

Novel Alkali-Stable, Cellulase-Free Xylanase from Deep-Sea *Kocuria* sp. Mn22

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A novel xylanase gene, *Kxyn*, was cloned from *Kocuria* sp. Mn22, a bacteria isolated from the deep sea of the east Pacific. *Kxyn* consists of 1,170 bp and encodes a protein of 390 amino acids that shows the highest identity (63%) with a xylanase from *Thermobifida fusca* YX. The mature protein with a molecular mass of approximately 40 kDa was expressed in *Escherichia coli* BL21 (DE3). The recombinant *Kxyn* displayed its maximum activity at 55°C and at pH 8.5. The K_m , V_{max} , and k_{cat} values of *Kxyn* for birchwood xylan were 5.4 mg/ml, 272 μ mol/min·mg, and 185.1/s, respectively. *Kxyn* hydrolyzed birchwood xylan to produce xylobiose and xylotriose as the predominant products. The activity of *Kxyn* was not affected by Ca^{2+} , Mg^{2+} , Na^+ , K^+ , β -mercaptoethanol, DTT, or SDS, but was strongly inhibited by Hg^{2+} , Cu^{2+} , Zn^{2+} , and Pb^{2+} . It was stable over a wide pH range, retaining more than 80% activity after overnight incubation at pH 7.5–12. *Kxyn* is a cellulase-free xylanase. Therefore, these properties make it a candidate for various industrial applications.

Keywords: *Kocuria* sp., alkaline stability, cellulase-free xylanase

Xylan is the major structural component of hemicelluloses, the second most abundant polysaccharide in nature after cellulose, and constitutes one-third of a total plant biomass. Xylan consists of a backbone of β -(1,4)-linked D-xylopyranosyl units that are then linked to different side groups, such as L-arabinose, D-galactose, acetyl, feruloyl, *p*-coumaroyl, and glucuronic acid residues. Xylanolytic enzymes include endo- β -1,4-xylanase (E.C. 3.2.1.8) and β -xylosidase (E.C. 3.2.1.37), both of which hydrolyze the

xylan backbone, and α -L-arabinofuranosidase (E.C. 3.2.1.39), acetyl xylan esterase (E.C. 3.1.1.72), and ferulic or *p*-coumaric acid esterase (E.C. 3.2.1.73), all of which are used for debranching [22]. As the hydrolysis of xylan is an important step towards the utilization of hemicelluloses, xylanolytic enzymes are widely used in the pulp and paper, animal feed, and baking industries.

As the main xylanolytic enzyme, endo- β -1,4-xylanase hydrolyzes the xylan backbone and generates a mixture of short xylooligosaccharides. Based on primary structure comparisons of the catalytic domains, most xylanases have been classified as family 10 or 11 of glycoside hydrolases, whereas a few have been classified as family 5, 7, 8, or 43 (<http://afmb.cnrs-mrs.fr/CAZY/>) [3].

Although many xylanase genes have already been cloned from various microorganisms, only a few are from marine bacteria and fungi. Marine microorganisms that live under extreme conditions are able to produce enzymes with particular characteristics that enable adaptation to an extreme environment. Thus, the possibility of exploiting these enzymes has generated considerable research interest in marine microorganisms. For example, Hou *et al.* [6] isolated a cold-adapted xylanase from a *Penicillium* strain existing in the Yellow Sea of China. A thermostable xylanase gene from a deep-sea thermophilic *Geobacillus* sp. was also cloned and expressed in *Escherichia coli* [30], whereas *Aspergillus niger*, which produces a cellulase-free alkaline xylanase, was isolated from a marine habitat [23]. To date, a small number of xylanase genes have been cloned from marine bacteria and fungi, yet this number is far smaller than the number of xylanase genes cloned from land microorganisms. Thus, marine microorganisms remain the largest resource from which to isolate novel xylanase genes.

Accordingly, this paper reports on the first xylanase gene to be cloned from *Kocuria* sp. The xylanase was

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characterized and shown to have desirable traits for the paper and pulp industry.

MATERIALS AND METHODS

Materials

Oat spelt xylan, birchwood xylan, locust bean gum, carboxymethyl cellulose, and Avicel were purchased from Sigma (St. Louis, MO, U.S.A.). The restriction endonucleases, T4 DNA ligase, DNA polymerase, CIAP, and GC buffer were purchased from Takara (Dalian, China). The DNA purification kit and Bradford protein assay kit were purchased from Sangon (Shanghai, China). All other chemicals used were analytical grade reagents, unless otherwise stated.

Bacterial Strains, Plasmids, and Culture Conditions

Kocuria sp. Mn22 was originally isolated from deep-sea polymetallic nodules collected from the eastern Pacific Ocean and supplied from the Marine Culture Collection of China (MCCC 1A00387). The strain was grown in a Luria–Bertani (LB) medium with 2% (w/v) NaCl at 28°C, and then for xylanase production, it was cultivated in a medium containing 2% NaCl, 0.1% peptone, 0.1% yeast extract, 0.2% K₂HPO₄, 0.03% MgSO₄, 0.02% CaCl₂, and 1% corn cob xylan (made in the authors' laboratory). The *E. coli* DH5 α strain and pUC18 vector were used for the library construction and gene subcloning, and the *E. coli* BL21 (DE3) strain and pGEX-6P-1 vector were used to express the xylanase. All the transformants were cultivated in an LB medium or on LB agar plates at 37°C with ampicillin (100 μ g/ml).

Genomic Library Construction and Screening for Xylanase Genes

To construct the genomic DNA library, the genomic *Kocuria* sp. Mn22 DNA was partially digested with Sau3AI and separated by 0.8% agarose gel electrophoresis. Fragments (4–9 kb sized) were then purified from the gel, ligated into a BamHI-digested and CIAP-treated pUC18 vector, and finally transformed into *E. coli* DH5 α . The transformants were grown on LB agar plates containing 40 μ l of a 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal) solution (20 mg/ml) and 40 μ l of 100 mM isopropyl-1-thio- β -D-galactoside (IPTG). The white clones were picked for cultivation on xylan plates and screened using the Congo red method [29]. The xylan plates were stained with 0.5% (w/v) Congo red and washed with 1 M NaCl. The clones that formed clear halos were then selected as xylanase-positive colonies. The recombinant plasmid pUC-Kxyn was extracted and digested with various restriction endonucleases to determine the size of the insert.

Gene Analysis

The recombinant plasmid pUC-Kxyn was sequenced by the Beijing Genomics Institute (BGI, Beijing, China), and the nucleotide sequences were analyzed using the Softberry Gene Finding tool (<http://linux1.softberry.com/berry/>). The presence of a signal peptide in the deduced amino acid sequence was predicted by SignalP (<http://www.expasy.org/>), the DNA and protein sequence alignments were carried out using the Blast program (<http://blast.ncbi.nlm.nih.gov/Blast>), and the multiple sequence alignments were performed using the ClustalW program.

Cloning the Xylanase Gene in *E. coli*

The predicted coding region, without a signal peptide, was amplified by a PCR using the following primers: forward primer, ACCGGATCCGCCCGGAGACCCCGCC, containing a BamHI site (underlined); and reverse primer, GCGGAATTCTCAGCGGAACGCGGGGT, containing an EcoRI site (underlined). The oligonucleotide primers were designed on the basis of the sequences supplied by the BGI. pUC-Kxyn was used as the template. The PCR was performed as follows: 95°C for 4 min, followed by 30 cycles at 95°C for 30 s, 60°C for 30 s, 72°C for 60 s, and a final extension step at 72°C for 10 min. The PCR products were then purified from the gel, digested with BamHI and EcoRI, and ligated into the corresponding sites of pGEX-6P-1. Thereafter, the ligation products were transformed into *E. coli* DH5 α competent cells, and the positive clones selected using the Congo red method. The recombinant plasmid pGEX-6P-Kxyn was transformed into *E. coli* BL21 (DE3) competent cells for protein expression and purification.

Expression and Purification of Kxyn

E. coli BL21 (DE3), harboring pGEX-6P-Kxyn, was grown overnight at 37°C in an LB medium supplemented with 100 μ g/ml ampicillin. A 1:100 dilution of this culture was then inoculated into a fresh LB medium containing 100 μ g/ml ampicillin. After growth at 37°C for 2–3 h to an A₆₀₀ of 0.6–0.8, IPTG was added to a final concentration of 0.2 mM. The culture was further incubated at 18°C for 10 h. The cells were then harvested by centrifugation, washed, and resuspended in a phosphate buffer (pH 7.4, 140.0 mM NaCl, 2.7 mM KCl, 10.0 mM Na₂HPO₄, and 1.8 mM KH₂PO₄). After disrupting the cells by sonication, the lysates were centrifuged at 10,000 \times g for 30 min at 4°C. The glutathione-S-transferase (GST)-tagged xylanase was then purified according to the manufacturer's instructions. The GST tag was removed by digestion with a 3C protease solution (10 U/ μ l, PreScission; Pharmacia) and the purified protein eluted with 1 ml of a PBS buffer. The molecular mass of the purified xylanase was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and a native PAGE. Zymogram analysis was performed using a 12% native PAGE gel containing 1% birchwood xylan [1]. The concentration of the enzyme was determined with a Bradford Protein assay kit.

Enzyme Assay

The xylanase activity was determined by measuring the release of reducing sugar from birchwood xylan using 3,5-dinitrosalicylic acid (DNS). The reaction mixture, consisting of 10 μ l of the appropriately diluted enzyme and 90 μ l of 1% xylan in a 50 mM Glycine–NaOH buffer (pH 8.5), was incubated for 30 min at 55°C. The reaction was then terminated by the addition of 100 μ l of DNS and boiled for 10 min, and the absorption at 540 nm was measured using a Multiskan Spectrum spectrophotometer (Thermo Scientific, Vantaa, Finland). One unit of xylanase activity was defined as the amount of enzyme that released 1 μ mol of reducing sugar from xylan per minute under the conditions above (with xylose as the standard).

Effects of pH and Temperature on Enzyme Activity

The optimum pH of the purified xylanase was determined within a pH range of 3–12. The buffers used were 0.2 M Na₂HPO₄/0.1 M citric acid buffer for pH 3.0–8.0, and 50 mM glycine–NaOH buffer for pH 8.0–12. To determine the pH stability, the enzyme was incubated in buffers from pH 5 to 12 at 4°C overnight and the residual activity

was measured using a standard assay. The optimum temperature of the enzyme was determined over a range from 30°C to 70°C in a Gly–NaOH buffer (pH 8.5). To determine the thermostability, the xylanase was incubated at 55°C, 60°C, and 70°C for different times without a substrate, and the remaining activity measured under standard assay conditions.

Hydrolysis of Xylan and Xylooligosaccharides by Purified Xylanase

The hydrolysis of birchwood xylan, xylotriase, and xyloetraose (10 mg/ml) was carried out in a Gly–NaOH buffer (pH 8.5) with the appropriately diluted purified xylanase enzyme at 55°C for 12 h. The products of the enzymatic hydrolyses were analyzed by thin-layer chromatography (TLC) on silica gel G-60 plates with a butan-1-ol/acetic acid/water (2:1:1, v/v) mobile phase system. Sugars were detected with 0.2% (w/v) orcinol in sulfuric acid/methanol (10:90) followed by heating for a few minutes at 100°C in an oven [16].

Effects of Various Reagents on Enzyme Activity

The recombinant xylanase was incubated with 2 mM of various metal ions and 5 mM of various chemical reagents at pH 8.5 with 1% birchwood xylan at 55°C for 30 min, and the xylanase activity measured under the standard conditions described above.

Kinetic Analysis

The K_m , V_{max} , and k_{cat} values of the purified xylanase were determined by measuring the enzymatic activity with various concentrations of birchwood xylan as the substrate. The concentrations ranged from 0.5 to 15 mg/ml. The enzyme activity was assayed in a Gly–NaOH buffer (pH 8.5) at 55°C. The data were plotted according to the Lineweaver–Burk method.

Substrate Specificity

The activities towards birchwood xylan, oat spelt xylan, carboxymethyl cellulose, Avicel, and locust bean gum were tested. The enzyme was incubated with 1% (w/v) of the different substrates in a Gly–NaOH buffer (pH 8.5) at 55°C for 30 min. The release of reducing sugars was measured using the DNS method and calculated from the standard curves for xylose, glucose, or mannose.

Nucleotide Sequence Accession Number

The nucleotide sequence of *Kxyn* from *Kocuria* sp. Mn22 was deposited in the GenBank database under Accession No. FJ458449.

RESULTS AND DISCUSSION

Isolation and Sequence Analysis of the Xylanase Gene

Nearly 3,000 transformants were screened by the Congo red method and only one clone that formed a clear zone was obtained. The recombinant plasmid, named pUC-Kxyn, was digested with EcoRI and HindIII and produced a 6 kb insert. This fragment was completely sequenced and its nucleotide sequence analyzed using DNASTRA, Exspasy, Softberry, and Blast.

The insert fragment contained four open reading frames (ORFs). The first truncated ORF encoded a putative permease component of the ABC-type sugar transport system, the

second ORF encoded β -xylosidase, the third ORF encoded endo- β -1,4-xylanase, defined as *Kxyn*, and the last truncated ORF encoded a putative xylose repressor. However, this paper focuses on the second ORF encoding a hypothetical xylanase gene.

The xylanase ORF consisted of 1,170 bp and encoded a 390-residue polypeptide. It was GC-rich, and the overall GC content was 70%. A potential Shine–Dalgarno sequence (5'GAAGGAA3') was found 8 bp upstream of the ATG initiation codon. A putative signal peptide of 30 amino acid residues was located at the N terminus, with a cleavage site located between Ala30 and Ala31. The mature peptide was predicted by DNASTAR to have a mass of 40,825 Da.

The deduced amino acid sequence of the xylanase ORF was used to search for homologous sequences using the Blast program. On the basis of its sequence homology and hydrophobicity, *Kxyn* was found to belong to family 10 of glycosyl hydrolases. It showed the highest identity (63%) with a putative xylanase from *Thermobifida fusca* YX (AAZ56824). It also exhibited a 48% identity with a xylanase from *Thermobifida alba* (CAB02654), 57% identity with Umxyn10A from *Thermobifida fusca* YX (ABL73883), 44% identity with a xylanase from *Cellulosimicrobium* sp. HY-12 (ABX88978), and 32% identity with an extracellular xylanase from *Geobacillus stearothermophilus* (ABI49951). According to its sequence homology, *Kxyn* was found to be genetically closely related to the xylanases from *Thermobifida*, as *Kocuria* and *Thermobifida* both belong to Actinomycetales, and the sequence of the 16S ribosomal RNA gene of *Kocuria* sp. Mn22 (DQ336242) exhibited the highest identity (89%) with that of *Thermobifida fusca* YX (AM932257). Further alignment indicated that *Kxyn* contained the most highly conserved catalytic residues (Glu-180 and Glu-288, marked with ※) that are crucial for the activity of family 10 glycosyl hydrolases [26]. The alignments of these sequences are given in Fig. 1.

Most of the xylanases in family 10 contain a catalytic domain, carbohydrate-binding domain, and/or other functional domains, where examples include Xyn from *Cellulomonas pachnodae*, XynA from *Thermotoga maritima*, XynA from *Clostridium stercorarium*, and Xyn10B from *Clostridium thermocellum* [2, 10, 25, 31]. However, *Kxyn* only included a catalytic domain with no carbohydrate-binding domain. Similar structures have been reported in other studies [5, 15].

Enzyme Expression and Purification

Kxyn, without the coding sequence for the predicted signal peptide, was cloned into the vector pGEX-6P-1 and expressed in *E. coli* BL21 (DE3). The xylanase was then harvested and purified from the *E. coli* BL21 (DE3)/pGEX-6P-Kxyn lysates.

After induction with IPTG at 18°C, the induced and non-induced recombinant bacterium (harboring pGEX-6P-Kxyn) and control bacterium (harboring empty pGEX-6P-1 vector)

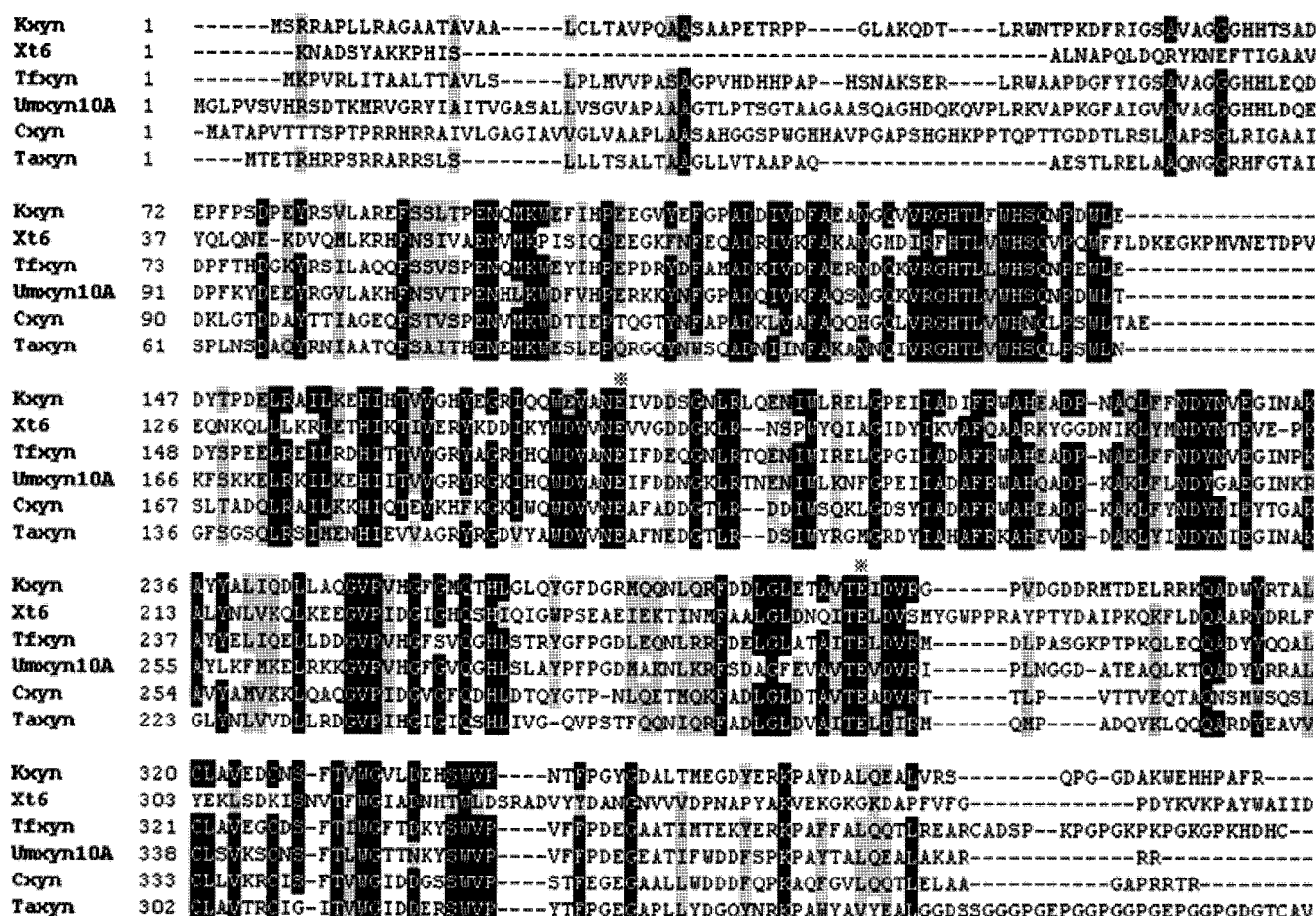


Fig. 1. Amino acid alignment of Kxyn with most similar xylanases from glycosyl hydrolase family 10 using the ClustalW program. Kxyn from *Kocuria* sp. Mn22; Xt6, xylanase from *Geobacillus stearothermophilus*; Tfxyn, putative xylanase from *Thermobifida fusca* YX; Umxyn10A, another xylanase from *Thermobifida fusca* YX; Cxyn, xylanase from *Cellulosimicrobium* sp. HY-12; Taxyn, beta-1,4-endoxylanase from *Thermobifida alba*. The conserved residues are shaded in black and similar residues are shaded in gray. Glu-180 and Glu-288 are marked with *.

were analyzed by SDS-PAGE. The results showed that the cell extracts from the induced recombinant bacterium exhibited a clear band (about 66 kDa) corresponding to the GST-Kxyn fusion protein (Fig. 2, lane 5). No band was observed in the same position in the extract from the non-induced recombinant bacterium, the induced, and the non-induced control bacterium (empty vector only). This showed that Kxyn was expressed. After purification by affinity chromatography and digestion with 3C protease, the recombinant xylanase was harvested and resolved to a single band of about 40 kDa following SDS-PAGE (Fig. 2, lane 6). Additionally, a clear zone was produced on a zymogram confirming the purified protein as a xylanase (Fig.3).

Effects of pH and Temperature on Enzyme Activity

The enzyme displayed an optimum activity at pH 8.5, and over 60% of its maximal activity was achieved between pH 6.5 and 9.5 (Fig. 4A). This is similar to the endoxylanase produced by the alkalophilic *Bacillus* sp. strain NG-27,

which has an optimum pH of 8.4, and to xylanase J from *Bacillus* sp. strain 41M-1, which is most active at pH 9.0 [5, 19]. The xylanase was sensitive at a low pH and displayed less than 20% of its maximal activity at pH 6.0 and nearly no activity below pH 5.5. However, it was very stable over a wide pH range, showing more than 80% of its maximal activity after incubation overnight at 4°C within a pH range of 7–12 (Fig. 4B). pH stabilities ranging from 4 to 10, 4 to 9.5, 3 to 9, and 5.5 to 10 have already been reported for xylanases from *Streptomyces fradiae*, *Streptomyces cyaneus* SN32, *Marasmius* sp., and *Geobacillus* sp., respectively [14, 20, 24, 30]. It has also been shown that salt bridges can contribute to the alkaline stability of xylanases. For example, the optimum pH of xylanase J from *Bacillus* sp. strain 41M-1 can be shifted by changing the salt bridges in the catalytic cleft [27]. Furthermore, it has been reported that a protein surface rich in acidic residues, such as Asp and Glu, and with a low number of Asn residues can enhance the stability of xylanase at an alkaline pH. This is probably because the acidic residues

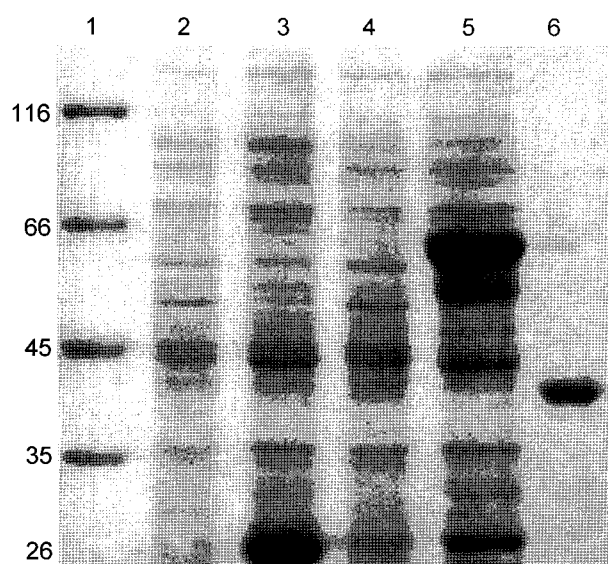


Fig. 2. SDS-PAGE of purified xylanase.

Lanes: 1, protein marker; 2, control bacterium (harboring empty pGEX-6P-1 vector) non-induced with IPTG; 3, control bacterium (harboring empty pGEX-6P-1 vector) induced with IPTG; 4, recombinant bacterium (harboring pGEX-6P-Kxyn) non-induced with IPTG; 5, recombinant bacterium (harboring pGEX-6P-Kxyn) induced with IPTG; 6, purified xylanase without GST (Kxyn).

on the surface protect the protein core from an OH⁻ attack [17]. Kxyn is rich in Asp and Glu (18.9%), especially compared with the percentage of Asn (3.9%), and therefore, this feature is likely to facilitate Kxyn activity at an alkaline pH.

The xylanase exhibited its maximal activity at 55°C (Fig. 5A). Most xylanases have been found to show their optimal activity at or near mesophilic temperatures (approximately 40–60°C) [3]. The xylanase also retained nearly 70% of its maximal activity after incubation for one hour at 55°C, and

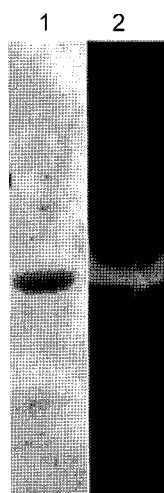


Fig. 3. Zymogram of Kxyn.

Lane 1, native PAGE of Kxyn; Lane 2, zymogram stained with Congo red.

still exhibited nearly 30% and 15% of its maximum activity after incubation at 60°C and 70°C, respectively (Fig. 5). XynAS9 from *Streptomyces* sp. S9 loses nearly all activity after incubation for one hour at 70°C [13], whereas Sfxyn 10 from *Streptomyces fradia* becomes totally inactive after incubation for less than 5 min at 70°C [14]. Although the xylanase from *Geobacillus* sp. exhibits its maximum activity at 70°C, it retains only 5% activity after incubation for 10 min at 70°C [30]. Thus, when compared with these xylanases, Kxyn exhibited a better thermal stability. However, its thermal stability still needs to be improved by genetic engineering technology.

Hydrolysis of Xylan and Xylooligosaccharides by Kxyn

As shown in Fig. 6, xylotriose could not be degraded by Kxyn (Lane X3), although xylo-tetraose was degraded to xylobiose (Lane X4). Moreover, Kxyn hydrolyzed birchwood xylan to produce xylobiose and xylotriose as the predominant products and no xylose was detected (Lane Xylan). This pattern of hydrolysis classified Kxyn as an endoenzyme (β -1,4-xylan xylanohydrolase). Most xylanases, such as Xyn10B from *Clostridium acetobutylicum*, Xyn10 from *Phanerochaete chrysosporium*, and xylanase from *Talaromyces thermophilus* hydrolyze the substrate xylan into xylobiose or a mixture of xylobiose and xylotriose [1, 4, 16]. However, XynAS9 from *Streptomyces* sp. S9 has been reported to hydrolyze xylan into xylose and xylobiose as the final products [13].

Effects of Various Reagents on Kxyn Activity

As shown in Table 1, the activity of Kxyn was affected by most of the metal ions tested. Its activity was nearly completely inhibited by Hg²⁺, Cu²⁺, Zn²⁺, and Pb²⁺, where less than 10% of its maximal activity remained after incubation with these reagents. As Hg²⁺ can oxidize indole rings, it most likely interacted with the tryptophan residues of the enzyme [13]. The inactivation of xylanases by Hg²⁺, Cu²⁺, Zn²⁺, and Pb²⁺ has also been reported in other studies [7, 19, 30]. Co²⁺, Mn²⁺, and Fe²⁺ also strongly inhibited the activity, whereas Mg²⁺, Ca²⁺, Na⁺, and K⁺ had little or no effect. Moreover, the activity of Kxyn was not affected by β -mercaptoethanol or DTT, showing that the S–S linkage between cysteine residues was not essential to maintain Kxyn activity. Furthermore, the xylanase was highly tolerant of SDS, showing 90% residual activity after incubation with SDS.

Kinetic Analysis

The K_m , V_{max} , and k_{cat} values when using birchwood xylan as the substrate were 5.4 mg/ml, 272 μ mol/min·mg, and 185.1/s, respectively. The K_m value was similar to that (5.1 mg/ml) of the xylanase from *Aspergillus oryzae* [9]. K_m values from 1.09 to 22.51 mg/ml have also been reported in previous studies [10, 11, 12]. The k_{cat} value for Kxyn

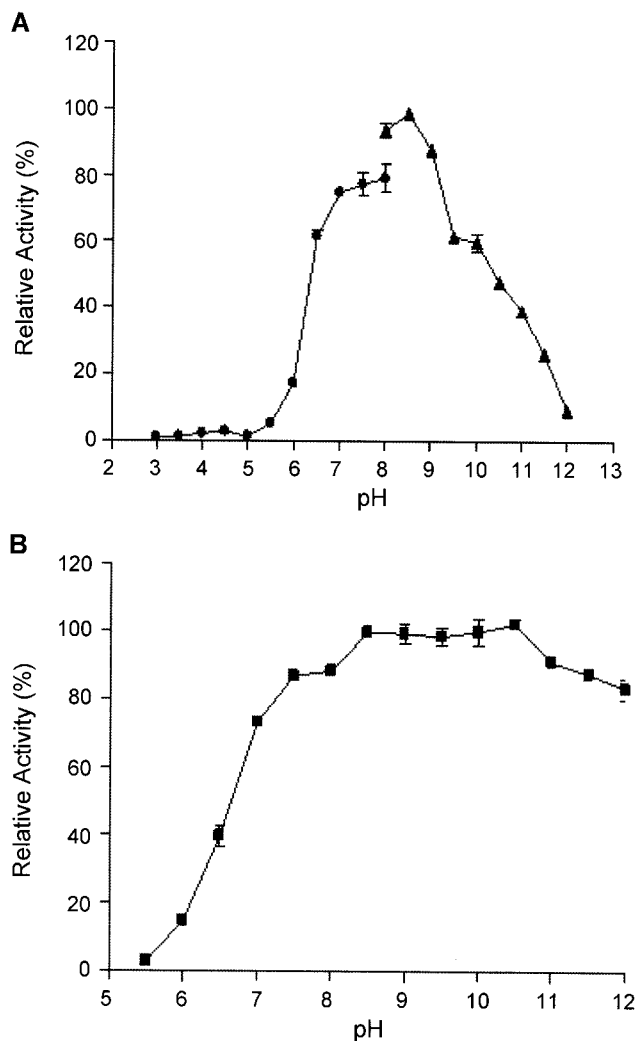


Fig. 4. Effect of pH on activity (A) and stability (B) of purified β -1,4-xylanase.

A. The activity was assayed at 55°C in a pH range of 3.0–12 with the following buffers: 0.2 M Na_2HPO_4 /0.1 M citric acid for pH 3.0–8.0 (●); 50 mM glycine–NaOH buffer for pH 8.0–12 (▲). The activity at the optimum pH was defined as 100%. B. After preincubation at 4°C overnight in buffers ranging from pH 5.5 to 12, the activity was measured in a glycine–NaOH buffer (pH 8.5) at 55°C. The initial activity was defined as 100%.

was lower than that for the xylanase from *Streptomyces* sp. S9 ($K_{\text{cat}}=376.33/\text{s}$) [13], yet higher than those for the xylanases from *Trichoderma reesei* ($k_{\text{cat}}=106/\text{s}$), *Flavobacterium* sp. ($k_{\text{cat}}=100/\text{s}$), and *Streptomyces fradiae* var. k11 ($k_{\text{cat}}=103/\text{s}$) [8, 12, 14].

Substrate Specificity

As shown in Table 2, Kxyn showed a strong specificity toward birchwood and oat spelt xylan. It could only hydrolyze birchwood xylan and oat spelt xylan, and exhibited a much higher activity with birchwood xylan. No activity was detected against carboxymethyl cellulose, Avicel, and locust bean gum, which confirmed it was a

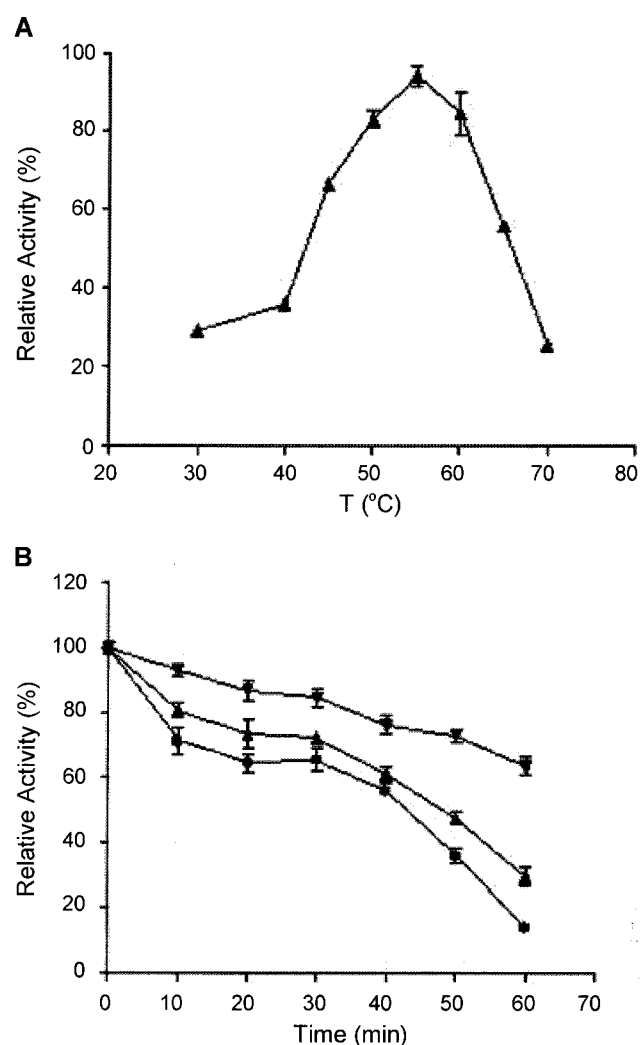


Fig. 5. Effect of temperature on activity (A) and stability (B) of xylanase.

A. The xylanase activity was measured in a glycine–NaOH buffer (pH 8.5) at different temperatures. The activity at the optimum temperature was defined as 100%. B. The thermostability was determined by measuring the activity after incubation at 55°C (▼), 60°C (▲), and 70°C (●) for various times. The initial activity was defined as 100%.

cellulase-free xylanase. Most of the xylanases reported in the literature exhibit significant cellulase activity, such as the xylanase from *Clostridium acetobutylicum*, *Trichoderma reesei*, *Cellulomonas flavigena*, *Marasmius* sp., and *Thermotoga maritima* [1, 8, 21, 24, 28], whereas only a few cellulase-free xylanases have been reported in *Talaromyces thermophilus*, *Thermomyces lanuginosus*, and *Aspergillus niger* isolated from a marine habitat. [16, 18, 23].

In the paper industry, chlorine and chlorine compounds are usually used in the bleaching process. However, these compounds are toxic and known to be persistent organic pollutants. Xylanase, which degrades the xylan on the surface of cellulose fibers, has been shown to improve the efficiency of chlorine bleaching, thereby allowing a reduction

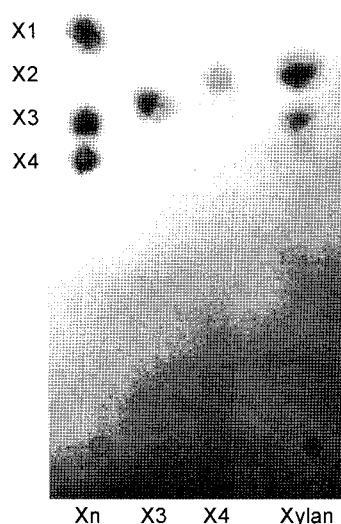


Fig. 6. TLC analyses of hydrolysis products from birchwood xylan and xylooligosaccharides.

Lanes: Xn, a mixture of xylose (X1), xylotriose (X3), and xylotetraose (X4); X3, xylotriose hydrolysate; X4, xylotetraose hydrolysate; Xylan, birchwood xylan hydrolysate.

in the use of chlorine, pollution, and damage to the environment [32]. However, the xylanase used in the paper industry must be free of cellulase activity to avoid degradation of the pulp fiber and ensure the quality of the pulp [23]. Thus, bifunctional cellulase/xylanase is less suitable for the pulp and paper industry. Furthermore, the pulp is alkaline with a pH of 8.0 or more and kept at a high temperature. Therefore, the need for large quantities of xylanases free of

Table 1. Effect of different metal ions and chemical reagents on enzyme activity.

Compounds (2 mM)	Relative activity (%)
Control	100
CoCl ₂ ·6H ₂ O	21.7±1.3 ^a
HgCl ₂	1.0±0.2
MgSO ₄	99.6±3
CuSO ₄ ·5H ₂ O	2.0±0.1
ZnSO ₄ ·7H ₂ O	7.2±0.3
MnCl ₂ ·4H ₂ O	57±5.6
NaCl	96±0.6
KCl	98±0.8
Pb (CH ₃ COO) ₂ ·3H ₂ O	6.3±2.9
CaCl ₂	100.8±1.2
FeSO ₄ ·7H ₂ O	27.8±0.6
DTT (5 mM)	100±2.6
β-Mercaptoethanol (5 mM)	94.5±1.5
SDS (5 mM)	90±0.3

^aRelative activity±standard deviation.

Table 2. Activity of purified xylanase on different substrates.

Substrate	Activity (IU/mg) ^a
Birchwood xylan	132±1 ^b
Oat spelt xylan	22±2
Carboxymethyl cellulose	No activity
Locust bean gum	No activity
Avicel	No activity

^aAssay was performed under optimum conditions.

^bSpecific activity±standard deviation.

cellulase activity and stable at higher temperatures and pH values has necessitated a search for novel enzymes. Kxyn is a cellulase-free xylanase with a maximum activity at pH 8.5 and good thermal stability. As such, these properties are advantageous for the application of Kxyn in the biobleaching of paper pulp.

In conclusion, *Kocuria* sp. Mn22 was isolated from deep-sea polymetallic nodules collected from the eastern Pacific Ocean. Although it has already been reported that the genus *Kocuria* can produce xylanolytic enzymes [11], this is the first report of a cloned xylanase gene from *Kocuria*. Thus, as an alkali-stable, cellulase-free xylanase, Kxyn is a good candidate for various industrial applications, especially the biobleaching of paper pulp.

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