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Purification and Properties of a Psychrotrophic *Trichoderma* sp. Xylanase and its Gene Sequence

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Abstract A psychrotrophic fungus identified as *Trichoderma* sp. SC9 produced 36.7 U/ml of xylanase when grown on a medium containing corncob xylan at 20 °C for 6 days. The xylanase was purified 37-fold with a recovery yield of 8.2%. The purified xylanase appeared as a single protein band on SDS-PAGE with a molecular mass of approximately 20.5 kDa. The enzyme had an optimal pH of 6.0, and was stable over pH 3.5–9.0. The optimal temperature of the xylanase was 42.5 °C and it was stable up to 35 °C at pH 6.0 for 30 min. The xylanase was thermolabile with a half-life of 23.9 min at 45 °C. The apparent K_m values of the xylanase for birchwood, beechwood, and oat-spelt xylans were found to be 3, 2.1, and 16 mg/ml respectively. The xylanase hydrolyzed beechwood xylan and birchwood xylan to yield mainly xylobiose as end products. The enzyme-hydrolysed xylotriose, xylotetraose, and xylopentose to produce xylobiose, but it hardly hydrolysed xylobiose. A xylanase gene (*xynA*) with an open reading frame of 669 nucleotide base pairs (bp), encoding 222 amino acids, from the strain was cloned and sequenced. The deduced amino acid sequence of XynA showed 85% homology with Xyn2 from a mesophilic strain of *Trichoderma viride*.

Keywords Pshychrotrophic · Xylanase · Characterization · Cloning · *Trichoderma*

Abbreviations

BSA	Bovine serum albumin
CMC	Carboxymethylcellulose
DNS	Dinitrosalicylic acid
DTT	Dithiothreitol

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EDTA	Ethylenediaminetetracetic acid
GH	Glycoside hydrolase
MES	2-(N-Morpholino)ethane sulfonic acid
MOPS	3-(N-Morpholino)-propane sulphonic acid
ORF	Open reading frame
PCR	Polymerase chain reaction
PDA	Potato dextrose-agar
*p*NP	*p*-Nitrophenyl
RACE	Rapid amplification of cDNA ends
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TLC	Thin-layer chromatography

Introduction

As the major plant cell wall polysaccharide component of hemicellulose, xylan is the most abundantly available polysaccharide on earth after cellulose, whose complete degradation requires the synergistic action of several glycoside hydrolytic enzymes, such as endo-β-1,4-xylanase, β-D-xylosidase, and α-L-arabinofuranosidase. Xylanases (EC 3.2.1.8) as key enzymes for the complete degradation of xylan randomly hydrolyze the 1,4-β-D-glycosidic bonds of xylan to produce xylooligomers of different length [1, 2]. They have received considerable attention because of their application in the food, feed, pulp, and paper industries [2]. Cold-active xylanolytic enzymes have recently received much attention due to their potential applications in the textile and food industries in addition to bioremediation and basic research studies to understand the mechanism of their adaptation to low temperatures [3, 4].

Driven by industrial demands for enzymes that can operate under process conditions, a number of mesophilic, thermophilic, and hyperthermophilic xylanases have been reported, while little attention has been given to psychrophilic/psychrotrophic xylanases [1, 2, 5–7]. So far, only a few cold-active xylanases from the psychrophilic bacteria and fungi have been isolated, purified, and studied in detail [8–13]. Most of xylanases fall into two glycoside hydrolase (GH) families: family-10 xylanases with a high molecular mass of greater than 30 kDa and family-11 xylanases with a relatively low molecular mass ranging from 18 to 26 kDa [2]. Mesophilic xylanases characterized display optimal activity at temperatures between 45 and 65 °C [1, 5], whereas optimal temperatures of psychrophilic/psychrotrophic xylanases are generally lower [8–13]. Among mesophilic fungi, *Trichoderma* species are well known as excellent producers of cellulolytic and xylanolytic enzymes [14–17] and genes encoding xylanases from *Trichoderma* species have been reported [18, 19]. Recently, a psychrotrophic fungus *Trichoderma* sp. SC9 with an optimal growth temperature of 20 °C was isolated from a forest soil sample in Sichuan province, China [4]. In the present investigation, an extracellular xylanase of *Trichoderma* sp. SC9 was purified and characterized. Furthermore, the cloning of the xylanase gene from this strain and its gene sequence analysis was performed.

Materials and Methods

Materials

Birchwood xylan, beechwood xylan, oat-spelt xylan, and carboxymethylcellulose (CMC) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Chromatographic

resins, Sephadex G50 and DEAE 52 were obtained from Pharmacia (Pharmacia, Uppsala, Sweden) and Whatman Co. respectively. All other chemicals and reagents used were analytical grade reagents unless stated otherwise.

Fungal Strain and Xylanase Production

Soil samples were collected from forest soil in Sichuan province of China. Approximately 1 g of the sample was added to 100 ml of 0.85% saline solution, diluted to 10^{-5}, and spread on the xylan-agar plates containing (grams per liter) 10.0 g corncob xylan, 2.0 g peptone, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KH_2PO_4, 15.0 g agar. The incubation was carried out at 15 °C for 3 days. Xylan-degrading activity was detected as clear halos after staining with 0.5% (*w*/*v*) Congo red and washing with 1 M NaCl. The fungal strain showing high xylanase activity was identified as *Trichoderma* sp. SC9 by the Institute of Microbiology of Chinese Academy of Sciences and was deposited with the accession number CGMCC No.2680 at China General Microbiological Culture Collection Center. The fungus was grown at 20 °C for 5 days on potato dextrose-agar (PDA) plates and stored as stock cultures at 4 °C and sub-culturing of the fungus was carried out periodically every 6 weeks.

For xylanase production, the basal culture medium contained (grams per liter): corncob xylan, 25; yeast extract, 10; tryptone, 10; $MgSO_4 \cdot 7H_2O$, 0.3; $CaCl_2$, 0.3; $(NH_4)_2SO_4$, 0.3; $FeSO_4$, 0.3. A piece (1 cm^2) of 5-day-old fungus culture on PDA plate was used to inoculate into the above growth medium (50 ml) in 250 ml Erlenmeyer flasks and grown for 5 days at 20 °C in an orbital-shaker (160 rpm) incubator. The fungal mycelium was separated by centrifugation at 10,000×*g* for 10 min and the supernatant was used as crude enzyme for the xylanase purification.

Determination of Enzyme Activity and Protein Concentration

Xylanase activity was assayed by measuring the release of reducing sugar from birchwood xylan according to the method of Bailey et al. [20]. The reaction mixture containing 0.9 ml of 1.0% (*w*/*v*) birchwood xylan and 0.1 ml of a suitably diluted enzyme solution was incubated in 50 mM citrate buffer (pH 5.5) at 30 °C for 10 min and stopped the reaction by adding 1 ml of 1.0% (*w*/*v*) DNS (dinitrosalicylic acid). The amount of reducing sugar liberated was determined by DNS method using xylose (Sigma) as the standard [21]. One unit of xylanase activity is defined as the amount of enzyme that produces 1 μmol of xylose equivalent per minute. The concentration of protein in enzyme solution was measured by the Lowry method [22] using bovine serum albumin (BSA) as the standard. Specific activities of xylanase were expressed as units per mg of protein.

Purification of Xylanase

The crude extract was precipitated by slow addition of ammonium sulfate to a final concentration of 35–55% (*w*/*v*) with a constant stirring under cold condition. The precipitate formed was collected by centrifugation and redissolved in a small volume of 25 mM citrate buffer (pH 5.5). The sample was then loaded onto a DEAE 52 column (10× 1.0 cm) which was pre-equilibrated with the same buffer at a flow rate of 1 ml/min. The fractions showing xylanase activity were pooled and further purified on a Sephadex G50 column (100×1.0 cm) equilibrated with 50 mM citrate buffer (pH 5.5).

SDS-PAGE and Gel Filtration

The purity of xylanase after Sephadex G50 step was checked on 12.5% (*w*/*v*) SDS-PAGE as described by Laemmli [23] using Coomassie brilliant blue R-250 to visualize protein bands on gel. The following low molecular weight calibration kit (GE Healthcare) was used as protein markers in SDS-PAGE: phophorylase *b* (97.4 kDa), albumin (66.2 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa).

Native molecular mass of the purified xylanase was determined by gel filtration on a Superdex 75 column (40×1 cm) previously equilibrated with 50 mM citrate buffer (pH 6.0). Molecular weight standards containing phosphorylase *b* (97.2 kDa), albumin bovine V (68.0 kDa), albumin (45.0 kDa), chymotrypsinogen *a* (25.7 kDa), and cytochrome *c* (13 kDa) from Sigma were used to calibrate the Superdex 75 column.

Characterization of the Purified Xylanase

The influence of pH on xylanase activity was determined in 50 mM different buffers using 1.0% (*w*/*v*) of birchwood xylan at 30 °C. The buffers used were citrate (pH 2.5–6.5), acetate (pH 3–5.5), MES (pH 5–7), MOPS (pH 6–8.5), Tris–HCl (pH 7–9), and glycine-NaOH (pH 8.5–11). The pH stability was determined by incubating the enzyme in different buffers for 30 min at 30 °C over various pH ranges, and then measuring the remaining enzyme activity at optimal pH condition.

The temperature optimum for maximal enzyme activity was determined by incubating the enzyme in 50 mM citrate buffer (pH 6.0) with 1.0% (*w*/*v*) of birchwood xylan at different temperatures ranging from 4 to 70 °C. For determination of thermostability, the purified xylanase in 50 mM citrate buffer (pH 6.0) was incubated at 30 °C for 30 min. The thermal inactivation of the enzyme was further studied at 35, 40, and 45 °C by incubating the enzyme for 4 h in 50 mM citrate buffer (pH 6.0). Aliquots of reaction samples were drawn at different time intervals and cooled rapidly before checking the residual xylanase activities.

Substrate Specificity and Enzyme Kinetics

The substrate specificity of *Trichoderma* sp. SC9 xylanase was tested against various substrates. The purified enzyme was incubated with 10 mg/ml of each substrate in 50 mM citrate buffer (pH 6.0) at 40 °C for 10 min. The amount of reducing sugar produced was estimated using the DNS method as described above. Activities towards *p*-nitrophenyl derivatives were measured spectrophotometrically at 410 nm by the rate of *p*-nitrophenol formed from 1 mM of the substrates in 50 mM citrate buffer (pH 6.0) at 40 °C for 10 min during hydrolysis. One unit (U) of activity was defined as the amount of enzyme releasing 1 μmole of reducing sugar or *p*-nitrophenol per minute under the above conditions.

For the kinetic experiments, the purified xylanase was incubated with six different concentrations of each substrate ranging from approximately 0.5 to 2.0 times of the K_m values in 50 mM citrate buffer (pH 6.0) at 30 °C for 5 min. The K_m and k_{cat} values were calculated from kinetic data using "GraFit" software.

Analysis of Hydrolysis Products

Ten units of enzyme was added along with 20 mg of each xylan to final 2 ml of 50 mM citrate buffer (pH 6.0) and incubated for 24 h at 30 °C to hydrolyze the xylans. To further

determine the mode of action of xylanase, 1% (*w*/*v*) of different xylooligosaccharides such as xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4), and xylopentose (X_5) were separately incubated at 30 °C for 24 h with 10 units of xylanase in a reaction volume of 1.0 ml of 50 mM citrate buffer (pH 6.0). The aliquots were analyzed for products of hydrolysis at different time intervals on Thin-layer chromatography (TLC) plates 60 F 254 (E. Merck, Germany). The TLC plates were developed with two runs of butanol–acetic acid–water (2:1:1, *v*/*v*) solvent system followed by spraying the plates with a methanol-sulfuric acid mixture (95:5, *v*/*v*) and heating for a minute in an oven. A mixture of xylooligosaccharides consisting of xylose (X_1), xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4), and xylopentaose (X_5) was used as the standard.

Gene (*xynA*) Sequence Analysis

The molecular cloning procedure was performed by the standard method [24]. For isolation of genomic DNA, *Trichoderma* sp. SC9 was grown at 20 °C in a growth medium containing corncob xylan (2%, *w*/*v*) for 4 days. Fungal mycelium was collected by centrifugation (5,000×*g*, 10 min), washed twice with cold water and the genomic DNA was isolated using Fungal DNA Midi Kit (Omega Bioteck, Doraville, GA, USA). To isolate total RNA, fungal mycelium was collected as described above, frozen, and ground to fine powder in liquid nitrogen. The total RNA was isolated with the Trizol reagent (Invitrogen, Carlsbad, CA, USA) and mRNAs were purified using the Oligotex mRNA Midi Kit (Qiagen, Hilden, Germany).

Genomic DNA of *Trichoderma* sp. SC9 was used as template for subsequent polymerase chain reaction (PCR) amplification. To clone the xylanase gene (*xynA*), degenerate primers of XTP1 and XTP2 (Table 1) were designed based on the conserved sequences (FVGGKGWN and AVEGYFSSG) of known GH family 11 xylanases of *Trichoderma* species using the CODEHOP algorithm [25]. Genomic DNA of *Trichoderma sp* SC9 was used as template to amplify the gene of interest by polymerase chain reaction (PCR). The PCR conditions were as follows: a hot start at 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, followed by 1 cycle of 72 °C for 10 min. The PCR product was purified, ligated into pMD18-T vector (TakaRa, Japan) and sequenced.

Further, the full-length cDNA sequence of the xylanase was obtained by 5′ and 3′ RACE (rapid amplification of cDNA ends) using a SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). For the 5′ RACE, 5′ RACE Ready cDNAs were

Table 1 Oligonucleotide primers used for PCR amplification of the xylanase

Primers	Primer sequence (5′→3′)[a]	Size (bp, base pairs)
XTP1	TTCGTCGGCGGCAARGGNTGGMA	23
XTP2	TGCCGGAGGAGAAGTAGCCYTCNACNGC	28
XTF	ATGGTCGCCTTCACATCTCTC	21
XTR	TTAGCTGACGTTGATGTTGGCA	22
GSP1	CGACCAGTACTGGTAAAAGGTGGC	24
NGSP1	GGTTGTAGGTGCCAAAGTTCTCGA	24
GSP2	AGCGTCTACGACATTTACCGCACG	24
NGSP2	AGCGGGTCTGTCAACGTCGGCAAC	24

[a] Y=C/T, R=A/G, M=A/C, N=A/T/G/C

synthesized by reverse transcription in accordance with the manufacturer's instructions. To amplify the 5′ end of the cDNA, RACE product was amplified with primer GSP1 and adapter primer UPM, and then subjected to nested PCR using a nested gene-specific primer (NGSP1) and adapter primer NUP (Table 1). The PCR condition for RACE was: 1 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 68 °C, and 1 min at 72 °C, and finally 10 min at 72 °C. For the 3′ RACE, first-strand cDNAs were synthesized by reverse transcription as described above. The primary PCR using primers GSP2 and UPM was followed by a nested PCR using nested gene-specific primer (NGSP2) and NUP. The obtained PCR product was purified, cloned, and sequenced. The xylanase cDNA (designated as *xynA*) sequences from *Trichoderma* sp. SC9 were deposited in the GenBank with the accession No. ADB28453.1. To amplify this region from the *Trichoderma* sp. SC9 genomic DNA, the same PCR conditions were performed using the specific primers XTF and XTR (Table 1). The amplified PCR product of the DNA was purified and cloned into the pMD18-T vector, and transformed into *E. coli* DH5α for sequencing. The nucleotide sequence of *xynA* was subjected to BLAST analysis at NCBI. At http://www.expasy.ch/tools/ the deduced amino acid sequence for *xynA* was obtained and its extent of homology was searched at NCBI database. Signal sequence for the protein was analyzed by Signal P 3.0 server (http://www.cbs.dtu.dk/services/SignalP). Search analysis of conserved domain and signature sequences was carried out using ScanProsite (http://www.expasy.ch/tools/ScanProsite). Prediction of *N*- and *O*-glycosylation sites on *xynA* of *Trichoderma* sp. SC9 were made using NetNGlyc1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetOGlyc3.1 (http://www.cbs.dtu.dk/ services/NetOGlyc/), respectively.

Results

Purification of Xylanase from *Trichoderma sp.* SC9

The xylanase production by *Trichoderma* sp. SC9 was studied in shake-flask cultures using corncob xylan as the sole carbon source. It showed maximal xylanase activity of 36.7 U/ml after 6 days of cultivation (data not shown). From the culture supernatant of *Trichoderma* sp. SC9, an extracellular xylanase was purified 37.5-fold using ammonium sulfate precipitation followed by two chromatographic steps to an apparent homogeneity with a recovery yield of 8.2% (Table 2). Under denaturing condition on SDS-PAGE gel, the protein migrated as a single band corresponding to a molecular mass of approximately

Table 2 Summary of purification of xylanase from *Trichoderma* sp. SC9

Purification step	Total activity (U)[a]	Total protein (mg)[b]	Specific activity (U/mg)	Purification factor (-fold)	Recovery (%)
Crude supernatant	8,535.1	878.5	9.7	1	100
35–55% Ammonium sulfate precipitation	6,921.6	87.9	78.7	8.1	81. 1
DEAE 52	989.5	6.9	143.4	14.8	11.6
Sephadex G50	691.4	1.9	363.9	37.5	8.2

[a] Activity was measured in 50 mM citrate buffer (pH 5.5) at 30 °C using 1.0% (*w*/*v*) birchwood xylan as substrate by the DNS method

[b] The protein was measured by the Lowry method [22], using BSA as the standard

20.5 kDa (Fig. 1). Native molecular mass of the xylanase as determined by gel filtration chromatography was found to be about 22 kDa (data not shown), which indicates the protein exits in monomeric form.

Effect of pH and Temperature on the Activity and Stability of Xylanase

The purified xylanase exhibited high activity in the pH range of 5.5–6.5 (Fig. 2a) with maximum activity at pH 6.0. It retained more than 90% of its maximal activity at 30 °C for 30 min when tested in the pH range of 3.5 to 9.0 (Fig. 2b). The xylanase was maximally active at 42.5 °C and lost more than 80% of the activity at 60 °C. It retained 22% of xylanase activity at 20 °C (Fig. 3a). The enzyme was stable up to 35 °C for 30 min. At 50 °C within 30 min, 92.5% of the enzyme activity was lost (Fig. 3b). The half-life of the enzyme was 161 min at 40 °C and 23.9 min at 45 °C (Fig. 3c).

Substrate Specificity and Kinetic Parameters of Xylanase

The purified *Trichoderma* sp. SC9 xylanase was assayed for activity against different natural and synthetic substrates. Hydrolytic activity was observed at high rate towards different xylans tested (data not shown). The highest activity (676.8 U/mg) for birchwood xylan followed by oat-spelt xylan (645 U/mg) and beechwood xylan (426 U/mg) was noticed. The enzyme did not act on other substrates such as Avicel, CMC, filter paper, starch, and locust bean gum. No detectable activity towards *p*NP-β-D-xylopyranoside, *p*NP-β-D-glucopyranoside, *p*NP-β-D-cellobioside, *p*NP-β-D-mannopyranoside, or *p*NP-α-L-arabinofuranoside was observed under the conditions tested. The Michaelis–Menten constants were determined for birchwood xylan, beechwood xylan, and oat-spelt xylan which are given in Table 3. The K_m and k_{cat} values were respectively 3 mg/ml and 46 s^{-1} for birchwood xylan, 2.1 mg/ml and 46.4 s^{-1} for beechwood xylan, 16 mg/ml and 83 s^{-1} for oat-spelt xylan.

Hydrolysis Properties of the Xylanase

Birchwood xylan and beechwood xylan were hydrolyzed with purified xylanase and their products were analyzed by TLC (Fig. 4a). Hydrolysis of birchwood xylan and beechwood xylan produced xylobiose as the main product especially when the reaction was allowed to proceed for a long duration (6 h). The mode of action of xylanase was determined using

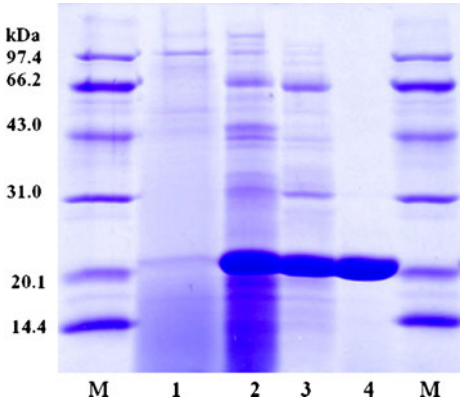

Fig. 1 SDS-PAGE analysis of a purified xylanase from *Trichoderma* sp. SC9. *Lane M*, low molecular weight calibration kit; *lane 1*, crude extract; *lane 2*, fraction of $NH_4(SO_4)_2$ precipitation; *lane 3*, after DEAE 52 column; *lane 4*, after Sephadex G50 step

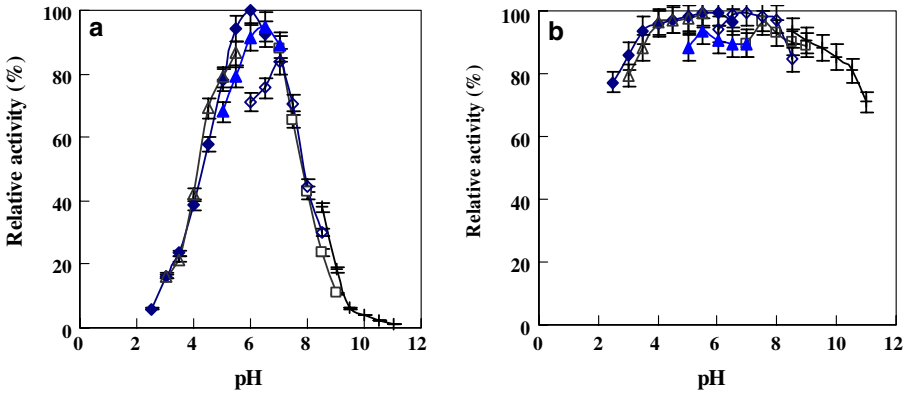

Fig. 2 Optimal pH (**a**) and pH stability (**b**) of the purified xylanase from *Trichoderma* sp. SC9. Buffers used: citrate (*barred dark blue diamond*), acetate (*barred unshaded up-pointing triangle*), MES (*barred blue up-pointing triangle*), MOPS (*barred unshaded blue diamond*), Tris–HCl (*barred unshaded square*), Glycine-NaOH (*barred plus sign*). The values given are the averages of separate experiments performed in triplicate

different xylooligosaccharides as substrates (Fig. 4b). Thin layer chromatographic analysis revealed the transglycosylation nature of the enzyme during first 15 min of xylanase activity on X_3, X_4, and X_5 substrates. As time proceeded, the enzyme rapidly hydrolyzed xylopentose, xylotetraose, and xylotriose to yield xylobiose. Xylobiose was hardly hydrolyzed by the enzyme. These results clearly show that *Trichoderma* sp. SC9 xylanase is an β-1,4-endoxylanase.

Cloning and Characterization of the Xylanase Gene of *Trichoderma sp.* SC9

Partial gene of *Trichoderma* sp. SC9 xylanase was amplified by PCR using degenerate primers XTP1 and XTP2 (Table 1). Nucleotide sequence analysis of the 473 bp PCR-amplified fragment showed highest (89%) sequence identity with GH family 11 xylanase of *Trichoderma viride*. The generated partial nucleotide sequence was used to design gene-specific primer for 5′and 3′ RACE to get full-length gene sequence. The 5′and 3′ RACE yielded 482- and 300-bp DNA fragments respectively (data not shown). Sequence analysis indicated the presence of a start codon (ATG) in the 5′-fragment and a stop codon (TAA) in the 3′-fragment. After assembling the sequence, a putative full-length xylanase cDNA of 902 bp was obtained. The nucleotide gene sequence (designated as *xynA*) has been deposited at NCBI GenBank with an accession number ADB28453.1.

A 736 bp consensus DNA fragment of the xylanase was obtained from genomic DNA of *Trichoderma* sp. SC9. The nucleotide sequence and deduced amino-acid sequences of the cDNA and genomic DNA of *Trichoderma* sp. SC9 *xynA* are shown in Fig. 5. One intron of 67 bp interrupted the coding region. The translational initiation codon, ATG, was present in nucleotide positions 65–67, and the termination codon, TAA, in positions 798–800 in the 5′-terminus region and at 3′-terminus region, a poly(A+) tail was present. The full-length ORF (669 bp) of the xylanase gene contained a predicted signal peptide of 19 amino acid residues and a catalytic domain belonging to GH family 11 xylanases. The predicted mature xylanase had 203 amino acids with a molecular mass of about 22.3 kDa. The protein sequence contained two possible *N*-glycosylation sites (consensus Asn-Xaa-Ser/Thr) at amino acid positions 92 and 128, respectively (Fig. 5). Sequence homology search at NCBI

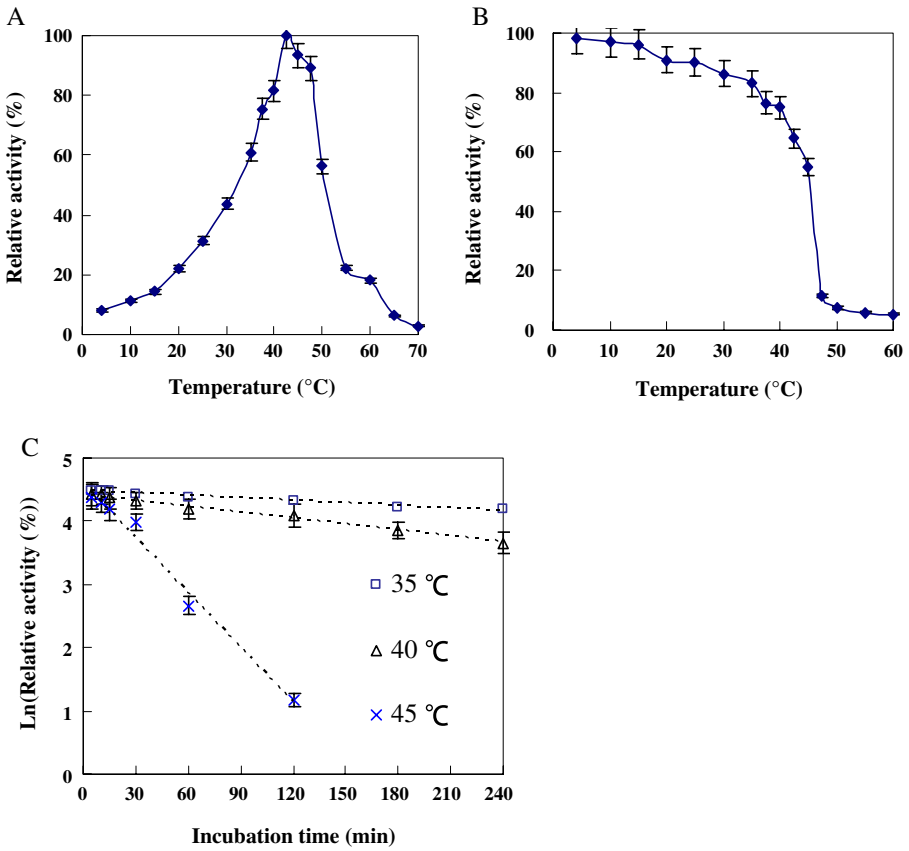

Fig. 3 Optimal temperature (**a**), thermostability (**b**), and thermal inactivation (**c**) of the purified xylanse from *Trichoderma* sp. SC9. For determination of thermostability, the residual activity of the treated xylanase was measured after pre-incubation of enzyme for 30 min at different temperatures at pH 6.0. Thermal inactivation of the xylanase was determined at 35 °C (*barred unshaded blue square*), 40 °C (*barred unshaded up-pointing triangle*), and 45 °C (*barred blue letter x*). The values given are the averages of separate experiments performed in triplicate

GenBank was performed using BLAST program. The amino acid identities of *Trichoderma* sp. SC9 XynA to xylanases from *T. viride* (AAP83925.1), *Trichoderma* sp. SY (AAN78423.1), *T. viride* (CAB60757.1), and *T. viride* Rut C-30 (ACB38137.1) were 92%, 91%, 88%, and 85%, respectively. However, their gene products have not been characterized with the exception of xylanase (*Xyn2*) of *T. viride* [19].

Table 3 Kinetic parameters for purified *Trichoderma* sp. SC9 xylanase[a]

Substrates	K_m (mg/ml)	k_{cat} (s^{-1})	k_{cat}/K_m ($mg^{-1} \cdot s^{-1}$ ml)
Birchwood xylan	3.0±0.25	46	15.3
Beechwood xylan	2.1±0.13	46.4	22. 1
Oat-spelt xylan	16±1.2	83	5.2

[a] Enzymatic reactions were carried out for 5 min at 30 °C in 50 mM citrate buffer (pH 6.0)

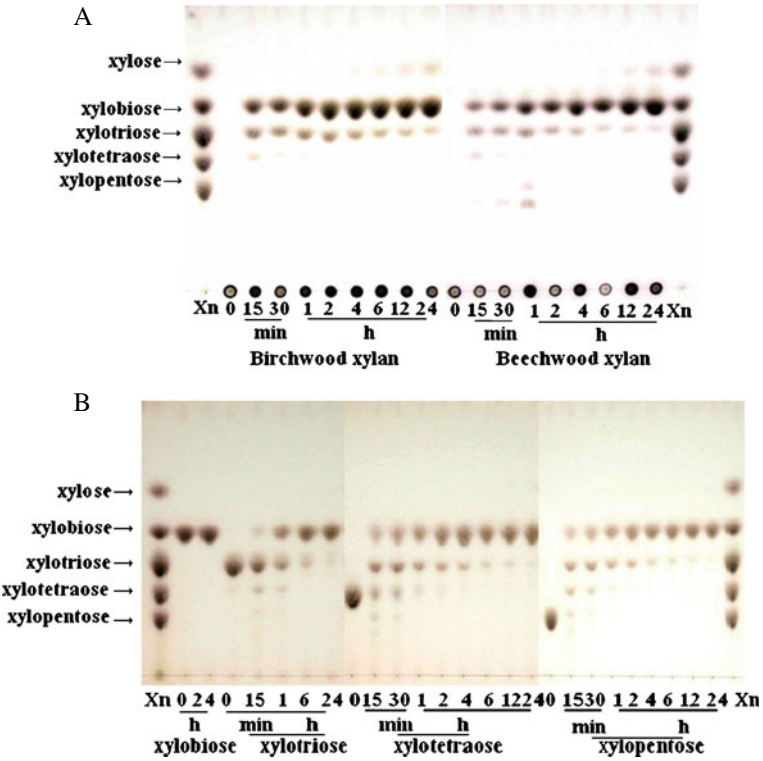

Fig. 4 TLC analyses of hydrolysis of xylans (**a**) and xylooligosaccharides (**b**) by the purified xylanase. Substrates and incubation times (hours or minutes) are indicated. *Lanes Xn*, a mixture of xylose to xylopentose. Each substrate (1%) was incubated with 10 units of xylanase for 24 h at 30 °C, and hydrolyzates were analyzed by TLC

Discussion

In this study, a xylanase from a psychrotrophic *Trichoderma* sp. SC9 was purified and characterized. In general, *Trichoderma* species strains produce two major kinds of xylanases, while the smaller xylanases have molecular masses of approximately 20 kDa [15]. The apparent molecular mass of xylanase (20.5 kDa) from *Trichoderma* sp. SC9 (Fig. 1) is similar to the molecular masses of some xylanases from *Trichoderma* species such as *T. viride* [26], *Trichoderma reesei* [14], *Trichoderma longibrachiatum* [15], and *Trichoderma harzianum* [16].

The properties of xylanases from different strains of *Trichoderma* species are compared with the xylanase of *Trichoderma* sp. SC9 (Table 4). The optimal pH for maximal xylanase activity (Fig. 2) was found almost similar for the xylanases of *Trichoderma* species in the pH range of 5–6 [15–17]. The purified xylanase showed apparent optimal activity at 42.5 °C and more than 40% of its activity was retained at 30 °C. In general, the activity of xylanases isolated from *Trichoderma* species will be maximal between 45 and 65 °C. Compared to xylanases from mesophilic *Trichoderma* species, the xylanase of *Trichoderma* sp. SC9 exhibited a lower optimum temperature for maximal enzyme activity (Table 4). This temperature-related profile is different from those of previously described xylanases from *Trichoderma* species, but similar to cold-active xylanases [2].

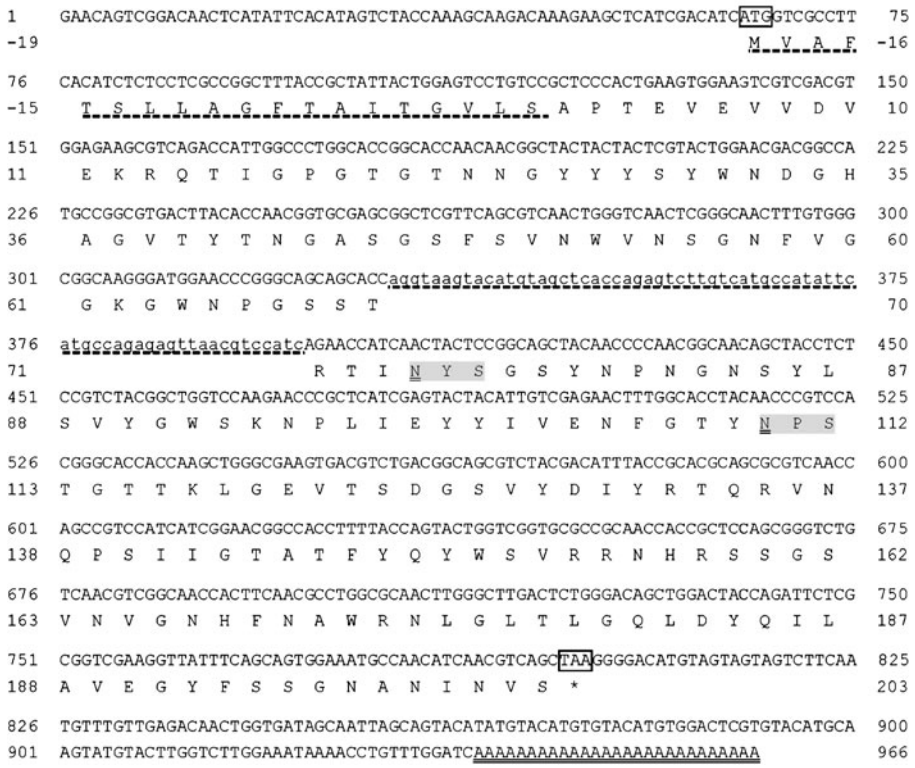

Fig. 5 Nucleotide and deduced amino acid sequences of the cDNA and genomic DNA of *Trichoderma* sp. SC9 xylanase. Conceptual translation of the ORF to the 222 amino acids is shown in a one-letter code below the respective codon. An intron sequence is shown in *lowercase letters*. The translational initiation codon ATG, and termination codon TAA marked by *asterisk* (*) are *boxed* in *black*. A putative signal peptide is indicated by a *dotted underline*. Two putative *N*-glycosylation sites are *underlined* and in *shaded boxes*

Table 4 Comparative analysis of physicochemical properties of xylanases from *Trichoderma* species

Species/strain	MW (kDa) [a]	Specific activity (U/mg)	pH_{opt} [b]	T_{opt} (°C) [c]	K_m (mg/ml)	Hydrolysis products	Reference
Trichoderma sp. SC9	20.5	676.8	6.0	42.5	3.0	*X*2	This work
T. reesei Rut C-30 Xyn2	24	1,080	5.0	50	0.11		19
T. longibrachiatum CS-185	18.6	6,630	5.0–6.0	45	10.14		5
T. koningii G39	21.5	6,100	5.5	60	0.7		30
T. viride	22	152.6	5.0	53	4.5	X1, *X*2 from larchwood	26
T. harzianum E52	20	–	5.0	50	6.3	X1, *X*2 from larchwood	26
T. harzianum C (XYL2)	18	–	5.0	45	8.34		16

[a] *MW*, molecular weight estimated by SDS-PAGE

[b] pH_{opt} optimum pH

[c] T_{opt} optimum temperature

The optimal temperature (42.5 °C) reported for the *Trichoderma* sp. SC9 xylanase is similar to the values reported for several thermolabile xylanases [8, 27]. Some of the cold-active xylanases have shown maximal activity at 30 °C or less [10–13]. Most cold-active xylanases are thermolabile and lose more than 50% of their activity at 40 °C within 30 min [6, 8, 28, 29]. Similar to the xylanase (*Xyn*8) of glycosyl hydrolase family 8 [11] and recombinant *Xyn*A19 from *Sphingobacterium* sp. TN19 [27] the xylanase of *Trichoderma* sp. SC9 retained more than 50% of its initial activity after incubation at 40 °C for 120 min. The half-life of a cold-active xylanase enzyme from *Flavobacterium* sp. was reported to be 40 min at 45 °C [12], which is longer than that of the *Trichoderma* sp. SC9 xylanase (24 min at 45 °C).

The xylanase did not show β-xylosidase, β-glucanase, α-arabinofuranosidase, β-mannosidase, β-mannanase, or cellulase activity. But some of the xylanases from *Trichoderma* species exhibit low activities towards cellulosic substrates [5, 19]. Variations in affinity of fungal xylanases towards xylans and hence a wide range of K_m values (0.1–15 mg/ml) have been reported [1, 5, 7]. As shown in Table 4, the K_m values determined for *Trichoderma* sp. SC9 xylanase were found lower than those measured for many other purified *Trichoderma* xylanases. Many xylanases upon hydrolysis of xylans produce a mixture of xylose and xylooligosaccharides [5, 11, 27, 30]. Xylanases of *T. viride* and *T. harzianum* produce xylose and xylobiose from larchwood xylan [26]. Some of the reported xylanases cannot hydrolyze xylotriose [11, 13, 14]. It is noted that the hydrolysis products of xylans and xylooligosaccharides by the xylanase were mainly xylobiose, similar to that of XynB [31] and XynBS27 [32]. These results suggest that the xylanase can perform transglycosylation reactions. The gene (*xynA*) sequence analysis of the purified endoxylanase revealed an open reading frame of 669 bp encoding 222 amino acids. The deduced amino acid sequence of XynA shares the highest identity (85%) with its characterized counterpart from mesophilic *T. viride* [19].

This is the first report on the purification, characterization and gene cloning of a xylanase from the psychrophilic *Trichoderma* sp. SC9. The enzyme showed optimal enzyme activity at 42.5 °C and was stable up to 35 °C. The low molecular weight, high specificity for xylans, and gene sequence analysis suggest that XynA belongs to the family 11 of glycosyl hydrolases. The xylanase may be suitable for its commercial use in low- to moderate-temperature processes, particularly in food industries.

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