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The identification, purification, and characterization of STXF10 expressed in *Streptomyces thermonitrificans* NTU-88

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Abstract Multiple xylanolytic enzymes of *Streptomyces thermonitrificans* NTU-88 were induced by oat-spelt xylan and separated by two-dimensional polyacrylamide and zymogram gels. Nineteen clear spots differed in p*I* and molecular weight values were found on the zymogram, and only spot one was seen on the corresponding silver-stained gel. These results revealed that multiple xylanases were secreted when *S. thermonitrificans* NTU-88 was induced and the spot (STXF10), identified as being a glycosyl hydrolase family 10 xylanase, was the predominant one among xylanases. STXF10 showed a tolerance for high temperatures and broad pH ranges and high affinity and hydrolysis efficiency for xylans. Furthermore, it also featured the minor ability to degrade different lignocellulosic substrates. Although *S. thermonitrificans* NTU-88 possesses multiple xylanases, our results suggest that the major form of xylanase might be selectively and specifically induced depending on the type of substrate to which the microorganism is exposed.

Keywords Endo-β-1 · 4 xylanase · Glycosyl hydrolase family 10 · *Streptomyces thermonitrificans* NTU-88 · Two-dimensional zymogram

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Introduction

Xylan, a complex and highly branched heteropolysaccharide, is the major component of hemicellulose in plant cell walls. It varies in structure among different plant species. The homopolymeric backbone chain of β-1,4-D-xylopyranosyl units can be substituted to varying degrees with glucuronopyranosyl, 4-*O*-methyl-D-glucuronopyranosyl, α-L-arabinofuranosyl, acetyl, feruloyl and/or *p*-coumaroyl side-chain residues. Because of its heterogeneity and complexity, the complete hydrolysis of xylan requires various xylanolytic enzymes. For example, endo-1,4-β-D-xylanases randomly degrade the xylan main backbone. β-D-xylosidases are able to cleave xylose monomers from the non-reducing end of xylooligosaccharides and xylobiose while the side groups are removed by α-L-arabinofuranosidases, α-D-glucuronidases, acetylxylan esterases, ferulic acid esterases and *p*-coumaric acid esterases (Saha 2003; Collins et al. 2005). Xylanolytic enzymes are not only essential for providing a source of carbon for hemicellulose-utilizing microbes, but they are also involved in plant cell infection by plant pathogens. These enzymes play important roles in the breakdown of xylan and could be applied in the bioconversion of plant biomass, biobleaching etc.(Saha 2003; Polizeli et al. 2005; Calero-Nieto et al. 2007).

Many microorganisms, including bacteria, actinomyces as well as fungi have been investigated for xylanolytic ability. The ecological niches of these microorganisms are diverse and widespread, but they generally survive in the environments where plant material accumulates and deteriorates. Among xylanolytic enzymes, endo-1,4-β-D-xylanases, commonly referred to as xylanases or endoxylanases, are expressed by microorganisms in the presence of xylan-containing substrates. These enzymes are predominantly found in two discrete sequence families known as GH10 (glycosyl hydrolase family 10) and GH11 (glycosyl hydrolase family 11). Xylanases have also been found in glycosyl hydrolase families 5, 8, 43 (Collins et al. 2005). An inverse relationship between the p*I* and molecular mass values has been found in xylanases belonging to GH10 and GH11. Furthermore, the structure and substrate specificity has been reported to be dissimilar between these two families (Liu et al. 2004; Collins et al. 2005). In addition to the production of various enzymes with debranching activities, many microorganisms are able to produce multiple endoxylanases in order to acclimatize to various plant structural polysaccharides. For instance, *Aspergillus niger* produces five types of extracellular xylanases, and *Streptomyces* sp. 3137 also expresses three types of xylanases (Wong et al. 1988). The crude enzyme of *Paenibacillus curdlanolyticus* B-6 also exhibited 12 proteins with xylanase activity (Pason et al. 2006). It appears that multiple xylanases may have specialized functions that result in more effective xylan hydrolysis, thereby permitting saprophytes to utilize a wider range of plant debris (Wong et al. 1988; Elegir et al. 1994).

Zymography is the most effective way of studying xylanases with different molecular weights (Elegir et al. 1994; Sachslehner et al. 1998). However, xylanases of similar size but with different p*I* values cannot be differentiated from one another on the gel. Immunoblotting has also been used to detect xylanase production (Li et al. 1993), but the antibody preparation necessary for this method of analysis is time consuming. RT-PCR (reverse transcriptase PCR) is another method for examining xylanase gene expression; however, the results of RT-PCR do not directly represent the amount of xylanase production (Jun et al. 2003; Calero-Nieto et al. 2007). Recently, 2DE (two-dimensional electrophoresis) has been widely used for the separation of protein samples on an acrylamide gel according to p*I* and molecular weight. The amount of expressed protein also can be estimated according to the intensity of the spot on the gel (Oda et al. 2006). However, the catalytic ability of protein is difficult to present on the gel. Therefore, integrating zymogram and 2DE will facilitate a more complete examination of xylanase multiplicity.

Mesophilic *Streptomyces* is one of the major bacteria found within plant debris composts(Yang et al. 2001). It had been found that the ability of this bacterial species to degrade and utilize hemicellulose was due to the expression of multiple xylanases; however, the xylanolytic systems of *Streptomyces* were rare to be completely realized (Chen et al. 2006). In the present study, the xylan-degrading enzymes of *S. thermonitrificans* NTU-88 were separated, and their activities on two-dimensional acrylamide and zymogram gels were determined. The xylanase expressed in the greatest quantity, STXF10, was excised and identified by mass spectrometry. The properties of the purified STXF10 were then characterized in order to better understand the roles that STXF10 plays in the degradation of oat-spelt xylan in the mesophilic environment.

Methods

Microorganism and the production of xylanolytic enzymes

S. thermonitrificans NTU-88 was isolated from agricultural-waste compost (Jang and Chen 2003). The isolate was grown for eight days at 50°C in Mandels–Reese broth supplemented with 1% (wt./vol.) oat-spelt xylan in order to induce expression of xylanases. The broth was collected by centrifugation (4°C, 10,000×*g*, 30 min) and filtered through a membrane (0.45 μm, Millipore, Ireland) as the crude enzyme solution(Cheng et al. 2008).

Two-dimensional electrophoresis and zymogram

The sample for protein electrophoresis was concentrated in a centrifugal device by passage through a 3×10^3 molecular weight cutoff membrane (Amicon ultra, Millipore). The salts in the concentrate were removed by dialysis. The dialyzed samples were dehydrated using a freeze dryer, and the dried samples were dissolved in denaturing solution before two-dimensional analysis. Two 7-cm-long pH gradient IPG strips (pH 3 to 10, Immobiline^{TM} DryStrip, GE Healthcare Bio-Sciences AB, Sweden) were used in the first dimensional separation. The denaturing solution containing the protein sample was used to passively hydrate the IPG strips. The IPG strips were then placed on an IPGphor (GE Healthcare Bio-Sciences AB) for the isoelectric focusing separation procedure. The IPG strips were equilibrated for 20 min in 10 mL of equilibration solution and subsequently separated on polyacrylamide gels for second dimensional electrophoresis. The electrophoresis was carried out using a Hoefer mini VE system (GE Healthcare Bio-Sciences AB; Sabounchi-Schutt et al. 2000). The polyacrylamide gel was visualized by a protein silver-staining kit (GE Healthcare Bio-Sciences AB).

The zymogram gel was prepared by incorporating 0.5% (wt./vol.) of oat-spelt xylan into the 10% (wt./vol.) polyacrylamide gels used for second dimension electropho-

resis. After electrophoresis, the gels were washed three times with 1% (vol./vol.) Triton X-100, and equilibrated with potassium phosphate buffer (50 mM, pH 6.5) containing 50 mM DTT (MDBio, Inc., Taiwan) at 4°C for 2 h and followed by incubation for 12 h at 37°C in 100 mM potassium phosphate buffer (pH 6.5). The zymogram slab was stained with 0.1% (wt./vol.) Congo red solution and destained with 1 M sodium chloride (Theather and Wood 1982; Cheng et al. 2008). For image documentation and computer analysis, gels were scanned with a scanner (SZW3300U, Banq Crop), and Adobe Photoshop software, version 6.0 (Adobe Systems Incorporated), at 600-dpi resolution.

Protein identification

The induced spot was excised and subjected to in-gel reduction and trypsin hydrolysis. Mass spectrometry analysis of the peptides was performed on an ESI-Q-TOF mass spectrometer (ABI QSTAR Pulsar i System, Applied Biosystems, CA, USA) fitted with an UltiMate Capillary LC System (Thermo Finnigan, CA, USA). Mass spectrometry data were compared with data in the NCBInr and SwissProt databases using the Mascot search algorithm (Perkins et al. 1999). Search parameters included: trypsin with one missed cleavage site; taxonomy of *Streptomyces coelicolor*; possible carbamidomethyl, deamidated, oxidation, and pryo-carbamidomethyl modifications; an MS/MS mass tolerance of 1.2 Da; a peptide charge of 2+; assumed monoisotopic masses; instrument ESI-TRAP.

Amplification of the GH10 xylanase gene from *S. thermonitrificans* NTU-88

The PCR reaction adopted herein was used for the amplification of GH10 xylanase genes obtained from the genomic DNA samples extracted from the biomass of *S. thermonitrificans* NTU-88. Two primers, GH10F (5′-CACGCCGCCGAGAGCACG-3′) and GH10R (5′-TCAGGTGCGGGTCCAGCGTTGG-3′), were designed based upon the *Streptomyces* GH10 xylanase sequences. The PCR products were cloned into the pGEMT-easy vector. The ligation mixture was used to transform *E. coli* DH5α (Invitrogen, Carlsbad, CA) by electroporation. The resultant plasmids were purified and the sequence of the xylanase gene inserts was determined by automatic sequencing (MDBio Inc. Taipei; Cheng et al. 2008).

Enzyme purification

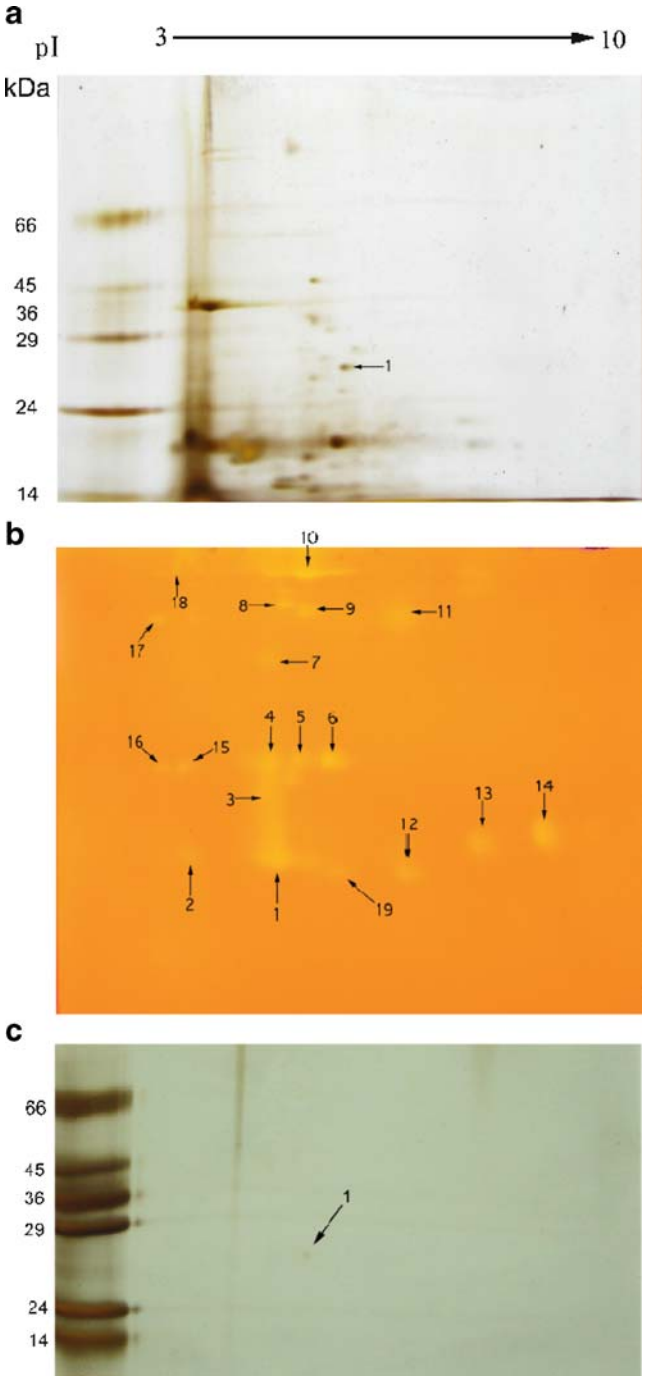
The sample for chromatography was concentrated and replaced its liquid by 20 mM Tris buffer (pH 8) containing 2 M ammonium sulfate by passage through a 5×10^3 molecular weight cutoff membrane (Amicon Ultra, Millipore) before being applied to a Hiprep^{TM} 16/10 phenyl FF column (GE Healthcare Bio-Sciences AB) equilibrated with the same buffer. Proteins were eluted from the column with an ammonium sulfate gradient (2 to 0 M). Fractions containing xylanase activity were combined, buffered with 20 mM Tris buffer (pH 8), and applied to an anion-exchange DEAE-Sepharose FF column (GE Healthcare Bio-Sciences AB). The bound proteins were eluted with the Tris buffer (20 mM, pH 8) containing sodium chloride with an ionic strength gradient from 0–1 M. Subsequently, the pool containing xylanase activity was concentrated by a centrifugal device (YM-10, Millipore), and purified by gel-filtration chromatography on a Superdex G75 column (GE Healthcare Bio-Sciences AB) using citrate buffer (50 mM, pH 6) as the mobile phase. The apparent values of p*I* and molecular weight of the purified sample were examined by 2DE as described above. The molecular weight was confirmed by mass spectrometry.

Enzyme activity assays

The activity of STXF10 was measured by a method described by Cheng et al. (2008). STXF10 was assayed by measuring the liberation of reducing sugar from 1% oat-spelt xylan suspended in citrate buffer (50 mM, pH 6). The mixture was incubated in a water bath at 50°C for 30 min. Reducing sugar, liberated as a result of enzymatic hydrolysis, was determined by the DNS method (Lin et al. 1999). One unit (U) is defined as the amount of enzyme that releases 1 μmol of reducing sugar per min. Protein concentration was determined using a BCA protein assay kit (Pierce, Rockford IL, USA).

Enzyme properties

For estimation of the temperature and pH optima, the relative activity was determined at several temperatures and at several pH levels. The range of buffers used in preparing 1% (wt./vol.) xylan solution for detecting xylanase activity comprised of citrate buffer (50 mM, pH 3–6), phosphate buffer (50 mM, pH 6–7), and Tris buffer (20 mM, pH 7–9; Lin et al. 1999). The reaction conditions were the same as those mentioned previously. The optimal temperature for enzyme activity was determined by performing the standard assay procedure at a range of temperatures (10 to 100°C). All subsequent enzyme assays were performed at the optimum temperature. The temperature stability of xylanase was determined by incubating the enzyme without substrate aseptically in 50 mM phosphate buffer, pH 6.0, at different temperatures (10–100°C) for 30 min; the activity was then determined by a standard assay (Saraswat and Bisaria 2000). The kinetic parameters of STXF10 were derived



◀ **Fig. 1** Two-dimensional silver-stained gels and zymogram. The gel images are oriented with the IEF in the horizontal dimension and the SDS-PAGE in the vertical dimension. **a** Extracellular proteins induced by oat-spelt xylan were separated on a two-dimensional gel with 10% polyacrylamide with IEF ranging from pH 3 to 10. **b** Composition of the xylanolytic enzymes of *S. thermonitrificans* NTU-88 on the zymogram gel. Proteins were separated on the same two-dimensional gels with additional 0.5% oat-spelt xylan. The gel was stained with 0.1% Congo red solution after buffer equilibration. The clear spots are indicated by arrows, and 19 clear spots were found on the zymogram gel including *spot 1* (pI5.2, Mr: 26 kDa), *spot 2* (pI3.5, Mr: 26 kDa), *spot 3* (pI5.5, Mr:38 kDa), *spot 4* (pI5.0, Mr: 50 kDa), *spot 5* (pI5.5, Mr: 50 kDa), *spot 6* (pI6.0, Mr: 50 kDa), *spot 7* (pI5.0, Mr: >66 kDa, *spot 8* (pI5.0, Mr: >66 kDa), *spot 9* (pI5.8, Mr: >66 kDa), *spot 10* (pI5.8, Mr: >66 kDa), *spot 11* (pI7.2, Mr: >66 kDa), *spot 12* (pI7.5, Mr: 26 kDa), *spot 13* (pI8.5, Mr: 28 KDa), *spot 14* (pI9.8, Mr: 30 KDa), *spot 15* (pI3.5, Mr: 45 KDa), *spot 16* (pI3.0, Mr: 48 kDa), *spot 17* (pI3.0, Mr: >66 kDa), *spot 18* (pI3.2, Mr:>66 kDa), and *spot 19* (pI6.5, Mr: 24.5 kDa). **c** STXF10 migrated on the two-dimensional polyacrylamide gel after chromatographic purification. The STXF10 is indicated by an *arrow*

from Lineweaver–Burk plots constructed using the program Enzfitter (version 2.0.18.0, Biosoft, UK; Cheng et al. 2002).

Enzyme specificity

Enzyme was tested for its specificity to hydrolyze a variety of substrates, including 1% (wt/vol) oat-spelt xylan, 1% (wt./vol.) beechwood xylan, 1% (wt./vol.) birchwood xylan, 1% (wt./vol.) carboxylmethyl cellulose, and 0.2% (wt./vol.) Avicel (PH101, Asahi, Japan). These substrates were suspended in citrate buffer (50 mM, pH6). Soluble xylan was prepared according to the description by Chen et al. (2001). The reaction mixture consisting of equal volumes of substrate solution and STXF10 solution in 50 mM citrate buffer (pH 6.0) was incubated at 50°C for 30 min. Reducing sugars were determined by DNS methods (Saraswat and Bisaria 2000). Each experiment was conducted in triplicate.

Analysis of hydrolysis products

The reaction mixture (50 μL) consisting of equal volumes of a 3% (wt./vol.) suspension of oat-spelt xylan and the STXF10 solution (20 U mL^{-1}) in 50 mM citrate

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1   HAAESTLGAA AAQSGRYFGT AIASGRLSDS TYTSIAGREF NMVTAENEMK
51  IDATEPQQGR FDFTAGDRVY NWAVQNGKEV RGPTLAWDLQ QPAWMPNLGG
101 SALRQAMINH INGVMTHYKG KIAQWDVVNE AFADGNSGAR RDSNLQRTGN
151 DWIEVAFRTA RAADPSAKLC YNDYNVENWN AAKTQAMYAM VRDFKQRGVP
201 IDCVGFQAHF NSGNPYNSNF RTTLQNFADL GVDVAVTELD IQGAPASTYA
251 SVVNDCLAVS RCLGITVWGV RDSDSWRSEQ TPLLFNNDGS KKAAYTAVLN
301 ALNGDDTTPL PPEGGQIKGV GSGRCLDVPN ASTADGTQVQ LWDCHSGSNQ
351 QWTYTDAGEL RVYGNKCLDA AGTGNGAKVQ IYSCWGGDNQ KWGLNSDGSF
401 VGVQSGLCLD AVGAGTGNGT LIQLYSCSGG SNQRWTRT*
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Fig. 2 The search results of mass spectrometry data from spot 1. The deduced amino acid sequences of *S. thermonitrificans* NTU-88 GH10 xylanase are the most probable ones and are therefore used as reference sequences. The matched peptides between STXF10 and reference sequence are *boxed*. The consensus pattern of glycosyl hydrolase family 10 in the matched region is *bold*

Table 1 Purification of STXF10 from *S. thermonitrificans* NTU-88

Purification step	Volume (mL)	Total activity (U)	Specific activity (U mg^{-1} protein)	Purification fold	Yield (%)
Culture extract	40	2345.7	45.4	1.0	100
Phenyl hydrophobic chromatography	9	270.1	535.9	11.8	12
DEAE-Sepharose chromatography	6	95.1	609.8	13.4	4
Superdex G75-Gel filtration	3	26.5	885.2	19.5	1

buffer (pH 6.0) was incubated at 50°C for 4 h. Hydrolysis was stopped by boiling for 10 min, and the hydrolyzed products were analyzed by TLC (thin layer chromatography) on silica gel plates (Merck AG, Darmstadt, Germany). The TLC plates were developed twice at room temperature with a solvent system comprising ethyl-acetate, acetic acid, formic acid, and water (9:3:1:4, vol./vol.). Spots were stained by spraying the plates with orcinol–sulfuric acid reagent and then heating at 100°C for 5 min (Cheng et al. 2008). All chemicals in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless mentioned otherwise.

Results

Two-dimensional SDS-PAGE analysis and zymography of protein components

The extracellular xylan-induced proteins of *S. thermonitrificans* NTU-88 were concentrated and separated on a two-dimensional polyacrylamide gel and on a zymogram gel (Fig. 1). More than 16 spots were found on the two-dimensional polyacrylamide gel, and most were located in the region of p*I* 3–6 and molecular weight 14–45 kDa (Fig. 1a). No spots and no clear zones were seen on the gels of protein samples that had been collected from the first-day broth. Xylanase activity was also not detected on the zymogram gels when glucose and xylose were used as the sole carbon sources. As demonstrated in Fig. 1a, the level of protein expression represented by spot one increased after 8 days of fermentation, and only this spot was observed in the corresponding zymogram gel. Although distinct clear zones appeared on the zymogram gel, spots 2–19 were hard to visualize in the same position of silver-stained gel. These results indicate that 19 xylan-degrading enzymes were produced, and that spot one was the main product with xylanolytic ability in extracellular broth when *S. thermonitrificans* NTU-88 was grown on oat-spelt xylan-containing medium.

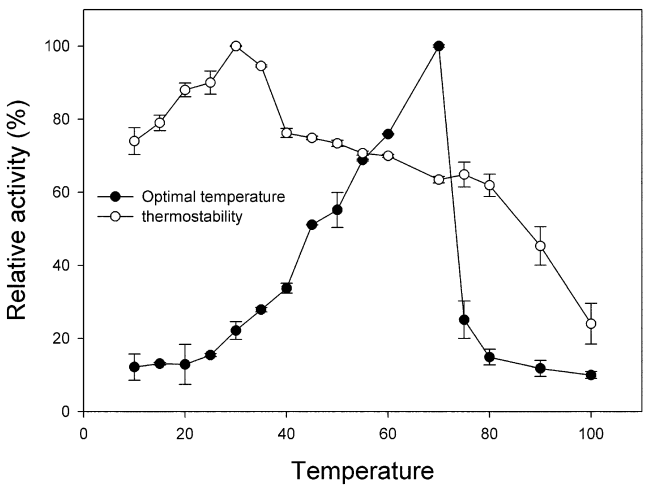

Fig. 3 Optimal temperature and thermostability of STXF10. Purified STXF10 in substrate solution was reacted at a range of temperatures (10 to 100°C) in order to examine temperature optima. Thermostability of STXF10 was analyzed following its incubation at elevated temperatures for 30 min. After that, the enzyme activity was measured by the standard protocol. Enzyme activities are compared to the largest activity value attained (i.e., 100%)

The identification of STXF10

Spot one was excised from the polyacrylamide gel for protein identification by mass spectrometry analyses and database searching. The proteins was identified with significant protein scores ($p<0.05$) from Mascot searches of peptide mass fingerprints. Three trypsin-digested peptides matched the amino acid sequence of *S. coelicolor* A3 xylanase A (GH10, accession number: NP_733679). The matched fragments also could be found in the GH10 xylanase of *S. thermonitrificans* NTU-88 (Fig. 2). One consensus pattern (GTA) of GH10 (Henrissat and Davies 1997) was also found in the first matched fragment. This result indicates that spot one is an endoxylanase belonging to the member of GH10.

Purification of STXF10

S. thermonitroficans NTU-88 xylanase reached a maximum extracellular specific activity of 45.46 U mg^{-1} protein after 8 days when cultivated on 10 g of oat-spelt xylan L^{-1}. The enzyme was collected from the culture supernatant by centrifugation and purified. Table 1 summarizes the procedures for the purification of xylanolytic enzyme. After

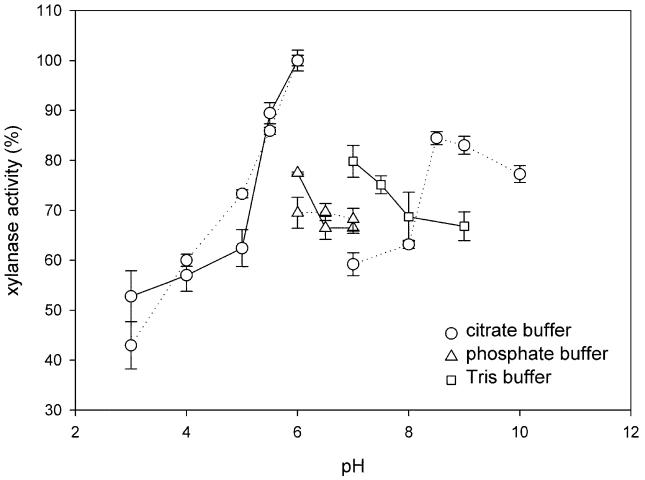

Fig. 4 pH optima and stability of STXF10. Optimal reaction pH of STXF10 was analyzed in different buffers with specific pH values (pH 3–9). STXF10 was incubated for 30 min with buffers of different pH values, and the activity was measured by the DNS method to determine the stability of the enzyme at different pH values. Enzyme activities are compared to the largest activity value attained (i.e., 100%)

hydrophobic interaction chromatography, xylanase was bound to matrix and eluted with phosphate buffer containing 0.4 M ammonium sulfate. After anion-exchange chromatography, xylanase activity was assigned to the unbound fraction at pH 7. Finally, size-exclusion chromatography yielded a single active peak. The yield of purified xylanase (1%) was 19.5-fold higher than the yield found in the crude culture supernatant (Table 1). The purified product had a specific activity of 885.29 U mg^{-1} protein when reacted with oat-spelt xylan. The protein was designated as STXF10 and subjected to further characterization.

STXF10 was separated by 2DE. The first purified product gave a single spot with an estimated p*I* of 5.2 and molecular weight of 26 kDa (Fig. 1c). The corresponding position of STXF10 conformed to spot one in Fig. 1a. The molecular weight of STXF10 was confirmed by mass spectrometry, which showed that the protein had a molecular weight of 25.97 kDa. The trypsin-digested fragments of STXF10 was also analyzed by ESI-Q-TOF MS and searched in mascot using the previously described parameters. The search results showed that three peptides also matched the amino acid sequences of *S. coelicolor* A3 xylanase A and *S. thermonitrificans* NTU-88 GH10 xylanase (Fig. 2).

The properties of STXF10

The effects of temperature variation and thermostability upon the activity of STXF10 are illustrated in Fig. 3. The activity of STXF10 remained above 50% between 30°C and 70°C and exhibited a maximum activity at 70°C in a 30-min assay with oat-spelt xylan as the substrate. The enzyme was stable when exposed to temperatures ranging from 10°C to 80°C, but the enzyme activity decreased above 90°C. The activity of STXF10 at different pH values is given in Fig. 4. Examination of the effect of pH upon STXF10 activity revealed the following features. The optimum pH value of xylanase was pH 6.0. The pH stability was compared in a range of pH 3–9 at 50°C during 30 min incubation periods (Fig. 4). STXF10 exhibited good stability at pH 4–9. These biochemical characteristics of STXF10 are similar to those reported for many of the enzymes of *Streptomyces* strains isolated from compost (Ruiz-Arribas et al. 1998). The thermostability of the enzyme appears to be quite pronounced within the mesophilic range.

Kinetic experiments at 50°C with different concentrations of two xylans are listed in Table 2. The K_m values of STXF10 for oat-spelt xylan and birchwood xylan were 3.06 and 7.6 mg mL^{-1}, indicating that STXF10 is able to bind oat-spelt xylan with greater affinity than birchwood xylan. The catalytic efficiency of STXF10 for oat-spelt xylan was 1.8-fold higher than that of birchwood xylan. Although multiple xylanases were found in the extracellular fraction of *S. thermonitroficans* NTU-88, the results suggest that STXF10 was specifically induced by oat-spelt xylan.

Xylan hydrolytes of STXF10

The hydrolyzed products released from oat-spelt xylan by STXF10 are presented in Fig. 5. The resultant end products consisted of xylotriose and xylobiose and a mixture of large xylooligosaccharide fragments; however, xylose was not visualized on the TLC plate in the early period. The xylotriose was further split into xylobiose and xylose by STXF10 after 16 h hydrolysis. This indicates that STXF10

Table 2 Kinetic parameters of STXF10

Substrate	K_m (mg mL^{-1})	V_{max} (U mg^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (mL min^{-1} mg^{-1})
Oat-spelt xylan	3.06±0.29	1.21±0.03	10,807±268	3,532
Birchwood xylan	7.60±0.82	1.63±0.08	14,559±715	1,915

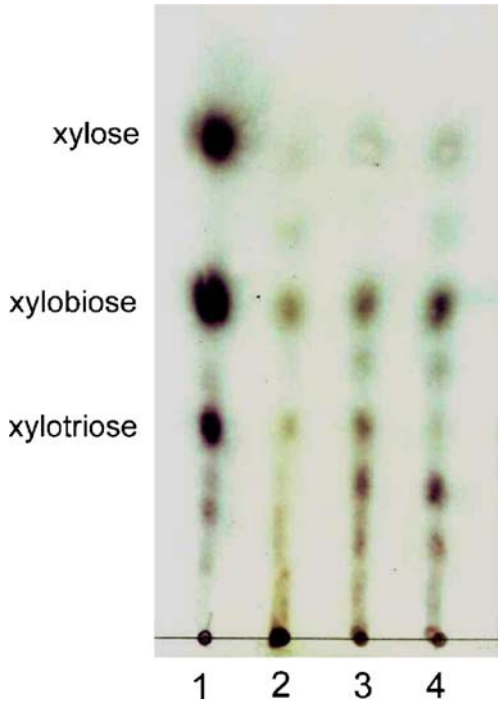

Fig. 5 Thin layer chromatography of hydrolysates produced by STXF10. The enzyme was incubated at 50°C for 2 h (*lane 2*), 4 h (*lane 3*) and 16 h (*lane 4*) in a 2 mL solution containing 1% xylan in 50 mM phosphate (pH 6.0). At the times shown, samples were heated to 100°C for 10 min to inactivate the enzyme and then spotted onto a chromatographic plate. Xylose, xylobiose, and xylotriose were used as standards and separated in *lane 1*

is an endo-type xylanase, and its mode of action was also similar to that of GH10 (Kluepfel et al. 1990; Ducros et al. 2000).

The enzyme specificity of STXF10

STXF10 appeared to be specific for xylan degradation, the highest activity being found on oat-spelt and birchwood xylans (Table 2). However, the enzyme showed a relative activity of 67% when oat-spelt was replaced by beechwood xylan. Xylan from different sources exhibits considerable variation in composition and structure. The most abundant xylan in hardwood (e.g. beechwood) is *O*-acetyl-(4-*O*-methylglucurono) xylan. In softwood plants such as birchwood and cereal, the main type of xylan is arabino-4-*O*-methyl glucuronoxylan, but the degree of polymerization of softwood xylans is higher than that of cereals (Saha 2003). It appears that arabino-4-*O*-methyl glucuronoxylan was more specific than *O*-acetyl-(4-*O*-methylglucurono) xylan for the digestion of STXF10.

In addition to xylanolytic activity, STXF10 also displayed slight activity toward carboxylmethyl cellulose and avicel as compared to xylan substrates (Table 3). According to previous studies of substrate specificity, xylanases of GH10 may not be highly specific for xylan and may also be active on cellulose substrates of low molecular mass (Collins et al. 2005).

Discussion

The multiplicity of xylanase in microorganisms has widely investigated. As many as five distinct xylanases have been characterized in streptomycetes using protein purification or zymogram technologies (Wong et al. 1988; Elegir et al. 1994; Arias et al. 2007). This number of xylanases in *S. thermonitrificans* NTU-88 found in this study is much higher than those previously reported (Peters et al. 2000; Jang and Chen 2003; Ryckeboer et al. 2003). These clear spots on the zymogram gel belonged to one of the enzymes responsible for degrading main and side chains of xylan. The reducing ends would be detectible while the xylooligosaccharides or side groups would be removed by these enzymes. The multiplicity of xylanase might be also caused by protease degradation or posttranslational modification; however, the results still demonstrate a better resolution of the multiple xylanases of *S. thermonitrificans* NTU-88 than previous studies of streptomycetes using protein purification or zymogram technologies (Wong et al. 1988; Elegir et al. 1994). In addition to STXF10, the other clear spots on the zymogram gel (Fig. 1) were also excised and subjected to in-gel reduction and trypsin hydrolysis, but no detectable peptide information was obtained from the analysis of mass spectrometry. The more complete information of bacterial xylanase systems would be demonstrated if sensitive detection methods of peptide fragments were developed.

Xylanases are predominantly found in two discrete sequence families known as glycoside hydrolase families 10 and 11. These two types of xylanase not only differed in p*I* value and molecular weight but also in catalytic properties. It has been suggested that xylanases belonging to GH10 exhibit greater catalytic versatility and lower substrate specificity than do those belonging to family 11 (Collins et al. 2005). Biely et al. also indicated that endoxylanases of GH10, in contrast to the members of GH11, are capable of attacking the glycosidic linkages next

Table 3 Substrate specificity of STXF10

Substrate(1%)	Relative activity (%)
Oat-spelt xylan	100
Soluble oat-spelt xylan	86
Birchwood xylan	82
Beechwood xylan	67
Avicel	11
Carboxylmethyl cellulose	15

to the branch points and toward the non-reducing end (Biely et al. 1985). In this study, multiple xylanases including a GH10 xylanase (STXF10) were detected after the induction of oat-spelt xylan. The GH11 xylanase gene of *S. thermonitrificans* NTU-88 was also characterized in our previous study (Cheng et al. 2008). Of the 19 xylanolytic enzymes induced, STXF10 is not only expressed at higher level than others (Fig. 1) but also demonstrated a broad range of pH (Fig. 4), good thermostability (Fig. 3) and insignificant substrate specificity (Table 3). Therefore, this enzyme appears to be the xylanase primarily responsible for the degradation of xylan in natural lignocelluloses in compost.

In conclusion, we successfully demonstrated the presence of multiple xylanases in *S. thermonitrificans* NTU-88 on a two-dimensional zymogram profile. Of the 19 xylanolytic enzymes expressed, STXF10, a GH10 xylanase was the predominant one when *S. thermonitrificans* NTU-88 was exposed in oat-spelt xylan. STXF10 possessed low specificity for its substrate and good adaptation to various reaction conditions. These properties allow *S. thermonitrificans* NTU-88 to utilize the xylan of lignocellulose during the periods of composting. Although *S. thermonitrificans* NTU-88 possesses multiple xylanases, our results suggest that the major form of xylanase might be selectively and specifically induced depending on the type of substrate to which the microorganism is exposed.

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References

Arias E, Li H, Morosoli R (2007) Effect of protease mutations on the production of xylanases in *Streptomyces lividans*. Can J Microbiol 53:695–701

Biely P, Markovic O, Mislovicova D (1985) Sensitive detection of endo-1,4-beta-glucanases and endo-1,4-beta-xylanases in gels. Anal Biochem 144:147–151

Calero-Nieto F, Di Pietro A, Roncero MI, Hera C (2007) Role of the transcriptional activator xlnR of *Fusarium oxysporum* in regulation of xylanase genes and virulence. Mol Plant Microbe Interact 20:977–985

Chen YL, Tang TY, Cheng KJ (2001) Directed evolution to produce an alkalophilic variant from a *Neocallimastix patriciarum* xylanase. Can J Microbiol 47:1088–1094

Chen YC, Tsai CY, Wang PM, Chiang YC, Huang HK, Yang SS, Cheng HL (2006) The studies of xylanases produced by *Streptomyces thermonitrificans* NTU-88. J Biomass Energy Soc China 25:33–39

Cheng HL, Tsai LC, Lin SS, Yuan HS, Yang NS, Lee SH, Shyur LF (2002) Mutagenesis of Trp(54) and Trp(203) residues on *Fibrobacter succinogenes* 1,3-1,4-beta-D-glucanase significantly affects catalytic activities of the enzyme. Biochemistry 41:8759–8766

Cheng HL, Wang PM, Chen YC, Yang SS, Chen YC (2008) Cloning, characterization and phylogenetic relationships of stxI, a endoxylanase-encoding gene from *Streptomyces thermonitrificans* NTU-88. Bioresour Technol 99:227–231

Collins T, Gerday C, Feller G (2005) Xylanases, xylanase families and extremophilic xylanases. FEMS Microbiol Rev 29:3–23

Ducros V, Charnock SJ, Derewenda U, Derewenda ZS, Dauter Z, Dupont C, Shareck F, Morosoli R, Kluepfel D, Davies GJ (2000) Substrate specificity in glycoside hydrolase family 10. Structural and kinetic analysis of the *Streptomyces lividans* xylanase 10A. J Biol Chem 275:23020–23026

Elegir G, Szakacs G, Jeffries TW (1994) Purification, characterization, and substrate specificities of multiple xylanases from *Streptomyces* sp. strain B-12-2. Appl Environ Microbiol 60:2609–2615

Henrissat B, Davies G (1997) Structural and sequence-based classification of glycoside hydrolases. Curr Opin Struct Biol 7:637–644

Jang HD, Chen KS (2003) Production and characterization of thermostable cellulases from *Streptomyces* transformant T3-1. World J Microbiol Biotechnol 19:263–268

Jun HS, Ha JK, Malburg LM Jr., Verrinder GA, Forsberg CW (2003) Characteristics of a cluster of xylanase genes in *Fibrobacter succinogenes* S85. Can J Microbiol 49:171–180

Kluepfel D, Vats-Mehta S, Aumont F, Shareck F, Morosoli R (1990) Purification and characterization of a new xylanase (xylanase B) produced by *Streptomyces lividans* 66. Biochem J 267:45–50

Li XL, Zhang ZQ, Dean JF, Eriksson KE, Ljungdahl LG (1993) Purification and characterization of a new xylanase (APX-II) from the fungus *Aureobasidium pullulans* Y-2311-1. Appl Environ Microbiol 59:3212–3218

Lin J, Ndlovu LM, Singh S, Pillay B (1999) Purification and biochemical characteristics of beta-D-xylanase from a thermophilic fungus, *Thermomyces lanuginosus*-SSBP. Biotechnol Appl Biochem 30:73–79

Liu L, Zhang J, Chen B, Shao W (2004) Principle component analysis in F/10 and G/11 xylanase. Biochem Biophys Res Commun 322:277–280

Oda K, Kakizono D, Yamada O, Iefuji H, Akita O, Iwashita K (2006) Proteomic analysis of extracellular proteins from *Aspergillus oryzae* grown under submerged and solid-state culture conditions. Appl Environ Microbiol 72:3448–3457

Pason P, Kyu KL, Ratanakhanokchai K (2006) *Paenibacillus curdlanolyticus* strain B-6 xylanolytic-cellulolytic enzyme system that degrades insoluble polysaccharides. Appl Environ Microbiol 72:2483–2490

Perkins DN, Pappin DJ, Creasy DM, Cottrell JS (1999) Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 20:3551–3567

Peters S, Koschinsky S, Schwieger F, Tebbe CC (2000) Succession of microbial communities during hot composting as detected by PCR-single-strand-conformation polymorphism-based genetic profiles of small-subunit rRNA genes. Appl Environ Microbiol 66:930–936

Polizeli ML, Rizzatti AC, Monti R, Terenzi HF, Jorge JA, Amorim DS (2005) Xylanases from fungi: properties and industrial applications. Appl Microbiol Biotechnol 67:577–591

Ruiz-Arribas A, Zhadan GG, Kutyshenko VP, Santamaria RI, Cortijo M, Villar E, Fernandez-Abalos JM, Calvete JJ, Shnyrov VL (1998) Thermodynamic stability of two variants of xylanase (Xys1) from *Streptomyces halstedii* JM8. Eur J Biochem 253:462–468

Ryckeboer J, Mergaert J, Vaes K, Klammer S, de Clercq D, Coosemans J, Insam H, Swings J (2003) A survey of bacteria and fungi occurring during composting and self-heating processes. Ann Microbiol 53:349–410

Sabounchi-Schutt F, Astrom J, Olsson I, Eklund A, Grunewald J, Bjellqvist B (2000) An immobiline DryStrip application method

enabling high-capacity two-dimensional gel electrophoresis. Electrophoresis 21:3649–3656

Sachslehner A, Nidetzky B, Kulbe KD, Haltrich D (1998) Induction of mannanase, xylanase, and endoglucanase activities in *Sclerotium rolfsii*. Appl Environ Microbiol 64:594–600

Saha BC (2003) Hemicellulose bioconversion. J Ind Microbiol Biotechnol 30:279–291

Saraswat V, Bisaria VS (2000) Purification, characterization and substrate specificities of xylanase isoenzymes from *Melanocarpus albomyces* IIS 68. Biosci Biotechnol Biochem 64:1173–1180

Theather RM, Wood PJ (1982) Use of Congo red–polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. Appl Environ Microbiol 37:777–780

Wong KK, Tan LU, Saddler JN (1988) Multiplicity of beta-1, 4-xylanase in microorganisms: functions and applications. Microbiol Rev 52:305–317

Yang X, Chen H, Gao H, Li Z (2001) Bioconversion of corn straw by coupling ensiling and solid-state fermentation. Bioresour Technol 78:277–280