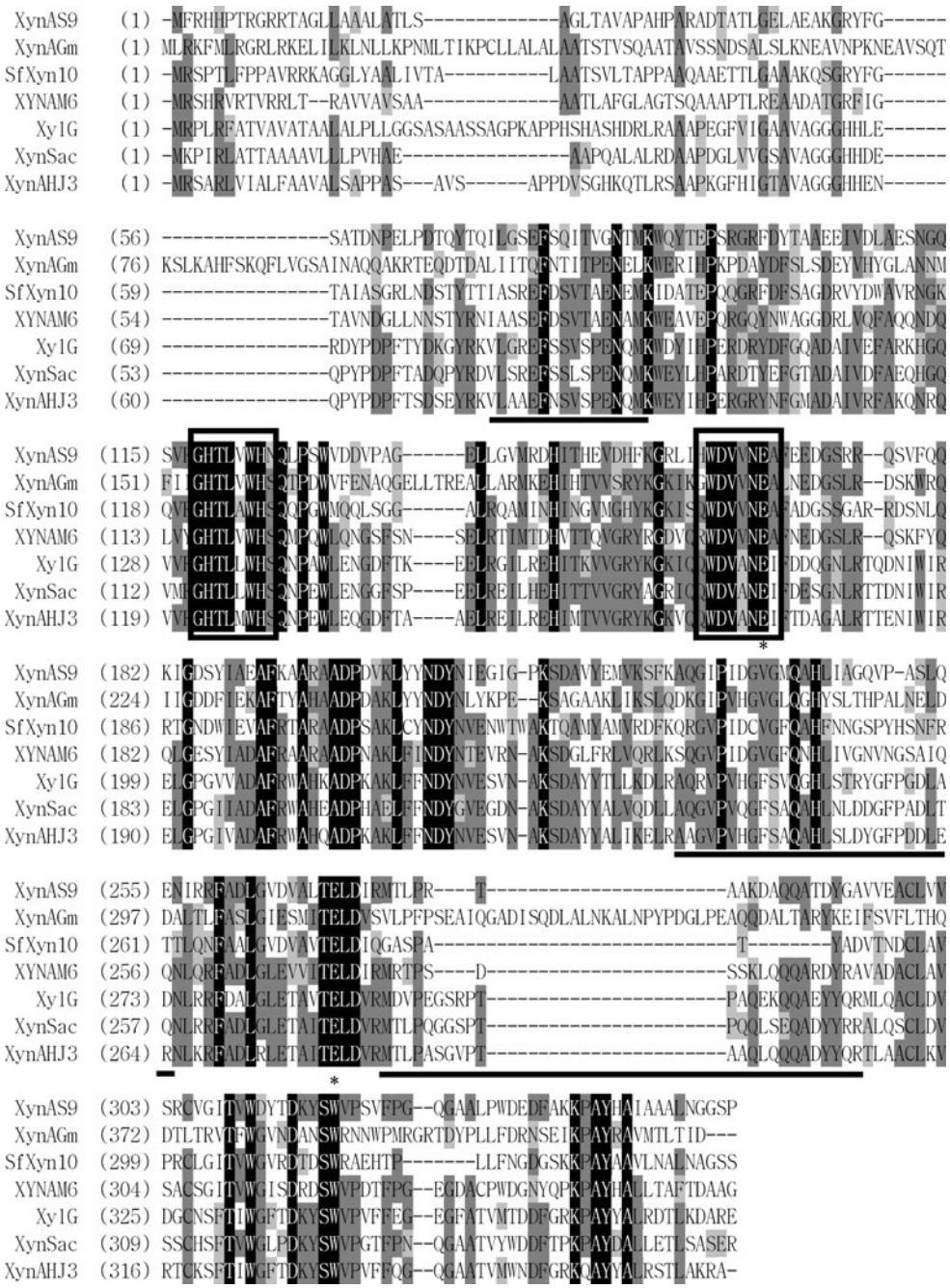


**Fig. 1** Amino acid sequence alignment of XynAHJ3 with GH 10 endoxylanases. Sequences are as follows (including the microbial source and accession number): XynAS9, *Streptomyces* sp. S9 xylanase (ABX71815) tolerant to SDS [16]; XynAGm, *Glaciecola mesophila* KMM 241 xylanase (ACN76857) tolerant to salt [9]; SfXyn10, *Streptomyces fradiae* var. k11 xylanase (ABR14270) tolerant to proteases [17]; XYNAM6, *Streptomyces megasporus* DSM 41476 xylanase (ADE37527) tolerant to proteases and used in the brewing industry [27]; XylG, *Streptomyces thermocarboxydus* HY-15 xylanase (ACJ64840) [13] most identical to XynAHJ3; XynSac, *Saccharopolyspora* sp. S582 xylanase (ADL60499) [30]. Identical residues are shaded in *black* and conserved residues are shaded in *gray*. The *boxes* indicate blocks used for designing degenerate primers. The *asterisks* show the putative catalytic residues. The internal peptides identified by MALDI–TOF/MS are underlined with *black bars*

The full-length gene *xynAHJ3* based on the partial endoxylanase gene was obtained using a time-saving and reduced-cost GC TAIL-PCR procedure (thermal asymmetric interlaced-PCR specific for GC-rich genes) [36, 38] with 4 nested insertion-specific primers (SP) (Table 1). The annealing temperatures used for high stringency are shown in Table 1. PCR products of the expected size appeared between the second and third rounds of amplification were gel purified and directly sequenced by Beijing Genomics Institute.

Multiple sequences were assembled and aligned using Vector NTI 10.3 software (InforMax, Gaithersburg, MD, USA). The signal peptide in the amino acid sequence (XynAHJ3) deduced from *xynAHJ3* was predicted using SignalP [3]. The identity values of DNA or protein sequences were calculated with BLASTN and BLASTP [1], respectively. The classification of the glycosyl hydrolase family of XynAHJ3 was determined with the InterProScan online tool [28].

### Expression of *xynAHJ3* in *E. coli*

The coding sequence of the mature protein (without the predicted signal peptide) of *xynAHJ3* was amplified by PCR using primers rxynAHJ3EF and rxynAHJ3XR (Table 1) including added *Eco*RI and *Xho*I sites at the 5′ terminal, respectively. The PCR product and pET-28a(+) vector were individually digested with *Eco*RI and *Xho*I and gel purified. The purified products were ligated and transformed into *E. coli* BL21 (DE3) competent cells. The transformant harboring the recombinant plasmid (pET-*xynAHJ3*) was identified by PCR analysis and further confirmed by DNA sequencing. After growing overnight at 37 °C in LB medium supplemented with 100 μg $ml^{-1}$ kanamycin, the culture of the positive transformant was inoculated at a 1:100 dilution into fresh LB medium containing kanamycin and induced with a final concentration of 0.7 mM IPTG (at 20 °C for 20 h) when the value of $A_{600}$ was approximately 0.7.

### Purification and identification of recombinant xylanase

To purify the recombinant XynAHJ3 (rXynAHJ3; $His_6$-tagged), cells were harvested by centrifugation at 10,000×*g* for 10 min at 20 °C, washed with sterile distilled $H_2O$, and resuspended in sterilized ice-cold buffer A (20 mM Tris–HCl, 0.5 M NaCl, pH 7.2). The cells were disrupted by sonication (7 s, 150 W) on ice and then centrifuged at 10,000×*g* for 10 min at 20 °C. Purification of the enzyme from the supernatant was performed with a $Ni^{2+}$-NTA agarose gel column with a linear imidazole gradient of 20–500 mM in buffer A.

The purified protein was detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; 12 % running gel) and verified using matrix-assisted laser desorption/ionization time-of-flight/mass spectrometry (MALDI–TOF/MS) performed by Tianjin Biochip (Tianjin, China). The Bradford method [5] was employed to determine protein concentration, using bovine serum albumin as standard.

### Enzyme assay

Xylanase activity was determined by measuring the release of reducing sugar from substrate using the 3,5-dinitrosalicylic acid (DNS) reagent. The standard reaction contained 0.1 ml of appropriately diluted enzyme and 0.9 ml of McIlvaine buffer (pH 6.0) containing 0.5 % (w/v) birchwood xylan as substrate. After incubation at 37 °C for 10 min, the reaction was stopped with 1.5 ml DNS reagent and boiled for 5 min. The absorption at 540 nm was measured when the aforementioned mixture had cooled to room temperature. One unit (U) 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 was assayed by following this standard procedure unless otherwise noted.

### Biochemical characterization

To identify the activity of purified rXynAHJ3, various substrates including 0.5 % (w/v) oat spelt xylan, beechwood xylan, birchwood xylan, barley β-glucan, pullulan (from *A. pullulans*) or carboxymethyl cellulose sodium salt (determined at pH 6.0 and 70 °C), or 1.0 % (w/v) cassava meal, corn bran, wheat bran, soybean meal, cottonseed meal or rapeseed meal (determined at pH 6.0 and 37 °C) were individually added to the reaction solution.

Further characterization of the purified rXynAHJ3 activity was determined using birchwood xylan as substrate. The optimal pH for xylanase activity of purified rXynAHJ3 was determined at 37 °C in buffers with pH ranging from 4.0 to 12.0. The buffers used were McIlvaine buffer for pH 4.0–8.0, 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 for purified rXynAHJ3 activity was determined over the range of 20–90 °C in McIlvaine buffer (pH 6.0). To investigate the effects of different metal ions and chemical reagents on the purified rXynAHJ3 (0.002 mg) activity measured in McIlvaine buffer (pH 6.0) at 37 °C, 10 mM (final concentration) KCl, $CaCl_2$, $CoCl_2$, $NiSO_4$, $CuSO_4$, $MgSO_4$, $FeSO_4$, $FeCl_3$, $MnSO_4$, $ZnSO_4$, $Pb(CH_3COO)_2$, $HgCl_2$, EDTA or β-mercaptoethanol, or 10 or 100 mM SDS, or 1 or 10 % ethanol (v/v; pH 5.0) was individually added to the reaction solution. The effect of 0.2–4.5 M NaCl on the purified rXynAHJ3 (0.002 mg) was also determined at pH 7.5 and 20 °C.

The stability of purified rXynAHJ3 at different pH values was estimated by measuring the residual enzyme activity after incubating the enzyme (0.02 mg) solution in various buffers at 37 °C for 1 h, again using birchwood xylan as substrate. The enzyme thermostability was determined after preincubation of the enzyme (0.02 mg) in McIlvaine buffer (pH 6.0) at 50, 60, or 70 °C without substrate for various periods. To examine resistance to ethanol, salt, and different proteases, purified rXynAHJ3 (0.02 mg) was incubated at 20 °C for 24 h with 0.2 mg trypsin (pH 7.5) or proteinase K (pH 7.5), or at 30 or 37 °C for 72 h with 10 % ethanol (v/v) (pH 5.0), or at 37 °C for 1 h with 80 or 95 % ethanol (v/v), or at 20 °C for 72 h with 0.6 or 3 M NaCl (pH 7.5), and the residual enzyme activity was measured in buffer and at temperature corresponding to treated condition.

$K_m$, $V_{max}$, and $k_{cat}$ values for purified rXynAHJ3 were determined using 0.5–10 mg $ml^{-1}$ birchwood xylan as the substrate in McIlvaine buffer (pH 6.0) at 70 °C. The data were plotted according to the Lineweaver–Burk method [18].

### Nucleotide sequence accession numbers

The nucleotide sequences for the *Lechevalieria* sp. HJ3 16S rDNA and xylanase gene (*xynAHJ3*) were deposited in GenBank under the accession numbers JF745866 and JF745868, respectively.

## Results

### Strain identification

The comparison of the partial 16S rDNA sequence from strain HJ3 (683 bp; JF745866) with that in GenBank showed a nucleotide identity of 99.9 % with *Lechevalieria xinjiangensis* R24 (accession no. DQ898283), 99.1 % with *Lechevalieria aerocolonigenes* HBUM 174553 (EU841595), and 98.4 % with *L. aerocolonigenes* ISP 5034 (AB020030). Thus, strain HJ3 was classified into the genus *Lechevalieria* and deposited in the China General Microbiological Culture Collection Center under CGMCC 4.6960. The distance tree created by the neighbor-joining method also revealed the same classification (data not shown).

### Gene cloning and sequence analysis

A fragment of *xynAHJ3* (153 bp) was amplified by PCR using the degenerate primers Xyn10F and Xyn10R. DNA fragments amplified by GC TAIL-PCR were assembled with the *xynAHJ3* fragment. As a result, the full-length *xynAHJ3* (JF745868), which is 1,104 bp, starts with the putative codon ATG, ends with TGA, and encodes a 367-residue polypeptide (XynAHJ3) with a calculated mass of 41.2 kDa.

The signal peptide was predicted from M1 to S22, followed by a catalytic domain of GH 10 from T36 to L363 in XynAHJ3 on the basis of sequence analysis. The amino acid sequence of XynAHJ3 showed the highest identities with the identified GH 10 endoxylanases from actinomycetes, as follows: 74.0 % identity with XylG from *Streptomyces thermocarboxydus* HY-15 (ACJ64840) [13], 71.3 % with XynSac from *Saccharopolyspora* sp. S582 (ADL60499) (Fig. 1) [30], 60.0 % with Kxyn from *Kocuria* sp. MN22 (ACJ73932) [15], 49.6 % with XynA119 from *Streptomyces* sp. TN119 (ACR61563) [36], 47.2 % with $XylA_{CspHY\text{-}12}$ from *Cellulosimicrobium* sp. HY-12 (ABX88978) [25], and 46.1 % with XylA from *Thermomonospora alba* ULJB1 (CAB02654) [4]. Furthermore, XynAHJ3 showed 46.2 % identity with the GH 10 endoxylanase XYNAM6 from *Streptomyces megasporus* DSM 41476 (ADE37527) [27], 44.0 % with the GH 10 endoxylanase XynAS9 from *Streptomyces* sp. S9 (ABX71815) [16], 34.0 % with the GH 10 endoxylanase SfXyn10 from *Streptomyces fradiae* var. k11 (ABR14270) [17], and 30.8 % with the GH 10 endoxylanase XynAGm from *Glaciecola mesophila* KMM 241 (ACN76857) (Fig. 1; Table 2) [9]. The conserved residues of GH 10 endoxylanases and putative active sites (E172 and E280) in XynAHJ3 are shaded in Fig. 1.

### Expression and purification of rXynAHJ3

The gene *xynAHJ3* was expressed in *E. coli* BL21 (DE3) and the crude enzyme extracted from *E. coli* BL21 (DE3)

**Table 2** Kinetic parameters of xylanases with specific characterization

| Xylanase | $K_m$ (mg ml$^{-1}$)[a] | $V_{max}$ (μmol min$^{-1}$ mg$^{-1}$)[a] | $k_{cat}$ ($s^{-1}$)[a] | Specific characterization | References |
|---|---|---|---|---|---|
| rXynAHJ3 | 0.8 | 400.0 | 292.6 | Tolerance to SDS, protease, ethanol, and salt | This study |
| Xyn | NR | NR | NR | Ethanol production | [22] |
| XynG2 | NR | NR | NR | Tolerance to ethanol | [29] |
| XynAM6 | 2.33 | 406.93 | NR | Mashing, and tolerance to proteases | [27] |
| SfXyn10 | 0.41 | 131.58 | 103.40 | Tolerance to proteases | [17] |
| Xyl11B | NR | NR | NR | Tolerance to proteases | [19] |
| XynB119[b] | 15.1 | 441.4 | 251.0 | Tolerance to proteases and SDS | [38] |
| XynAGm | 1.42 | 86.70 | 61 | Tolerance to salt | [9] |
| XynA-NTOU1 | NR | NR | NR | Tolerance to salt | [10] |
| XynFCB | 13 | 278 | 231 | Tolerance to salt | [11] |
| Xyn-GESF1 | 5.3 | 6,593.4 | NR | Tolerance to salt | [21] |
| XynAS9 | 2.43 | 490.87 | 376.33 | Tolerance to SDS | [16] |

*NR* not reported

[a] Values were determined using birchwood xylan as the substrate

[b] Data were obtained using oat spelt xylan as the substrate

cells showed 20.3 U $ml^{-1}$ xylanase activity, which is more than 200-fold higher than that of native xylanase from HJ3. The crude enzyme was purified to electrophoretic homogeneity by $Ni^{2+}$-NTA metal chelating affinity chromatography (Fig. 2), with a final purification of 196.5-fold and yield of 15.2 % (Table 3). The purified enzyme migrated as a single band on SDS-PAGE with a molecular mass of ca. 46.0 kDa, which is close to the calculated value of rXynAHJ3 (43.9 kDa). Three internal peptides from the purified enzyme (Figs. 1, 3), VLAAEFNSVSPENQMK, AAGVPVHGFSAQAHLSLDYGFPDDLER, and MTLPA SGVPTAAQLQQQADYYQR that were randomly selected from the results of MALDI–TOF/MS, matched the deduced amino acid sequence of rXynAHJ3, confirming that the purified enzyme was indeed rXynAHJ3.

## Enzyme characterization

At pH 6.0 and 70 °C, the specific activity of purified rXynAHJ3 towards substrates of 0.5 % (w/v) oat spelt xylan, beechwood xylan and birchwood xylan was 346.6, 328.1 and 287.3 U $mg^{-1}$, respectively, and at pH 6.0 and 37 °C, the specific activity of the purified xylanase towards substrates of 1.0 % (w/v) cassava meal, corn bran, wheat bran, soybean meal, cottonseed meal, and rapeseed meal was 0.6, 0.5, ca. 0.1, less than 0.1, less than 0.1, and less than 0.1 U $mg^{-1}$, respectively. However, no activity of rXynAHJ3 was detected towards substrates of 0.5 % (w/v) barley $\beta$-glucan, pullulan (from *A. pullulans*), and carboxymethyl cellulose sodium salt.

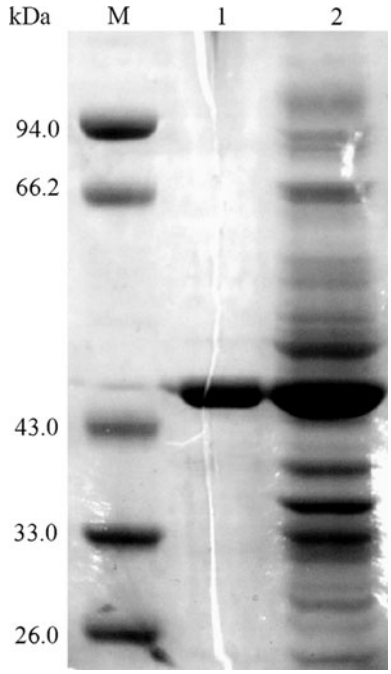


**Fig. 2** SDS-PAGE analyses of rXynAHJ3. *Lane M* low molecular weight markers; *lane 1* rXynAHJ3 purified by $Ni^{2+}$-NTA chelating affinity chromatography; *lane 2* cell extract of an induced transformant harboring pET-*xynAHJ3*

When assayed at 37 °C, purified rXynAHJ3 showed apparently optimal xylanase activity at pH 6.0, and retained greater than 60 % of the maximum activity between pH 5.5 and 8.0, and greater than 25 % between pH 9.0 and 12.0 (Fig. 4a). The thermal activity of purified rXynAHJ3 was apparently optimal at 70 °C when assayed at pH 6.0, and retained greater than 60 % of the maximum activity when assayed at 50–75 °C, 11 % at 20 °C, and 24 % at 80 °C (Fig. 4b). The activity of purified rXynAHJ3 was completely inhibited by 10 mM $Hg^{2+}$ and $Ag^{+}$, strongly inhibited (retaining less than 50 % activity) by 10 mM $Cu^{2+}$, $Zn^{2+}$, $Mn^{2+}$, $Fe^{3+}$ and $Fe^{2+}$, and 10 % (v/v) ethanol, and partial inhibited (retaining greater than 50 % activity) by 10 mM $Co^{2+}$, $Pb^{2+}$, $Ni^{2+}$, and $K^{+}$ (Table 4). Purified rXynAHJ3 retained greater than 55 % xylanase activity at concentrations of 0.2–2.0 M $Na^{+}$, and 26 % at 4.0 M $Na^{+}$ (Fig. 4c). Addition of other reagents, including 100 mM SDS, had little or no effect on the enzyme activity.

Purified rXynAHJ3 exhibited more than 90 % of the initial activity after incubation in buffers ranging from pH 4.0 to 11.0 at 37 °C for 1 h (Fig. 5a). The enzyme was stable at 50 °C for more than 60 min, whereas at 60 and 70 °C, the half-life of the enzyme was ca. 60 and ca. 4 min, respectively (Fig. 5b). Purified rXynAHJ3 was resistant to trypsin and proteinase K, retaining more than 97 % of the initial activity after incubation at 20 °C for 24 h (pH 7.5) (Fig. 5c). At 30 and 37 °C for 72 h with 10 % ethanol (v/v) (pH 5.0), the enzyme showed greater than 94 and greater than 50 % activity, respectively (Fig. 5d). In 80 % and 95 % ethanol (v/v) for 1 h, rXynAHJ3 retained ca. 98 and ca. 35 % of its activity, respectively. The stability of purified rXynAHJ3 at 0.6 M or 3 M NaCl (20 °C, pH 7.5) was estimated, showing more than 90 % xylanase activity after incubation for 72 h (Fig. 5e).

**Table 3** Purification of rXynAHJ3 (volume 3 l) expressed in *E. coli*

| Purification step | Total activity (U)[a] | Total protein (mg) | Specific activity (U $mg^{-1}$)[a] | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|
| Crude enzyme extracted from cells | 60,815.0 | 41,594.7 | 1.5 | 1 | 100 |
| $Ni^{2+}$-NTA chromatography | 9,243.9 | 32.2 | 287.3 | 196.5 | 15.2 |

[a] Xylanase activity was determined using 0.5 % (w/v) birchwood xylan as substrate in McIlvaine buffer (pH 6.0) at 70 °C

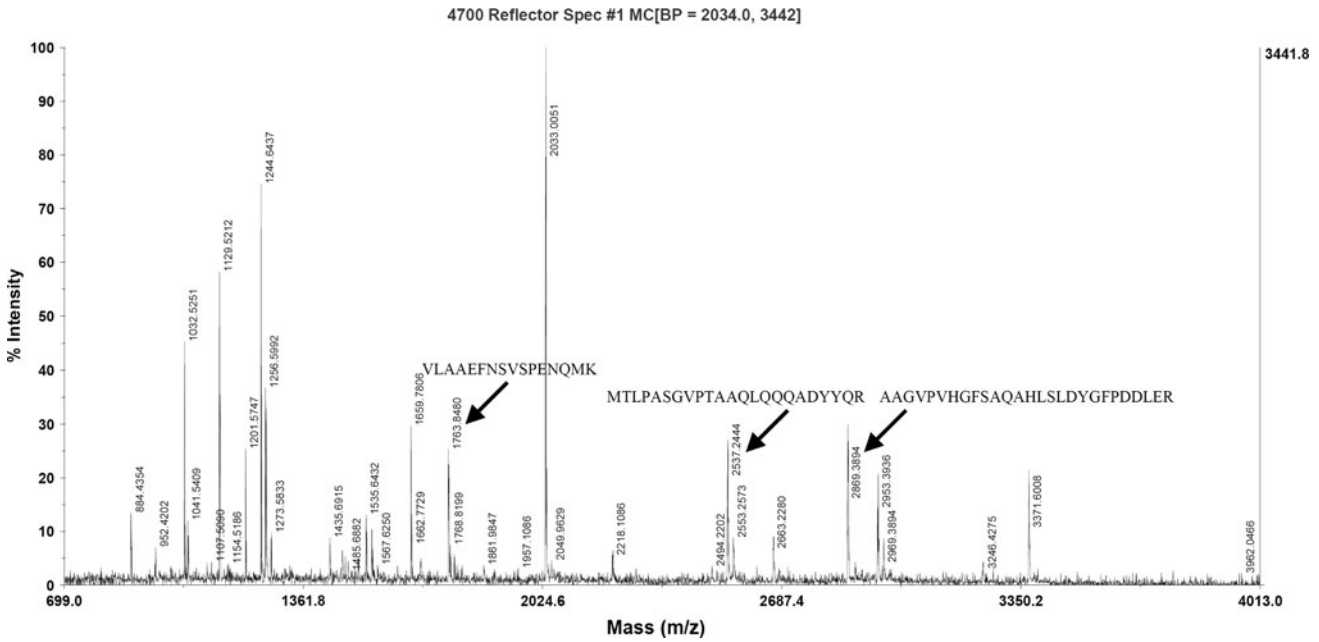


**Fig. 3** Identification of the purified rXynAHJ3 using MALDI–TOF/MS. *Arrows* indicate peaks of interest

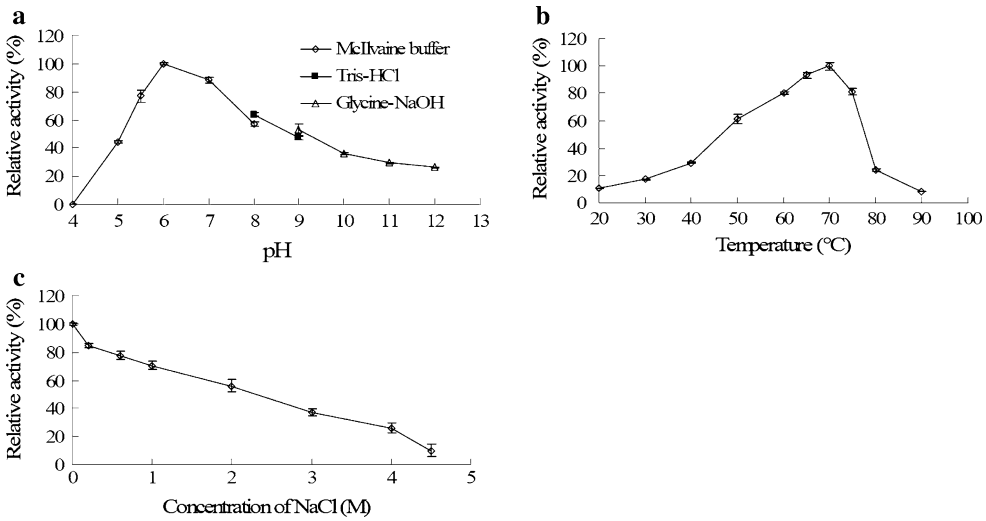


**Fig. 4** Activity of purified rXynAHJ3. **a** Effect of pH on xylanase activity. The enzyme activity was determined at 37 °C from pH 4.0 to 12.0. **b** Effect of temperature on rXynAHJ3 activity measured in McIlvaine buffer (pH 6.0) at 20–90 °C. **c** Effect of NaCl on rXynAHJ3 activity (pH 7.5, 20 °C). The *error bars* represent the means ± SD ($n = 3$)

Based on a Lineweaver–Burk plot, the $K_m$, $V_{max}$, and $k_{cat}$ values were 0.8 mg ml$^{-1}$, 400.0 μmol min$^{-1}$ mg$^{-1}$, and 292.6 s$^{-1}$, respectively, using birchwood xylan as the substrate (Fig. 6; Table 2).

## Discussion

Glycosyl hydrolases from actinomycetes have valuable applications in various industries [2, 16, 17, 27, 38]. To date, only the taxonomic information for *Lechevalieria* [26, 33] and genes involved in the biosynthesis of an antibiotic (rebeccamycin, accession no. AB071405; halogenase, FJ502204) from *L. aerocolonigenes* were reported. This study of xylanase XynAHJ3 is the first to present the cloning, heterologous expression, and biochemical characterization of a glycosyl hydrolase from a *Lechevalieria* sp.

Sequence analysis showed that XynAHJ3 was most similar to identified GH 10 xylanases from actinomycetes. The apparent optimal pH and temperature of rXynAHJ3 were also most similar to those of GH 10 xylanases from actinomycetes (most optimum at pH 6–8 and 60–70 °C) [2, 4, 13, 15–17, 25, 27, 30, 36]. However, to our knowledge, only a few GH 10 xylanases from actinomycetes showed activity at pH 11.0–12.0 [15, 27]. The thermo-alkali activity and stability (especially at pH 11.0) and freedom from cellulase activity suggested that rXynAHJ3 may have potential in the textile and paper and pulp industries [6, 7, 37].

**Table 4** Effect of metal ions and chemical reagents on the xylanase activity of purified rXynAHJ3 (0.002 mg)

| Reagent | Final concentration (mM) | Relative activity (%)[a] |
|---|---|---|
| None | 0 | 100.0 ± 2.8 |
| $Mg^{2+}$ | 10 | 98.2 ± 0.7 |
| $Ca^{2+}$ | 10 | 95.7 ± 5.0 |
| $K^{+}$ | 10 | 89.9 ± 2.7 |
| $Ni^{2+}$ | 10 | 87.8 ± 1.6 |
| $Pb^{2+}$ | 10 | 60.6 ± 17.2 |
| $Co^{2+}$ | 10 | 52.5 ± 4.4 |
| $Fe^{2+}$ | 10 | 44.4 ± 0.5 |
| $Fe^{3+}$ | 10 | 41.2 ± 1.3 |
| $Mn^{2+}$ | 10 | 39.3 ± 0.6 |
| $Zn^{2+}$ | 10 | 39.1 ± 0.8 |
| $Cu^{2+}$ | 10 | 30.6 ± 1.8 |
| $Ag^{+}$ | 10 | 0.0 |
| $Hg^{2+}$ | 10 | 0.0 |
| $\beta$-Mercaptoethanol | 10 | 102.3 ± 1.0 |
| EDTA | 10 | 89.9 ± 5.1 |
| SDS | 10 | 110.2 ± 1.8 |
| SDS | 100 | 102.3 ± 1.5 |
| Ethanol | 1 % (v/v) | 101.3 ± 4.3 |
| Ethanol | 10 % (v/v) | 44.2 ± 2.7 |

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

The utilization of xylanase could shorten the overall process time for ethanol production [22], improve sake mash fermentations [29], and reduce the filtration rate and viscosity under simulated brewery mashing [27] (Table 2). However, reports on the influence of ethanol on xylanase activity or stability were extremely limited [29]. The xylanase XynG2 from *Aspergillus oryzae* retained 80 % of its original activity after incubation in 80 % ethanol for 30 min [29]. The high stability of this xylanase in ethanol was proposed to be the primary effect in the improvement of material utilization under sake mash conditions [29]. Compared to XynG2, rXynAHJ3 had better stability in 80 % ethanol. Moreover, rXynAHJ3 was stable below 60 °C, active in 10 % ethanol (v/v) (pH 5.0), and stable in 10 % ethanol (v/v) (pH 5.0) at 30 or 37 °C for 72 h, the conditions for fermentation of materials by *Saccharomyces cerevisiae*. In view of these characteristics, rXynAHJ3 might be a new candidate for use directly as a xylanase additive, or as a heterologously expressed xylanase in yeast, for application in the fermentation processes of the brewing industry and ethanol production.

Salt-tolerant xylanases isolated from microorganisms harbored in marine or saline environments have potential uses in the processing of marine materials, which is a new, but seldom reported application of xylanases [9–11, 21, 34] (Table 2). The average sea salinity is 3.5 % (w/v). At 3.5 % (w/v) final concentration of NaCl (ca. 0.6 M; average sea salinity simply equivalent to NaCl % here), salt-tolerant xylanases XynAGm from marine *G. mesophila* KMM 241 [9] and Xyn-GESF1 from marine *Bacillus pumilus* GESF-1 [21] showed specific activities of less than 180 U $mg^{-1}$ towards various xylans, whereas xylanase XynFCB from *Thermoanaerobacterium saccharolyticum* NTOU1 [11] retained ca. 400 U $mg^{-1}$ towards birchwood xylan and ca. 180 U $mg^{-1}$ towards oat spelt xylan at a 0.5 M final concentration of NaCl (putatively ca. 330 U $mg^{-1}$ towards birchwood xylan and ca. 140 U $mg^{-1}$ towards oat spelt xylan at 0.6 M NaCl). rXynAHJ3, having 223 and 269 U $mg^{-1}$ xylanase activities towards birchwood xylan and oat spelt xylan in the presence of 0.6 M NaCl, respectively, shared much lower (less than 33 %) amino acid sequence identities with these salt-tolerant xylanases. Furthermore, The $K_m$ value of rXynAHJ3 is lower than that reported for salt-tolerant xylanases (Table 2); therefore, rXynAHJ3 is a novel salt-tolerant xylanase with potential application in the processing of sea food and saline food.

Only a few reported xylanases have strong protease resistance (i.e., retained ca. 100 % activity after incubation with the neutral proteases trypsin and proteinase K) [17, 19, 27, 38] (Table 2), and these shared less than 50 % amino acid sequence identities with XynAHJ3. The $K_m$ value of xylanase XynAM6 was 2.33 mg $ml^{-1}$ [27], whereas that of rXynAHJ3 was 0.8 mg $ml^{-1}$. The xylanase SfXyn10 had a $V_{max}$ value of 131.58 μmol $min^{-1}$ $mg^{-1}$ [17], far lower than the 400.0 μmol $min^{-1}$ $mg^{-1}$ of rXynAHJ3. The protease resistance and low- to moderate-temperature (body temperature of domestic animals and fish) activity suggested rXynAHJ3 may be a new candidate for feed supplements.

SDS is an anionic detergent and causes strong denaturation of proteins when the concentration is above ca. 7 mM in water [20]. In the presence of 10 mM SDS, only the reported GH 10 xylanase XynAS9 [16] and GH 11 xylanase XynB119 from *Streptomyces* sp. TN119 [38] exhibited ca. 100 % xylanase activity (Table 2). rXynAHJ3 is a novel SDS-resistant xylanase, showing significant resistance to even 100 mM SDS, and has a low $K_m$ value and only limited amino acid sequence identities with XynAS9 and XynB119.

SDS and protease resistances have been proposed as common properties of kinetically stable proteins, which owe their unusually high unfolding barrier to the limited access to partially and globally unfolded conformations [12] or high structural rigidity [20]. Accordingly, rXynAHJ3 is probably a kinetically stable protein. The physical basis for kinetic stability has been suggested to be related to the increasing numbers of hydrophobic residues

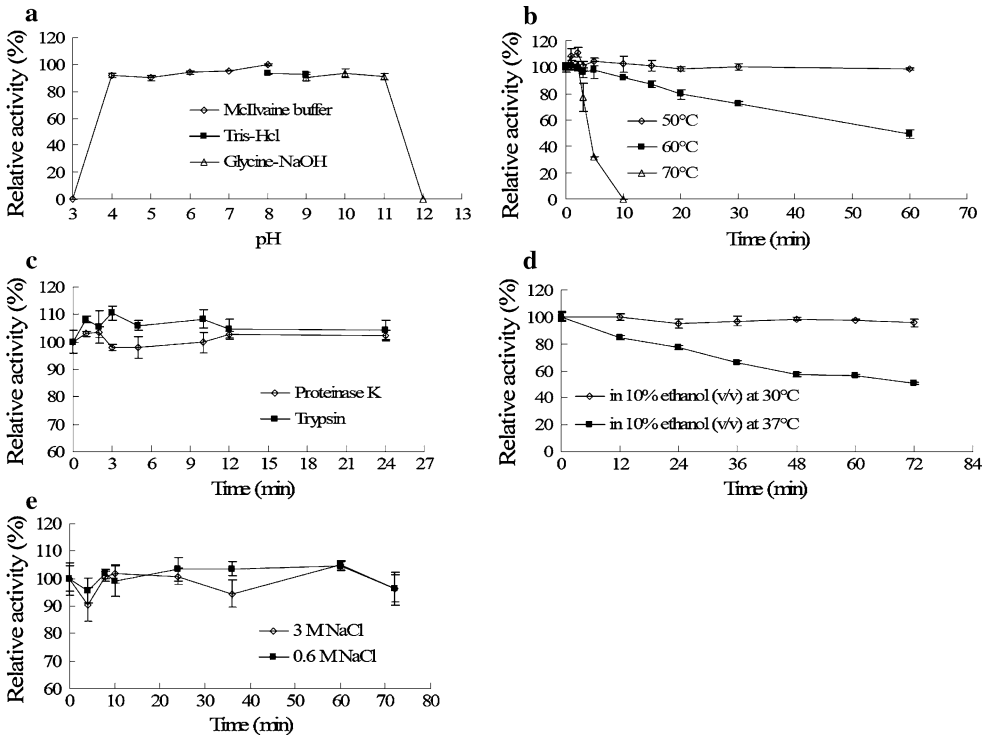


**Fig. 5** Stability of purified rXynAHJ3. **a** pH stability assay. After preincubation of the enzyme (0.02 mg) at pH 3.0–12.0 at 37 °C for 60 min, the enzyme activity was determined in McIlvaine buffer (pH 6.0) at 37 °C. **b** Thermostability assay. Purified rXynAHJ3 (0.02 mg) was preincubated in McIlvaine buffer (pH 6.0) at 50, 60, or 70 °C. Aliquots were removed at specific time points for the measurement of residual activity at 37 °C. **c** Protease resistance. Purified rXynAHJ3 (0.02 mg) was incubated at 20 °C for 24 h with 0.2 mg trypsin (pH 7.5) or proteinase K (pH 7.5), and the residual enzyme activity was determined in Tris–HCl buffer (pH 7.5) at 20 °C. **d** Stability in ethanol. The enzyme (0.02 mg) was incubated at 30 or 37 °C for 72 h with 10 % ethanol (v/v) (pH 5.0), and the residual enzyme activity was determined in McIlvaine buffer (pH 5.0) at 30 °C. **e** Stability in NaCl. The enzyme (0.02 mg) was incubated at 20 °C for 72 h with 0.6 M or 3 M NaCl (pH 7.5), and the residual enzyme activity was determined in Tris–HCl buffer (pH 7.5) at 20 °C. The *error bars* represent the means ± SD ($n = 3$)

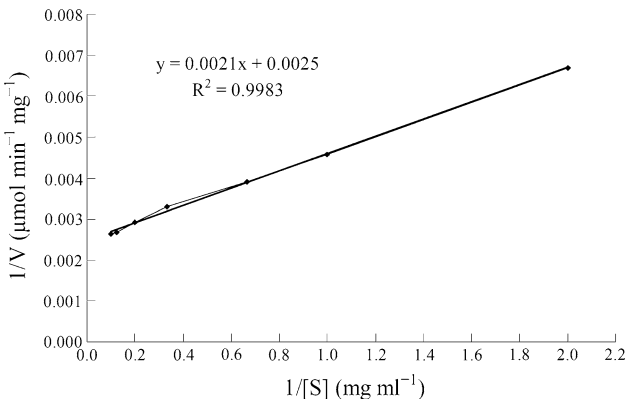


**Fig. 6** Lineweaver–Burk plot of purified rXynAHJ3 determined using 0.5–10 mg $ml^{-1}$ birchwood xylan as the substrate in McIlvaine buffer (pH 6.0) at 70 °C

on the protein surface, ion pairs, disulfide bonds, the protein pro-region, the presence of predominantly $\beta$ sheet, and other properties [20]. More hydrophobic amino acids engineered on the protein surface resulted in the stabilization of the enzyme in organic solvent [24]. Therefore, the kinetically stable nature of rXynAHJ3 might also account for the ethanol tolerance. However, the surfaces of halophilic proteins are characterized by a high occurrence of amino acids with low hydrophobicity [32]. Owing to the low identities of XynAHJ3 with SDS-, protease-, ethanol-, or salt-tolerant xylanases (Fig. 1), mutagenesis targeting these specific conserved residues or regions distinct from other xylanases and crystallization analysis of rXynAHJ3 might be useful for clarifying these assumptions.

In addition, the xylanase rXynAHJ3 was active towards various substrates of cheap agricultural by-products, especially cassava meal, which is the third largest source of calories in the world [23]. Addition of xylanase and cellulase was an efficient treatment for linamarase release or detoxification of cassava products during processing, but has rarely been reported [31].

In this study, a GH 10 xylanase XynAHJ3 from *Lechevalieria* sp. HJ3 harbored in a saline soil was cloned and expressed in *E. coli*. The purified recombinant XynAHJ3 showed excellent enzyme characteristics that have never been reported before—combined tolerance to ethanol, salt, protease, SDS, heat, and alkali. Thus rXynAHJ3

might represent a good material to study the relationship between structure and versatile functions and an alternative to various potential industrial applications.

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## References

1. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215(3):403–410. doi:10.1006/jmbi.1990.9999
2. Beg QK, Kapoor M, Mahajan L, Hoondal GS (2001) Microbial xylanases and their industrial applications: a review. Appl Microbiol Biotechnol 56(3):326–338. doi:10.1007/s002530100704
3. Bendtsen JD, Nielsen H, von Heijne G, Brunak S (2004) Improved prediction of signal peptides: SignalP 3.0. J Mol Biol 340(4):783–795. doi:10.1016/j.jmb.2004.05.028
4. Blanco J, Coque JJR, Velasco J, Martin JF (1997) Cloning, expression in *Streptomyces lividans* and biochemical characterization of a thermostable endo-$\beta$-1,4-xylanase of *Thermomonospora alba* ULJB1 with cellulose-binding ability. Appl Microbiol Biotechnol 48(2):208–217
5. 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(1–2):248–254. doi:10.1016/0003-2697(76)90527-3
6. Collins T, Gerday C, Feller G (2005) Xylanases, xylanase families and extremophilic xylanases. FEMS Microbiol Rev 29(1):3–23. doi:10.1016/j.femsre.2004.06.005
7. Dhiman SS, Sharma J, Battan B (2008) Pretreatment processing of fabrics by alkalothermophilic xylanase from *Bacillus stearothermophilus* SDX. Enzyme Microb Technol 43(3):262–269. doi:10.1016/j.enzmictec.2008.03.016
8. Finn RD, Tate J, Mistry J, Coggill PC, Sammut SJ, Hotz HR, Ceric G, Forslund K, Eddy SR, Sonnhammer ELL, Bateman A (2008) The Pfam protein families database. Nucleic Acids Res 36:D281–D288. doi:10.1093/nar/gkm960
9. Guo B, Chen XL, Sun CY, Zhou BC, Zhang YZ (2009) Gene cloning, expression and characterization of a new cold-active and salt-tolerant endo-$\beta$-1,4-xylanase from marine *Glaciecola mesophila* KMM 241. Appl Microbiol Biotechnol 84(6):1107–1115. doi:10.1007/s00253-009-2056-y
10. Hung KS, Liu SM, Fang TY, Tzou WS, Lin FP, Sun KH, Tang SJ (2011) Characterization of a salt-tolerant xylanase from *Thermoanaerobacterium saccharolyticum* NTOU1. Biotechnol Lett 33(7):1441–1447. doi:10.1007/s10529-011-0579-7
11. Hung KS, Liu SM, Tzou WS, Lin FP, Pan CL, Fang TY, Sun KH, Tang SJ (2011) Characterization of a novel GH10 thermostable, halophilic xylanase from the marine bacterium *Thermoanaerobacterium saccharolyticum* NTOU1. Process Biochem 46(6):1257–1263. doi:10.1016/j.procbio.2011.02.009
12. Jaswal SS, Sohl JL, Davis JH, Agard DA (2002) Energetic landscape of $\alpha$-lytic protease optimizes longevity through kinetic stability. Nature 415(6869):343–346. doi:10.1038/415343a
13. Kim DY, Han MK, Oh HW, Park DS, Kim SJ, Lee SG, Shin DH, Son KH, Bae KS, Park HY (2010) Catalytic properties of a GH10 endo-$\beta$-1,4-xylanase from *Streptomyces thermocarboxydus* HY-15 isolated from the gut of *Eisenia fetida*. J Mol Catal B Enzym 62(1):32–39. doi:10.1016/j.molcatb.2009.08.015
14. Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. Wiley, New York, pp 115–175
15. Li CJ, Hong YZ, Shao ZZ, Lin L, Huang XL, Liu PF, Wu GB, Meng X, Liu ZD (2009) Novel alkali-stable, cellulase-free xylanase from deep-sea *Kocuria* sp. Mn22. J Microbiol Biotechnol 19(9):873–880. doi:10.4014/jmb.0812.689
16. Li N, Meng K, Wang YR, Shi PJ, Luo HY, Bai YG, Yang PL, Yao B (2008) Cloning, expression, and characterization of a new xylanase with broad temperature adaptability from *Streptomyces* sp. S9. Appl Microbiol Biotechnol 80 (2):231–240. doi:10.1007/s00253-008-1533-z
17. Li N, Yang PL, Wang Y, Luo HY, Meng K, Wu NF, Fan YL, Yao B (2008) Cloning, expression, and characterization of protease-resistant xylanase from *Streptomyces fradiae* var. k11. J Microbiol Biotechnol 18(3):410–416
18. Lineweaver H, Burk D (1934) The determination of enzyme dissociation constants. J Am Chem Soc 56(3):658–666. doi:10.1021/ja01318a036
19. Luo HY, Wang Y, Li J, Wang H, Yang J, Yang YH, Huang HQ, Fan YL, Yao B (2009) Cloning, expression and characterization of a novel acidic xylanase, XYL11B, from the acidophilic fungus *Bispora* sp. MEY-1. Enzyme Microb Technol 45(2):126–133. doi:10.1016/j.enzmictec.2009.05.002
20. Manning M, Colon W (2004) Structural basis of protein kinetic stability: resistance to sodium dodecyl sulfate suggests a central role for rigidity and a bias toward $\beta$-sheet structure. Biochemistry 43(35):11248–11254. doi:10.1021/bi0491898
21. Menon G, Mody K, Keshri J, Jha B (2010) Isolation, purification, and characterization of haloalkaline xylanase from a marine *Bacillus pumilus* strain, GESF-1. Biotechnol Bioprocess Eng 15(6):998–1005. doi:10.1007/s12257-010-0116-x
22. Menon V, Prakash G, Prabhune A, Rao M (2010) Biocatalytic approach for the utilization of hemicellulose for ethanol production from agricultural residue using thermostable xylanase and thermotolerant yeast. Bioresour Technol 101(14):5366–5373. doi:10.1016/j.biortech.2010.01.150
23. Nassar N, Ortiz R (2010) Breeding cassava to feed the poor. Sci Am 302(5):78–84. doi:10.1038/scientificamerican0510-78
24. Ogino H, Ishikawa H (2001) Enzymes which are stable in the presence of organic solvents. J Biosci Bioeng 91(2):109–116. doi:10.1007/BF01022602
25. Oh HW, Heo SY, Kim DY, Park DS, Bae KS, Park HY (2008) Biochemical characterization and sequence analysis of a xylanase produced by an exo-symbiotic bacterium of *Gryllotalpa orientalis*, *Cellulosimicrobium* sp. HY-12. Antonie Leeuwenhoek 93(4):437–442. doi:10.1007/s10482-007-9210-2
26. Okoro CK, Bull AT, Mutreja A, Rong XY, Huang Y, Goodfellow M (2010) *Lechevalieria atacamensis* sp. nov., *Lechevalieria deserti* sp. nov. and *Lechevalieria roselyniae* sp. nov., isolated from hyperarid soils. Int J Syst Evol Microbiol 60(2):296–300. doi:10.1099/ijs.0.009985-0
27. Qiu Z, Shi P, Luo H, Bai Y, Yuan T, Yang P, Liu S, Yao B (2010) A xylanase with broad pH and temperature adaptability from *Streptomyces megasporus* DSM 41476, and its potential application in brewing industry. Enzyme Microb Technol 46(6):506–512. doi:10.1016/j.enzmictec.2010.02.003
28. Quevillon E, Silventoinen V, Pillai S, Harte N, Mulder N, Apweiler R, Lopez R (2005) InterProScan: protein domains identifier. Nucleic Acids Res 33:W116–W120. doi:10.1093/nar/gki442
29. Sato Y, Fukuda H, Zhou Y, Mikami S (2010) Contribution of ethanol-tolerant xylanase G2 from *Aspergillus oryzae* on Japanese sake brewing. J Biosci Bioeng 110(6):679–683. doi:10.1016/j.jbiosc.2010.07.015

30. Sinma K, Khucharoenphaisan K, Kitpreechavanich V, Tokuyama S (2011) Purification and characterization of a thermostable xylanase from *Saccharopolyspora pathumthaniensis* S582 isolated from the gut of a termite. Biosci Biotechnol Biochem 75(10):1957–1963. doi:10.1271/bbb.110353
31. Sornyotha S, Kyu KL, Ratanakhanokchai K (2010) An efficient treatment for detoxification process of cassava starch by plant cell wall-degrading enzymes. J Biosci Bioeng 109(1):9–14. doi:10.1016/j.jbiosc.2009.06.021
32. Tadeo X, Lopez-Mendez B, Trigueros T, Lain A, Castano D, Millet O (2009) Structural basis for the amino acid composition of proteins from halophilic Archea. PLoS Biol 7(12):e1000257. doi:10.1371/journal.pbio.1000257
33. Wang W, Zhang ZD, Tang QY, Mao J, Wei D, Huang Y, Liu ZH, Shi YH, Goodfellow M (2007) *Lechevalieria xinjiangensis* sp. nov., a novel actinomycete isolated from radiation-polluted soil in China. Int J Syst Evol Microbiol 57(12):2819–2822. doi:10.1099/ijs.0.65134-0
34. Wi SG, Kim HJ, Mahadevan SA, Yang DJ, Bae HJ (2009) The potential value of the seaweed *Ceylon moss* (*Gelidium amansii*) as an alternative bioenergy resource. Bioresour Technol 100(24):6658–6660. doi:10.1016/j.biortech.2009.07.017
35. Wu J, Guan T, Jiang H, Zhi X, Tang S, Dong H, Zhang L, Li W (2009) Diversity of Actinobacterial community in saline sediments from Yunnan and Xinjiang, China. Extremophiles 13(4):623–632. doi:10.1007/s00792-009-0245-3
36. Zhou JP, Huang HQ, Meng K, Shi PJ, Wang YR, Luo HY, Yang PL, Bai YG, Yao B (2010) 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. Appl Biochem Biotechnol 160(5):1277–1292. doi:10.1007/s12010-009-8642-8
37. Zhou JP, Huang HQ, Meng K, Shi PJ, Wang YR, Luo HY, Yang PL, Bai YG, Zhou ZG, Yao B (2009) Molecular and biochemical characterization of a novel xylanase from the symbiotic *Sphingobacterium* sp. TN19. Appl Microbiol Biotechnol 85(2):323–333. doi:10.1007/s00253-009-2081-x
38. Zhou JP, Shi PJ, Zhang R, Huang HQ, Meng K, Yang PL, Yao B (2011) Symbiotic *Streptomyces* sp. TN119 GH 11 xylanase: a new pH-stable, protease- and SDS-resistant xylanase. J Ind Microbiol Biotechnol 38(4):523–530. doi:10.1007/s10295-010-0795-5