

Full Length Research Paper

# Isolation, cloning and molecular characterization of a thermotolerant xylanase from *Streptomyces* sp. THW31

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A xylan-degrading *Streptomyces* sp. THW31 was isolated from rubbish compost in Thailand. Analysis of a genomic library of nucleotide sequences from *Streptomyces* sp. THW31 revealed that the complete open reading frame (ORF) of xylanase (*xlnW31*) was 999 bp, and this gene encoded a member of the glycosyl hydrolase family 11. Sequence homology of the predicted amino acid sequence encoded by *xlnW31* demonstrated that the enzyme consists of a signal peptide, catalytic and substrate-binding domains. The XlnW31 enzyme shared the highest identity (90%) to a xylanase family 11 member from *Streptomyces lividans* TK24. Cloning and expression of *xlnW31* in *Escherichia coli* resulted in the production and purification of a 31.0 kDa enzyme. Purified XlnW31 show the highest activity at pH 6.0 and at a temperature 65 to 70°C. Enzyme stability tests indicated that XlnW31 retained its activity over a broad pH range (5.0 to 11.0) and at temperatures reaching 60°C for 2 h. Purified XlnW31 also exhibited endo-1,4-β-xylanase activity using xylan as a substrate and bound to insoluble xylan. Hydrolysis of xylan by the xylanase yielded xylobiose as the principal product.

**Key words:** Gene expression, hemicellulase, *Streptomyces*, xylan, xylanase.

## INTRODUCTION

The lignocellulosic plant cell wall is comprised of the three major components (cellulose, hemicellulose and lignin). Xylan, a major component of hemicelluloses, is a heteropolysaccharide that contains a backbone of β-1, 4-linked xylopyranose units with substituent groups of acetyl, arabinosyl and glucuronosyl residues (Puls, 1997). Endoxylanases (1,4-β-D-xylan xylanohydrolase; E.C 3.2.1.8) randomly hydrolyze 1,4-β-D-xylosidic linkages of the main chain of xylan to xylooligosaccharides, which are then further degraded to xylose monomers from the non-reducing end of xylooligosaccharides and xylobiose by β-D-xylosidase

(EC 3.2.1.37) (Blely et al., 1997). The side groups that are present in heteroxylan are liberated by accessory enzymes, such as α-L-arabinofuranosidases (EC3.2.1.55), α-D-glucuronidases (EC 3.2.1.139) and acetyl xylan esterases (EC 3.1.1.72).

Xylanases are fast becoming a major group of industrial enzymes with significant application in the paper and pulp industry. These enzymes hydrolyze xylan and facilitate the release of lignin from paper pulp, which helps to reduce the use of toxic chlorine as a bleaching agent (Viikari et al., 1994). Potential applications of xylanase also include the bioconversion of xylan in lignocellulosic material and agro-wastes to fermentative sugar and food products (Coughlan and Hazlewood, 1993). Moreover, the xylan hydrolysis products can be subsequently converted to liquid fuel (Dodd and Cann, 2009). Many thermotolerant microorganisms including

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bacteria (actinomycetes, eubacteria and archaea) and fungi are known to produce thermostable xylanases. In addition, *Streptomyces* has been reported as the dominant xylanolytic species of actinomycetes producing the hemicellulose degradation enzymes (Subramaniyan and Prema, 2002).

The cellulase free-family 11 xylanases containing cellulose binding domains (CBDs) or xylans binding domains (XBDs) are normally used in hydrolysis applications in which low-molecular-mass xylanase is effective for penetrating and increasing destruction by enzyme attachment to its substrate (Gill, 1999). Thermo- and alkaline-stable xylanases from several *Streptomyces* species have been cloned, sequenced and biochemically characterized (Wang et al., 2007; Georis et al., 2000; Li et al., 2009; Shin et al., 2009). However, there are few reports regarding the cloning and expression of xylanase gene from thermotolerant *Streptomyces* that can produce many hemicellulases using agricultural waste materials as substrates.

In this study, the extracellular thermophilic hemicellulase-producing *Streptomyces* sp. strain THW31 was isolated and catalytic domains including CBD type 2 (CBD2) sequences in the xylanase enzyme were identified and analyzed. The deduced amino acid sequences suggested that this thermotolerant xylanase was classified in xylanase family 11. The xylanase gene was cloned and expressed in *Escherichia coli* and the expressed recombinant enzyme was purified and characterized.

## MATERIALS AND METHODS

### Vectors, reagents and mediums

*E. coli* TOP10 (Invitrogen, Carlsbad, CA, USA) and pZErO-2 (Invitrogen) were used as the host and vector, respectively, for the construction of a genomic library from *Streptomyces* sp. THW31. The pGEM<sup>®</sup>-T easy vector (Promega, Madison, WI, USA) and *E. coli* DH5 $\alpha$  (Takara, Kyoto, Japan) were used for subcloning. pQE80L (Qiagen, Valencia, CA, USA) and *E. coli* Rosetta gami B (DE3) (Novagen, Madison, WI, USA) were used for cloning and expression of a xylanase gene. Xylan-degrading bacterial were grown in an actinomycetes medium (Techapun et al., 2001) and in Luria Bertani (LB) medium at 60°C for three to five days. *E. coli* were grown in LB medium at 37°C for 18 to 24 h. Oat spelt xylan, birchwood xylan, beechwood xylan and *p*-nitrophenyl derivatives were purchased from Sigma-Aldrich (St. Louis, MO., USA). AZCL-oat spelt xylan and xylooligosaccharides were purchased from Megazyme Company (Bray, Ireland). Corncob, corn husk, sugarcane bagasse and rice straw were cut to a small size, grounded with a blender and transferred through a sieve to collect 40-mesh particles, which were washed three times with warm distilled water, dried at 50°C and used as inducers for hemicellulases production.

### Microorganism isolation

*Streptomyces* sp. strain THW31 was collected from rubbish compost in Thailand and isolated using a minimal xylan medium.

The 0.1 g soil sample was transferred to 100 ml of minimal xylan medium in a 1 000 ml Hinton flask and incubated on a rotary shaker at 200 rpm at 60°C for 5 days. The minimal xylan medium contained 10 g oat spelt xylan, 0.2 g yeast extract, 1.0 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.6 g K<sub>2</sub>HPO<sub>4</sub>, 0.2 g KH<sub>2</sub>PO<sub>4</sub>, 0.2 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.1 g NaCl, 0.02 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.01g FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.0005 g Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 0.0005 g Na<sub>2</sub>WO<sub>4</sub>·2H<sub>2</sub>O and 0.0005 g MnSO<sub>4</sub> in 1 L of distill water and was adjusted to pH 7.0. The diluted enriched culture (0.1 ml) was spread on a minimal xylan agar plate and incubated at 60°C for 5 days. Clear zones generated by xylan-degrading strains were visualized using 0.1% Congo red and destained with 1 M NaCl. The strongest xylan-degrader strain THW31 was selected and used for taxonomic identification based on 16S rDNA analysis using the method of Keiichi et al. (2000). A permanent stock of strain THW31 was generated by suspending growing cells in culture medium containing 15% glycerol and storing the resulting stock at -80°C.

### Induction of hemicellulose-degrading enzymes production using agricultural waste

The actinomycetes medium that contained 1.5 g/l KH<sub>2</sub>PO<sub>4</sub>, 2 g/l K<sub>2</sub>HPO<sub>4</sub>, 1.4 g/l (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 g/l yeast extract, 1 g/l peptone, 2 ml Tween 80 and 1 ml trace element solution was supplemented with agricultural waste and used for hemicellulase enzyme production. *Streptomyces* sp. strain THW31 was cultured in 50 ml of actinomycetes medium containing 3% of each kind of agriculture waste such as corncob, corn husk, rice straw and sugarcane bagasse. Each culture was inoculated in 250 ml Hinton flasks and shaken at 60°C for five days. The 3% of oat spelt xylan was added to the same medium for hemicellulase enzyme production. The culture supernatants were collected and assayed for xylanase,  $\beta$ -xylosidase,  $\alpha$ -arabinofuranosidase and acetyl xylan esterase activities.

### Enzymes assay

Xylanase activity was determined by the detection of reducing sugars that were liberated from 1% oat spelt xylan using the method of Bailey et al. (1992). The reaction mixture (0.7 ml) was consisted of 0.1 ml enzyme and 0.6 ml 1% xylan in Mcllvaine buffer (pH 6.0). After incubation for 10 min at 65°C, the reaction was inactivated for 5 min at 95 to 100°C and immediately placed on ice. Reducing sugars were estimated using the dinitrosalicylic acid method. Xylose was used as a standard for the xylanase activity test. One unit of enzyme activity was defined as the amount of enzyme releasing 1  $\mu$ mole reducing sugar per min.

$\beta$ -xylosidase,  $\alpha$ -arabinofuranosidase and acetyl xylan esterase activities were measured using *p*-nitrophenyl- $\beta$ -D-xylopyranoside (pNPX), *p*-nitrophenyl- $\alpha$ -L-arabinofuranoside (pNPA) and *p*-nitrophenyl-acetate (pNA) as the substrate, respectively. All assay mixture consisted of 0.9 ml of *p*-nitrophenyl-derivative (2 mM) in 100 mM Mcllvaine buffer (pH 6.0) and an appropriate volume of enzyme sample to result in a final volume of 1.0 ml. The reaction mixture was incubated at 65°C for 15 min then 1.0 ml of 0.5 M sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>) was added to terminate the reaction. The amount of nitrophenol liberated from a substrate was determined by absorbance measurements at 410 nm. One unit of enzyme activity was defined as the amount of an enzyme to yield 1  $\mu$ mole nitrophenol per min.

### Genomic DNA library construction, PCR and nucleotide sequence analysis

Chromosomal DNA from *Streptomyces* sp. strain THW31 was extracted according to a modification of the method of Marmur

(1961). The purified chromosomal DNA was partially digested with *Sau3AI* at 37°C for different lengths of time to obtain a size range of 2 to 10 kb. The linearized *Bam*HI-digested pZErO-2 vector was ligated to the *Sau3AI*-digested chromosomal DNA and transformed into competent *E. coli* TOP10 cells. Low salt LB agar that was supplemented with 0.5% AZCL-oat spelt xylan, 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) and 50 µg/ml kanamycin was used to screen for transformants that contained xylan-degrading activity. The plates were incubated at 37°C for 24 to 48 h, and positive transformants were identified by the presence of blue zones derived from the hydrolyzed AZCL-xylan around the transformants. Positive colonies were selected and re-analyzed for xylanase activity using the same procedure. The plasmid harboring the xylanase gene was designated as pZErO-X3. The nucleotide sequences of the inserted DNA were analyzed by using primer walking and the open reading frame (ORF) was analyzed using NCBI sequence analysis (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). A homology search was performed using the GenBank BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and multiple alignments were carried out with the CLUSTAL W2 program (<http://www.ebi.ac.uk/Tools/clustalw2/>). The signal peptide was predicted using the SignalP 3.0 server (<http://www.cbs.dtu.dk/services/SignalP/>). Three dimensional models of xylanase were generated using the CPHmodels 3.2 server (<http://www.cbs.dtu.dk/services/CPHmodels/>).

The xylanase gene ORF consisted of a 123 base pair (bp) signal peptide and 876 bp nucleotide sequences of the mature xylanase. The predicted mature xylanase gene was amplified by PCR using KOD Plus Taq Polymerase (Toyobo, Osaka, Japan) in the presence of 5% dimethyl sulfoxide. Primers (F1, 5'-CGGGATCCGGGCACGGTCGTCACGACCAAC-3' and R1, 5'-CCAAGCTTTTCAGCCCGCGTGCAGGAGACC-3'; *Bam*HI and *Hind*III restriction sites are underlined in F1 and R1, respectively) were designed based on the xylanase ORF and were used for xylanase gene expression in *E. coli*. All PCR products were amplified under the following conditions: denaturation at 98°C for 5 min followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 65°C for 1 min and polymerization at 68°C for 1 min with a final extension at 72°C for 7 min. A polyA tail was added to PCR products using 10X A-Attachment Mix. The resulting products were then purified and ligated into a pGEM<sup>®</sup>-T vector and named pGEM-*xlnW31*. These plasmids contained restriction sites for cloning into pQE80L.

### Expression and purification of the xylanase gene

The xylanase gene fragment in pGEM-*xlnW31* was liberated by digestion with *Bam*HI and *Hind*III, and subsequently ligated into pQE80L expression vector. The pQE-*xlnW31* was then transformed into *E. coli* Rosetta-gami B (DE3) and the transformants were cultured on LB agar supplemented with ampicillin (100 µg/ml) and kanamycin (34 µg/ml) at 37°C for 24 h. Xylanase activities of positive transformants were screened on LB agar supplemented with 0.5% (w/v) AZCL-oat spelt xylan, 1 mM IPTG and 100 µg/ml of ampicillin. Selected *E. coli* transformants were grown in LB supplemented with 100 µg/ml of ampicillin and 34 µg/ml of kanamycin until the cultures reached an optical density at 600 nm 0.6 to 0.8. IPTG was added to a final concentration of 1 mM, and the culture was cultivated at 37°C for 6 h. The cells were collected by centrifugation at 5 000 rpm for 5 min and washed twice with 0.5 M NaCl prior to cell disruption by sonication. The suspension was centrifuged at 12 000 rpm for 30 min to remove cell debris and unlysed cells. The resulting supernatant was purified by immobilized metal ion affinity chromatography (IMAC) on a Sepharose 6 fast flow column (GE Healthcare, Japan) according to the manufacturer's instruction. The purity of fractions containing

xylanase activity was confirmed by sodium dodecyl sulphate (SDS-PAGE).

### Enzyme characterization

The purified xylanase enzyme expressed in *E. coli* (XlnW31) was used for enzyme characterizations. The optimum pH was determined using three different buffers that spanned pH 3.0 to 11.0 at a final concentration of 100 mM: Mcllvaine buffer (pH 3.0 to 7.0); Tris-HCl buffer (pH 7.0 to 9.0) and glycine-NaOH (pH 9.0 to 11.0). The optimum temperature was determined by incubating purified enzymes in 100 mM Mcllvaine buffer (pH 6.0) at different temperatures ranging from 30 to 100°C. Xylanase activity assays were achieved according to the method of Bailey et al. (1992) and the relative activities of enzyme served as a control. The stability at different pHs (3.0 to 11.0) was determined by initially incubating the diluted enzyme in buffers of each pH at 60°C for 1 h then determining the residual activities at optimum temperature under the standard assay conditions. Thermal stability was determined by pre-incubation of the enzyme in 100 mM Mcllvaine buffer (pH 6.0) at various temperatures ranging from 50 to 70°C for 2 h prior to the addition of substrate. The residual activities were determined at optimum temperature under the standard assay conditions.

The effect of metal ions (1 mM) and the influence of other agents on xylanase activity were investigated by pre-incubation of the purified enzyme in the presence of each metal ion and other agent in parallel with an untreated control. Purified enzyme was pre-incubated in 100 mM Mcllvaine buffer (pH 6.0) at 60°C for 1 h. The residual activities at optimum temperature under the standard assay conditions were then determined. Substrate specificity of the purified enzyme was determined using various xylans as substrates.

### Thin layer chromatography (TLC) of xylan hydrolysis products

Purified enzyme (5 U/mg) was incubated in a reaction mixture including a substrate of commercial xylans (50 mg/ml) and 100 mM Mcllvaine buffer (pH 6.0) at 50°C. An aliquot of the reaction mixture was withdrawn at appropriate intervals and the reaction was stopped by boiling for 5 min. The mixture was centrifuged to remove precipitates and subjected to TLC (silica gel 60 F<sub>254</sub>, Merck Ltd., Tokyo, Japan). TLC plates were developed with chloroform-acetic acid-H<sub>2</sub>O (6:7:1, v/v/v) and sprayed with a mixture of methanol-sulfuric acid (95:5, v/v) followed by heating at 150°C in an oven until spots appeared.

### Binding assay

The preparation of insoluble oat spelt xylan was performed by an alkali treatment method as previously reported (Ghangas et al., 1989). Binding experiments were performed by adding the purified enzyme (5 U/mg) to various amounts of avicel or insoluble oat spelt xylan in 1.0 ml of 50 mM Tris-HCl (pH 7.0) in 1.5 ml micro-centrifuge tubes. Samples were placed at 60°C for 1 h and then centrifuged. The xylanase activity in the supernatant was determined by standard method and the activity lost from the supernatant was assumed to be the bound enzyme.

### SDS-PAGE and zymography

SDS-PAGE was performed using a 12.5% polyacrylamide gel according to the method of Laemmli (1970). Protein was visualized by Coomassie Brilliant Blue (CBB) staining. Zymography was performed on SDS-PAGE with a 12.5% polyacrylamide gel that was

**Table 1.** Induction of hemicellulase-related enzymes of *Streptomyces* sp. THW31 by xylan-containing agricultural wastes.

Carbon source (3%)	Xylanase (U/ml)	$\beta$ -Xylosidase (U/ml)	$\alpha$ -Arabinofuranosidase (U/ml)	Acetyl xylan esterase (U/ml)
Oat spelt xylan	28.6 $\pm$ 1.2	0.12 $\pm$ 0.011	0.16 $\pm$ 0.014	0.13 $\pm$ 0.015
Corn cob	30.5 $\pm$ 1.5	0.16 $\pm$ 0.004	0.12 $\pm$ 0.023	0.15 $\pm$ 0.011
Corn hull	12.8 $\pm$ 1.3	0.08 $\pm$ 0.001	0.09 $\pm$ 0.001	0.05 $\pm$ 0.0013
Cane bagasse	25.7 $\pm$ 2.1	0.07 $\pm$ 0.001	0.07 $\pm$ 0.003	0.06 $\pm$ 0.005
Rice straw	19.1 $\pm$ 2.0	0.05 $\pm$ 0.003	0.08 $\pm$ 0.002	0.03 $\pm$ 0.001
Xylose	2.5 $\pm$ 0.05	ND	ND	ND
Glucose	0.9 $\pm$ 0.01	ND	ND	ND

*Streptomyces* sp. THW31 was cultivated at 60°C for five days on actinomycetes medium containing different substrate. Small amount of xylanase activity (0.9 to 2.5 U/ml) was observed, when xylose and glucose were used as carbon source in actinomycetes medium. The results are expressed as mean  $\pm$  standard deviation in culture supernatants from three independent experiments. ND, not done.

supplemented with 0.2% oat spelt xylan. The xylanase activity on the gel was detected following the method of Nakamura et al. (1993) with slight modification. SDS was removed from SDS-PAGE gels by gentle shaking in 20% (v/v) isopropanol at 4°C for 1 h, and the gel was washed twice with distilled water to remove isopropanol. The washed gel was incubated in 100 mM McIlvaine buffer (pH 6.0) at 60°C for 1 h, stained with 0.1% (w/v) Congo Red for 30 min to react with the remaining xylan and washed twice in 1 M NaCl at room temperature for 10 min with shaking. When a destained band appeared, the gel was fixed with 0.5% (v/v) acetic acid.

## RESULTS

### Screening and identification of a xylan-degrading *Streptomyces* strain

The *Streptomyces* strain THW31 that was capable of xylan degradation was isolated from rubbish compost and identified by 16S rDNA analysis, as described in the Materials and Methods. Strain THW31 cells produced clear and strong halos on a minimal xylan agar plate that was incubated at 60°C for three days. The amplified 16S rDNA fragment (approximately 1406 bp; accession no. GU929208) suggested that the strain was a *Streptomyces* species with 99.8 % identity to *S. rochei* strain HBUM174096 (accession no. EU841560), *S. enissocaesilis* strain NRRL B-16365 (DQ026641), *S. olivaceus* (AB184730), *Actinomycetales bacterium* R10 (2010) strain R10 (HM007159). Thus, this strain was designated *Streptomyces* sp. strain THW31.

### Induction of hemicellulose-degrading enzymes by xylans

*Streptomyces* sp. THW31 produced total xylanase activity such as xylanase,  $\beta$ -xylosidase,  $\alpha$ -arabinofuranosidase and acetyl xylan esterase in the culture supernatant when the cells were grown on an actinomycetes medium supplement with various agricultural wastes that contained xylan or commercially available xylan (oat spelt xylan) as a carbon source

(Table 1). The strain show significant xylanase activity when grown on all xylans, but the highest xylanase activity (30.5 U/ml) was obtained from cells grown with corncob as the xylan source. The strain also produced hemicellulases such as  $\beta$ -xylosidase,  $\alpha$ -arabinofuranosidase and acetyl xylan esterase, when the cells were grown on all carbon sources, with corncob serving as the best carbon source for enzymes production. However, cells grown with cane bagasse (25.7 U/ml) produced more xylanase compared with cells grown with rice straw (19.1 U/ml) or corn hull (12.8 U/ml) as the carbon source. Lower levels of xylanase production (0.9 and 2.5 U/ml) were observed after growth with glucose and xylose, respectively.

### Cloning and sequencing analysis of a xylanase gene from *Streptomyces* sp. THW31

A genomic library was constructed from *Streptomyces* sp. THW31 genomic DNA using a pZErO-2 vector and *E. coli* Top10 cells. A genomic library that consisted of approximately 8,000 transformants was obtained. From this library, a single transformant exhibited xylanase activity on low salt LB agar plates that were supplemented with 0.5% (w/v) AZCL-oat spelt xylan, 1 mM IPTG and 50  $\mu$ g/ml kanamycin. The transformant that displayed positive xylanase activity was designated pZErO-X3 and contained a plasmid with an approximate insert size of 2.3 kb. The nucleotide sequences of the xylanase gene (*xlnW31*) (Figure 1) show a complete ORF that was 999 bp in length (accession no. HQ286611). The xylanase ORF encoded a 333 amino acids was corresponded to a calculated molecular weight of 34,976 Da with a 69% G+C content. The molecular weight of mature xylanase without signal peptide was 30,794 Da. According to the SignalP 3.0 program, XlnW31 contained a signal peptide of 41 amino acids and a probable mature xylanase and CBD2 of 292 amino acids. Upon amino acid comparison, the conserved catalytic residues of XlnW31 was Glu 128 and Glu 218. A ribosome binding site (5'-AGGAAG-3') was identified 10

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cccagggagcgggggttcgaaaatttcggaggactccaccgccccttccgggtgcattcgatgagggtcgcctcggtggtgagggggacg
cctgtcgggggatggcttgagctgcggggtctcttccctccggctccaccgtcggtggcgccgaaggtttcgaaatattaccggaaactgt
tgacgcttgatgggtgcaggtcaacactggcgccgttgcgaaccccgtaggtccgctcaggacctccggagaggaagcaccctcccc

1 ATGaacctgctcgtaaacaggagcgctcgcgggcgggcccgctcgcctcgcctcgaagccgctgggcccgtcgcgctggccgcccctc
1 M N L L V Q P R R R G G G P V S L L V K A A W A V A L A A L
93 gccgcatgatgctgcccggcaccgcccggcgccggcaggtcgtcacgaccaaccaggagggcaccaacaacggctactactactcgttc
31 A A M M L P G T A R A G T V V T T N Q E G T N N G Y Y Y S F
183 tggaccgacagccagggcaccgtctccatggacatgggttccggcggccagtagcagcacctcgtggcgcaacaccggcaactcgtcgcc
61 W T D S Q G T V S M D M G S G G Q Y S T S W R N T G N F V A
273 ggcaaggggtggagcaacggcgacgcccggaccgtgcagtagctcgggacacccctcaaccgctccgggaacgctacctggccctgtacgga
91 G K G W S N G G R R T V Q Y S G T F N P S G N A Y L A L Y G
363 tggacgtccaaccgctcgtcgagtactacatcgtcgacaactggggcacctaccggcccacggcgagttcaagggcaccggtgaccacc
121 W T S N P L V ■ Y Y I V D N W G T Y R P T G E F K G G T V T T
453 gacggcgccactacgacatctacaagacgacccggttaccacgcccctcgcgtagggcaccgacacccgacacccagtagctggagcgtg
151 D G G T Y D I Y K T T R Y N A P S V E G T R T F D Q Y W S V
543 cgcagtcacaagcggaccggcgccaccatcaccaccggcaaccacttcgacgctggtcgcgggcccgggatgccgctgggcagcttcagc
181 R Q S K R T G G T I T T G N H F D A W S R A G M P L G S F S
633 tactacatgatcatgccaccgagggctaccagagcagcgagctccgacatcacggtcgggggacccgctccggggcgggggcggg
211 Y Y M I M A T ■ G Y Q S S G S S D I T V G G T G S G G G G G
723 ggcggtggcgggcggggtgcaccgacggtctccgcccgggagaagtggggcgaccggtacaacctcaacgtctcggtgagcggcgcc
241 G G G G G G C T A T V S A G E K W G D R Y N L N V S V S G A
813 agtgactggacggtgacgatgaacgtgcccgtccccggcgaaggtcatgtcgacgtggaacgtcaacgccacgtacccagcgcacaggtg
271 S D W T V T M N V P S P A K V M S T W N V N A T Y P S A Q V
903 ctaccgcccggcccaacggcagcgggaaacacctggggcgccaccatccaggccaacggcaactggacctggcctcgtcgtcctcgtcagc
301 L T A R P N G S G N T W G A T I Q A N G N W T W P S V S C S

993 gcgggctga 999
331 A G * 333

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**Figure 1.** Nucleotide and deduced amino acid sequences of XlnW31 xylanase from *Streptomyces* sp. THW31. The location of the ribosome-binding site is underlined and signal peptides are bold. The start codon is uppercase. The Gly-rich linker is boxed and the amino acid composing the CBD family 2 of XlnW31 is double-underlined. Two conserved catalytic glutamic acids (E128 and E218) are shaded. The transcriptional stop codons were indicated by an asterisk.

bp upstream of the *xlnW31* start codon (ATG) and the gene contained a TGA stop codon. Comparison of the probable full length xylanase with glycoside hydrolases family 11 indicated that XlnW31 displayed varying high identities to xylanases from different organisms, including identity to *S. lividans* TK24 (90%; ZP\_05526520), *S. viridosporus* (76%; AAF09501), *S. thermoviolaceus* (75%; BAD02383), *Nocardioopsis dassonvillei* DSM 43111 (73%; EEK35774), *Thermobifida fusca* (71%; AAV64879), *Thermopolyspora flexuosa* (67%; CAD48747) and *Cellulomonas pachnodae* (61%; AAD54767). In addition, homology modeling prediction demonstrated that XlnW31 model show the highest protein sequence and model structure similarity with xyl1 xylanase from *Streptomyces* sp. S38 (Wouters et al., 2001) (Figures 2a, b).

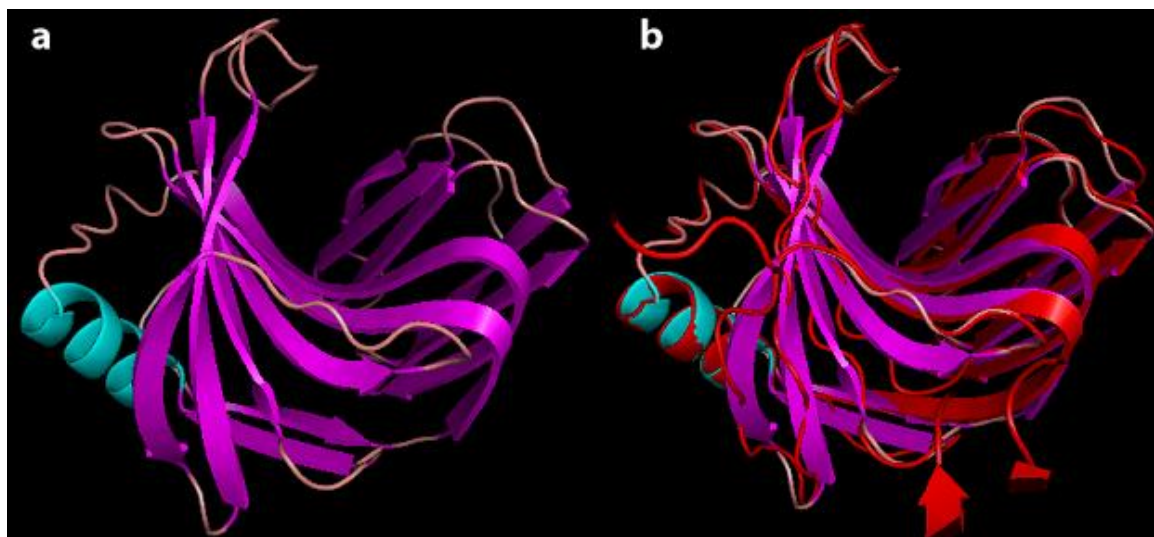
### Expression and purification of recombinant XlnW31 from *E. coli*

The gene products of pQE80L- *xlnW31* was expressed and purified from *E. coli* Rosetta-gami B (DE3) and subsequently characterized. SDS-PAGE analysis demonstrated that purified XlnW31 exhibited a clear band with a molecular weight of approximately 31.0 kDa.

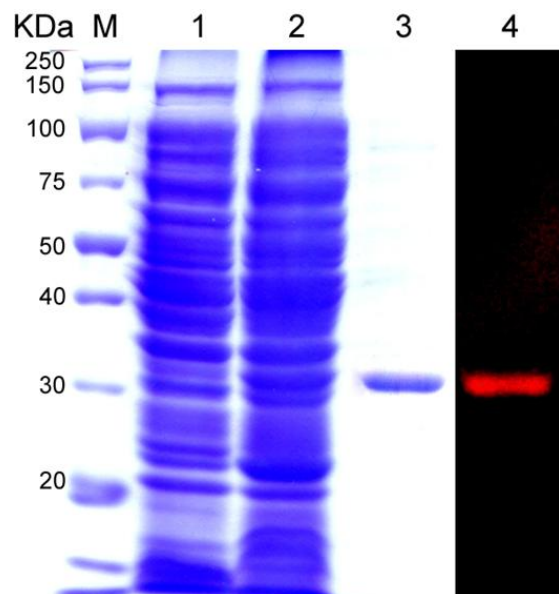
Similarly, a positive band on a zymogram was observed at the same position as that was presented on the SDS-PAGE gel (Figure 3). The highest specific activity of recombinant XlnW31 was 463.8 U/mg. The purification fold of recombinant XlnW31 from each purification step was listed in Table 2. The data indicated that purification fold and recovery yield of XlnW31 were 4.5 fold and 21.3%, respectively.

### Characterization of XlnW31

The optimum pH of XlnW31 was at pH 6.0, and the enzyme was stable over a pH range of 4.0 to 11.0 after 1 h incubation at 60°C (Figure 4). Assays determining the optimum temperature for xylanase activity revealed that XlnW31 shows the highest activity at 65 to 70°C (Figure 5). The enzyme was stable at 60°C and retained with approximately 53.7 % activity after 120 min while incubation at 70°C for 30 min resulted in rapid decrease in enzyme stability and activity to 50.5 % (Figure 6). A substrate specificity test indicated that XlnW31 hydrolyzed the soluble oat spelt xylan (119.2%), beech wood xylan (100%), insoluble oat spelt xylan (82.5%), and birch wood xylan (81.1%) (Table 3). In contrast, XlnW31 could not digest avicel, carboxymethyl cellulose



**Figure 2.** (a) Three-dimension structure modeling prediction of XynW31; (b) Superimposition of XynW31 (Pink) structure and template (Red) structure of Xyl1, a family 11 endo-xylanase from *Streptomyces* sp. S38 (Wouters et al., 2001).



**Figure 3.** Coomassie blue-stained SDS-PAGE gel and zymogram of purified XlnW31 from *E. coli* Rosetta gami (DE3) harboring pQE-*xlnW31*. Lane M, molecular mass markers; lane 1, cell free extract of *E. coli* harboring pQE80L; lane 2, cell free extract of *E. coli* harboring pQE-*xlnW31*; lane 3, purified XlnW31 with IMAC; lane 4, zymogram of purified XlnW31 by IMAC.

(CMC), glucomannan, spino gum, locust bean gum, pNPX, pNPA and pNA. These results suggested that recombinant XlnW31 was a cellulase-free xylanase and its CBD plays an important role in the hydrolysis of insoluble xylan.

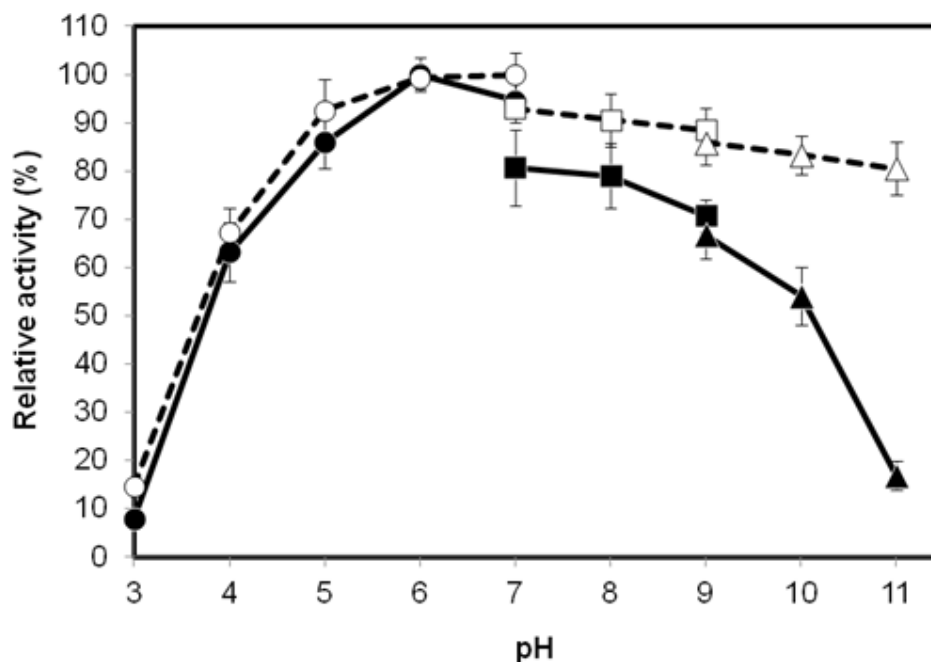
According to an enzyme inhibition test in the presence of various metal ions and other reagents, XlnW31 was totally and partially inhibited by  $\text{Hg}^{2+}$  and  $\text{Cu}^{2+}$ , respectively. The other metal ions (monovalent and divalent ions) that were tested, SDS and ethylenediaminetetraacetic acid (EDTA) at a final concentration of 1 mM did not inhibit xylanase activity (Table 4). Furthermore, XlnW31 exhibited similar binding efficiency to insoluble oat spelt xylan and avicel (Figure 7). Regarding the rate of xylan hydrolysis activity of XlnW31, the enzyme liberated xylooligosaccharides from oat spelt xylan in as short a time as 5 min (Figure 8). Based on this digestion, xylobiose was the final reaction product. Following an increased incubation time, xylose, xylobiose and xylotetraose production increased. These results suggested that this enzyme exhibited  $\beta$ -1,4-endoxylanase activity.

## DISCUSSION

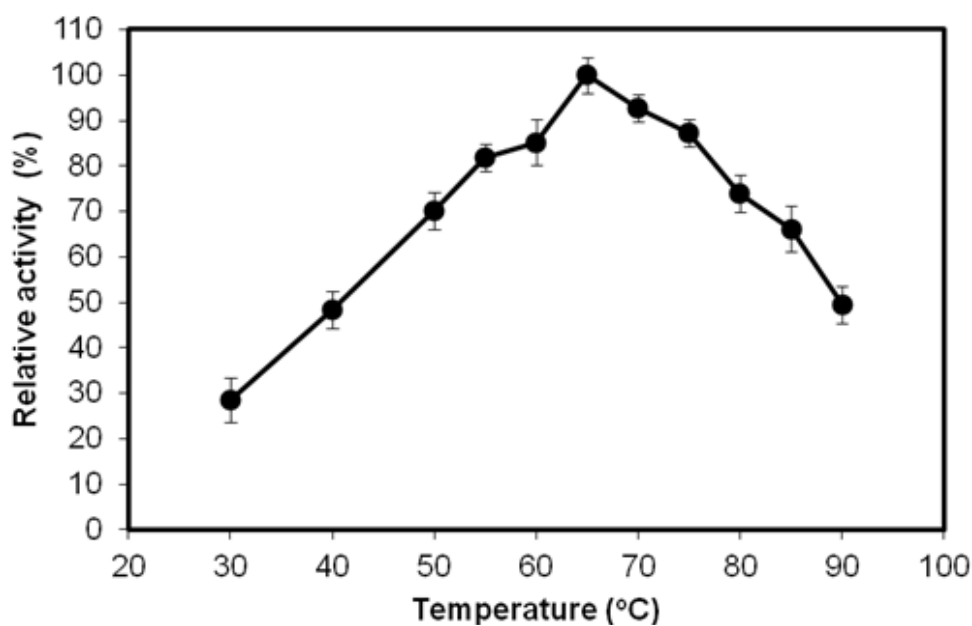
In this study, a *Streptomyces* sp. strain THW31 was isolated from rubbish compost that exhibited strong xylanase activity on a minimal xylan agar plate. The strain was subsequently identified as *Streptomyces* sp. strain THW31 based on 16S rDNA analysis and was closely related to *S. rochei*, *S. enissocaesilis* and *S. olivaceus*. *Streptomyces* sp. strain THW31 produced the highest xylanase activity at a temperature of 60°C. Actinomycete xylanases that have been studied so far, particularly those from *Streptomyces* genus, were mainly from thermophilic strains. *Streptomyces* sp. was reported to produce different enzymes with a wide variety of characteristics. However, comparatively little information

**Table 2.** Summary of the purification of XlnW31.

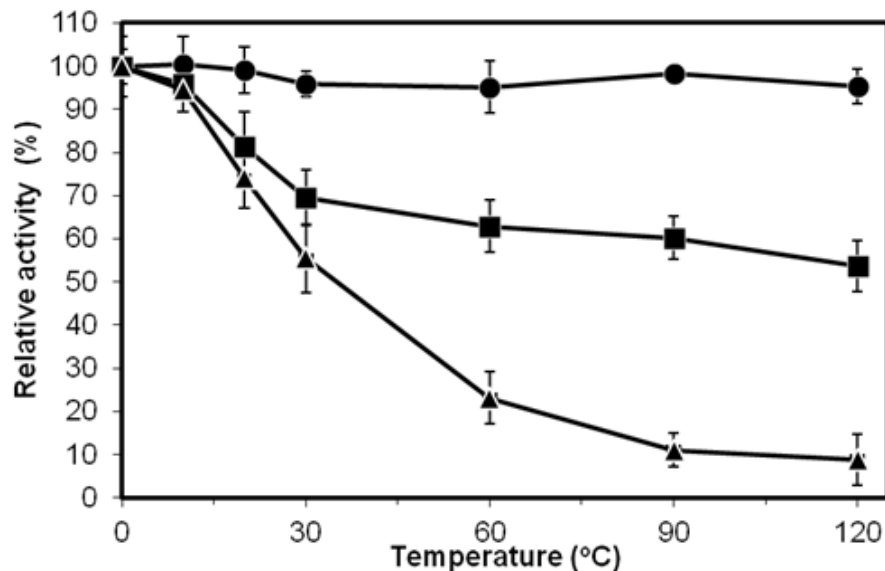
Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Cell-free extract	2608.5	25.3	103.1	1.0	100.0
IMAC	556.6	1.2	463.8	4.5	21.3



**Figure 4.** Effect of pH (—) and pH stability (-----) on xylanase activity assayed at 60°C for 1 h. The xylanase activity was performed in buffers ranging from pH 3.0 to 11.0. Symbols used: solid and open circles, Mcllvaine buffer for pH 3.0 to 7.0; solid and Open Square, Tris-HCl buffer for pH 7.0 to 9.0 and solid and open triangles, glycine-NaOH for pH 9.0 to 11.0.



**Figure 5.** Effect of temperature on the purified xylanase activity.



**Figure 6.** Thermostability profile of purified xylanase. The purified XlnW31 was incubated without substrate in 100 mM Mcllvaine buffer (pH 6.0) at different temperatures for 2 h and the residual activities were measured as described in Materials and Methods. Symbols used: solid circles, 50°C; solid squares, 60°C and solid triangles, 70°C.

**Table 3.** Substrate specificity of the XlnW31.

Substrate <sup>a</sup>	Relative activity (%) <sup>b</sup>
Birchwood xylan	81.1±3.3
Beechwood xylan	100.0±1.6
Soluble oat spelt xylan	119.2±5.2
Insoluble oat spelt xylan	82.5±2.4

a, No activity was detected towards Avicel, carboxymethyl cellulose (CMC), glucomannan, spino gum, locust bean gum, p-nitrophenyl- $\beta$ -D-xylopyranoside, p-nitrophenyl- $\alpha$ -L-arabinofuranoside, p-nitrophenyl- $\beta$ -D-glucopyranoside; b, The activities of purified XlnW31 towards beechwood xylan were 414.8 U/mg, which was defined as 100%. The results are expressed as mean  $\pm$  standard deviation from three independent experiments.

has been available regarding xylanases from thermotolerant *Streptomyces*, particularly an agro-wastes-degrading strain. Genes encoding a xylanase and other hemicellulase activities from this strain had not previously been cloned nor had the corresponding proteins been characterized.

Hemicellulase activity, such as xylanase,  $\beta$ -xylosidase,  $\alpha$ -arabinofuranosidase and acetyl xylan esterase, was detected in the culture supernatant of *Streptomyces* sp. strain THW31 following growth with agriculture waste or commercially available xylan in the medium. Corn cob induced the highest xylanase,  $\beta$ -xylosidase and acetyl xylan esterase activities, suggesting that strain THW31 could potentially be used for the production of hemicellulase and xylooligosaccharides from agricultural

**Table 4.** Effect of metal ions and reagents on the XlnW31.

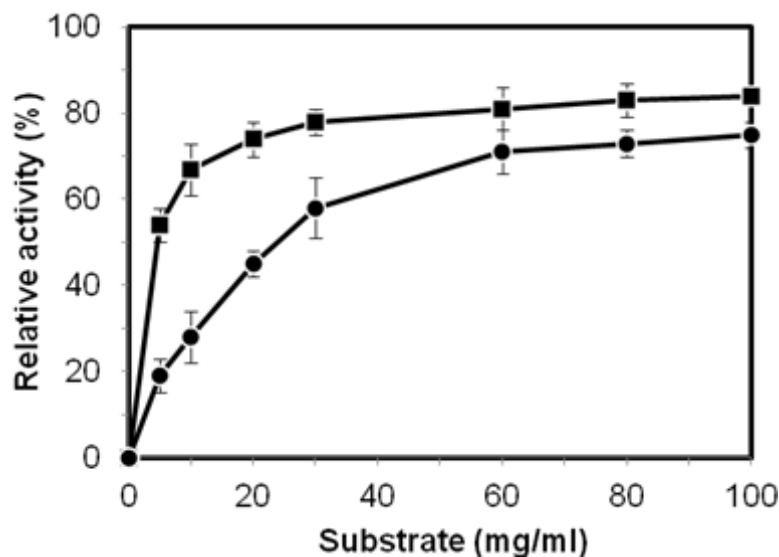
Reagent	Relative activity (%)
Control	100.0±1.6
K <sup>+</sup>	99.1±0.9
Li <sup>+</sup>	104.2±2.2
Na <sup>+</sup>	109.7±3.0
Ca <sup>2+</sup>	96.8±2.4
Co <sup>2+</sup>	129.7±7.1
Cu <sup>2+</sup>	83.7±2.9
Hg <sup>2+</sup>	4.6±0.05
Fe <sup>2+</sup>	114.7±3.0
Fe <sup>3+</sup>	110.5±4.5
Mg <sup>2+</sup>	100.6±2.7
Ni <sup>2+</sup>	108.2±3.7
Rb <sup>2+</sup>	110.9±3.0
Zn <sup>2+</sup>	116.8±6.5
EDTA	98.1±1.0
SDS	96.9±1.0

The activities of purified XlnW31 towards oat spelt xylan were 494.4 U/mg, which were defined as 100%. The results are expressed as mean  $\pm$  standard deviation from three independent experiments.

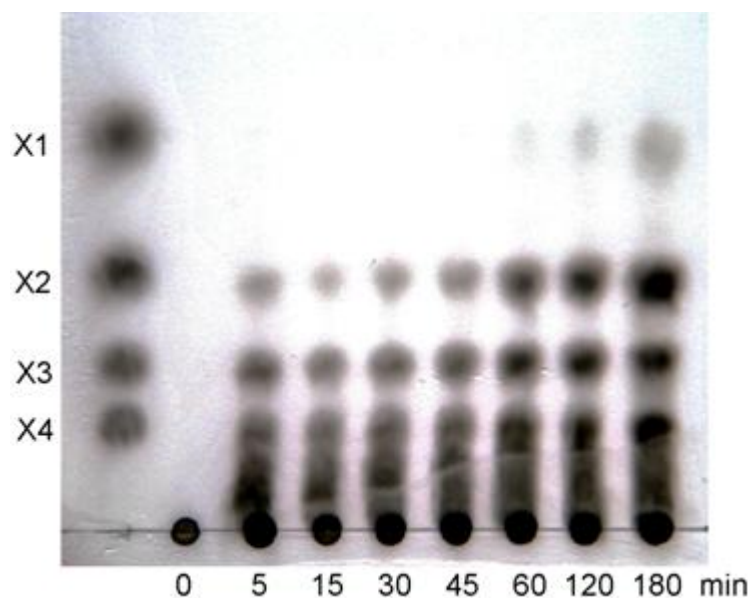
wastes. The data was corresponded to those of Abd El-Nasser et al. (2010) and Antonopoulos et al. (2001) found that wheat straw and oat spelt xylan were the suitable carbon sources for xylanase production, respectively.

Generally, *Streptomyces* have been reported to be the most active producers of these enzymes and to display the most potential for the production of hemicellulases





**Figure 7.** Binding of the purified XlnW31 to insoluble oat spelt xylan and Avicel. Binding experiments were performed at 60°C for 1 h. The residual activities in the supernatant were determined by as described in Materials and Methods. Symbols: solid squares, insoluble oat spelt xylan and solid circles, Avicel.



**Figure 8.** Xylan hydrolysis by the purified XlnW31 was identified by TLC. Abbreviations used: X1, xylose; X2, xylobiose; X3, xylotriose and X4, xylotetraose.

using cheap hemicellulosic substrates from agriculture wastes, such as corn cob, rice straw and cane bagasse (Beg et al., 2000; Techapun et al., 2001; Wang et al., 2003; Ninawe and Kuhad, 2005; Abd El-Nasser et al. 2010). The content of cellulose, hemicelluloses and lignin in common agricultural residues and wastes were

reported (Saha, 2003). Corncob was the best inducer because hemicelluloses are the main composition of corncob, accounting up to 30 to 35% of their dry mass. Following genomic library construction and xylanase gene identification, an ORF of 999 bp was identified and termed *XlnW31*. The XlnW31 protein contained a signal

peptide of 41 amino acids, and the mature xylanase was 292 amino acids. According to the predicted nucleotide and amino acid sequences, XlnW31 was classified into the xylanase family 11. The mature xylanase consisted of a Gly-rich linker and a type 2 CBD. The region downstream of *xlnW31* contained an ORF that was similar to acetyl xylan esterase gene (data not shown). Regions flanking the xylanase gene locus were similar to those of the known xylanase family 11 and acetyl xylan esterase genes of *Streptomyces* (Shareck et al., 1995; Tsujibo et al., 1997; Georis et al., 1999).

To characterize the xylanase of *Streptomyces* sp. THW31, a gene encoding the mature xylanase (*xlnW31*) was cloned and expressed in *E. coli*. Although many xylanases have been cloned from *Streptomyces* (Techapun et al., 2003), no xylanase expression has been previously reported from the closest relatives of *Streptomyces* sp. THW31, including *S. rochei*, *S. Enissocaeisilis* and *S. olivaceus*. An amino acid sequence comparison indicated that XlnW31 is 90% similar to XlnB from *S. lividans* strain 1326 (accession no. AAC06114) (Shareck et al., 1995; Kluepfel et al., 1990). Both XlnB and XlnW31 have the same molecular weight (31 kDa) but some of their enzyme characteristics were significantly different. Firstly, the optimal pH of XlnB and XlnW31 were 6.5 and 6.0, respectively. Secondly, the optimum temperature of XlnB and XlnW31 were 55 and 65°C, respectively. Despite these differences, they both displayed similar thermal stability.

Additionally, XlnW31 retained more than 50% activity after pre-incubation at 70°C for 30 min. Under the same condition, other family11 xylanases, such as XLNB from *S. olivaceoviridis* A1 (Wang et al., 2007), XlnBS27 from *Streptomyces* sp. S27 (Li et al., 2009), Xyl1 from *Streptomyces* sp. strain S38 (Georis et al., 2000) and purified xylanase from *S. matensis* (Yan et al., 2009), were almost inactive. The thermostability of XlnW31 at 70°C was similar to the stability of xylanases from *S. rameus* (Li et al., 2010) and *Streptomyces thermocyaneoviolaceus* (Shin et al., 2009). XlnW31 also displayed an optimum pH of 6.0 and retained more than 80% activity over a pH range of 4.0 to 11.0. This characteristic is similar to xylanases from other *Streptomyces* strains (Georis et al., 2000; Wang et al., 2007; Ninawe et al., 2008; Shin et al., 2009; Li et al., 2009). The broad pH profile of the XlnW31 xylanase together with its thermostability makes this enzyme an attractive candidate for industrial applications.

Upon the analysis of metal ions,  $\text{Co}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  have significant enhanced the effect of XlnW31 activity. Previously, the above metals have been reported to enhance the xylanase activity (Yan et al., 2009). The addition of  $\text{Hg}^{2+}$  at 1 mM almost completely inhibited xylanase activity while  $\text{Cu}^{2+}$  could partially inhibited the activity of the enzyme. Most xylanases are inhibited by sulfhydryl-enzyme inhibitors like  $\text{Hg}^{2+}$  and  $\text{Cu}^{2+}$  (Magnuson and Crawford, 1997; Li et al., 2009).

Sulfhydryl-enzyme inhibitors could modify sulfhydryl groups (thiol group) near the active site that could change the native structure of the enzyme resulting in a destabilization of enzyme folding (Rouvinen et al., 1990) or the formation of disulfide bonds at irregular position in the protein (Ohmiya et al., 1995). In addition, no significant effect on xylanase activity was observed when 1 mM EDTA was added. This result demonstrated that the enzyme did not contain any metal ions as prosthetic groups in the active site.

Hydrolysis of xylan by XlnW31, produced the major reaction product xylobiose from all of the xylan substrates that were tested (oat spelt xylan, birchwood xylan, beechwood xylan). Many member of xylanase family 11 hydrolyze xylans to xylo-oligosaccharides with the higher degree of polymerization  $\geq 2$  have been reported (Kluepfel et al., 1990; Yan et al., 2009; Li et al., 2009; Shin et al., 2009). This suggested that the enzyme preferentially cleave the internal glycosidic bonds of these xylo-oligosaccharides. Furthermore, XlnW31 hydrolyzed both soluble and insoluble xylans and also bound to both insoluble xylan and crystalline cellulose. These results suggested that the XBD was important for insoluble xylan digestion. Usually, a xylanase that contains a XBD acts on both insoluble and soluble xylans, and previous work shows that the CBD of family 2 binds to insoluble substrates (Tsujibi et al., 1997; Shin et al., 2009).

In conclusion, the specific characteristics of recombinant XlnW31 such as the broad pH profile, its thermostability and the efficacy of oligosaccharides production from commercial xylan and agriculture wastes, make this enzyme an attractive candidate for use in commercial production processes or industrial applications.

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