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Endo-1,4-β-xylanase B from *Aspergillus* cf. *niger* BCC14405 Isolated in Thailand: Purification, Characterization and Gene Isolation

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During the screening of xylanolytic enzymes from locally isolated fungi, one strain BCC14405, exhibited high enzyme activity with thermostability. This fugal strain was identified as *Aspergillus* cf. *niger* based on its morphological characteristics and internal transcribed spacer (ITS) sequences. An enzyme with xylanolytic activity from BCC14405 was later purified and characterized. It was found to have a molecular mass of *ca.* 21 kDa, an optimal pH of 5.0, and an optimal temperature of 55°C. When tested using xylan from birchwood, it showed K_m and V_{max} values of 8.9 mg/ml and 11,100 U/mg, respectively. The enzyme was inhibited by $CuSO_4$, EDTA, and by $FeSO_4$. The homology of the 20-residue N-terminal protein sequence showed that the enzyme was an endo-1,4-β-xylanase. The full-length gene encoding endo-1,4-β-xylanase from BCC14405 was obtained by PCR amplification of its cDNA. The gene contained an open reading frame of 678 bp, encoding a 225 amino acid protein, which was identical to the endo-1,4-â-xylanase B previously identified in *A. niger*.

Keywords: *Aspergillus* cf. *niger*, Endo-1,4-β-xylanase B, Hemicellulase, Thermostability, Xylanase

Introduction

Xylan (hemicellulose) is a polysaccharide composed of β-1,4-linked xylopyranose units. It is highly branched and tightly associates with other biopolymers (Eriksson *et al*., 1990). Xylanolytic enzymes are a group of enzymes that hydrolyze xylan and arabinoxylan polymers. This enzyme group includes endo-1,4-β-xylanase, (-xylosidase, (-arabinofuranosidase and acetylxylan esterase (Biely, 1993), and play important roles in the animal feed, pulp and paper, textile, and food industries. Other potential applications include the conversion of xylan in wastes from agriculture and food industries into xylose, and the production of fuel and chemical feedstocks (Sunna and Antranikian, 1997). Given their different modes of action and great variations in substrate/product specificities, xylanolytic enzymes are a major focus of applied research and of great interest for biotechnology applications. A number of xylanolytic enzymes from various sources, and especially from microorganisms, have been studied to understand their physical and biochemical characteristics (Sunna and Antranikian, 1997).

Due to their diversity, fungi have been recognized as a target for screening and as a source of new enzymes with useful and/or novel characteristics (Singh *et al.*, 2003), and since high temperatures are used in many industrial processes, thermostability is one of the most desirable enzyme characteristics. Moreover, it has long been recognized that thermophiles represent a source of novel thermostable enzymes (Bruins *et al.*, 2001).

The purpose of this study was to characterize the xylanolytic enzyme from a thermoresistant fungus (BCC14405). During the course of our studies we have tested 116 strains of thermoresistant fungi isolated from various sources in Thailand for xylanolytic activity and thermostability, and the strain BCC14405 exhibited the highest enzyme activity. In addition, its crude proteins retained high xylanolytic activity after heat treatment. Thus, strain BCC14405 was chosen for further investigation. The protein with this xylanase activity was initially purified and characterized to determine its biochemical and physical properties, and the full-length gene encoding this enzyme was cloned. Sequence analysis and homology modeling was performed to further classify the isolated enzyme.

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Materials and Methods

Microorganisms and plasmids Thermoresistant fungi were collected from several locations in Thailand. The strain BCC14405 was identified as a thermoresistant fungus with high xylanolytic activity, a voucher specimen has been deposited at the BIOTEC Culture Collection (BCC, Thailand). To induce enzyme production, the fungus was cultured in 5% wheat bran medium supplemented with 1.5% (w/v) xylan as an inducer. *Escherichia coli* DH5aFí (Fír(*lacZYA-argF*)U169 *deoR endA1 hsdR17 supE44 thi-1 recA1 gyr96 relA1* (*f80dlacZ rM15*) was grown at 37ºC in LB broth (Sambrook and Russell, 2001) or on agar (1.5% w/v) plates, and *E. coli* cells harboring a cloning vector, pGEM®-T Easy vector (Promega, Madison, USA), were cultured in LB medium supplemented with 100 µg/ml ampicillin.

Identification of the fungal strain The fungal strain BCC14405 was identified from its morphological characteristics and its internal transcribed spacer (ITS) sequences (Buchan *et al.*, 2002). The genomic DNA (gDNA) of this fungus was isolated by using CTAB and phenol/chloroform method (Velegraki *et al*., 1999). The partial DNA fragment of ITS was amplified by polymerase chain reaction (PCR) using gDNA as a template. The ITS-F primer (5'-GCGGAA GGATCATTACTGAG-3') and ITS-R primer (5'-GGGTATCCCTA CCTGATCCG-3') were designed from the conserved ITS sequences of fungi (Yeates *et al.*, 1998). The PCR product, 600 bp in length, was cloned into pGEM®-T Easy vector (Promega, Madison, USA), and its sequences were obtained using the dideoxynucleotide chain termination method (Sambrook and Russell, 2001) and were analyzed. For biosafety testing, the cytoxicity of this fungal strain was tested using the standard cell lines, L929, BHK(21), HepG2, and IEC6 for cell viability determination using the MTT assay (Plumb *et al.*, 1989).

Zymogram analysis The protein exhibiting xylanolytic activity was detected in crude extract by running it on native-PAGE. The gel was washed twice with distilled water and overlaid on AZCL-xylan agar (0.1% (w/v) xylan, 1.5% (w/v) agar in 50 mM sodium phosphate buffer, pH 5.8). The color zone of the azure dye released by the hydrolysis of AZCL-xylan was detected after incubation at 30ºC for 2 h. The protein band, corresponding to the position of the color zone, was then excised and subjected to the SDS-PAGE, and the SDS-PAGE gel was stained with Coomassie Brilliant Blue R250.

Purification of endo-1,4-β-xylanase BCC14405 was cultured in 5% (w/v) wheat bran broth in the presence of 1.5% xylan as an inducer for 6 days. The volume of the culture supernatant was adjusted with 2 M Tris, pH 8.0 to a final concentration of 60 mM and applied to a DEAE Sepharose Fast Flow column (Amersham Biosciences, Buckinghamshire, UK), which had been equilibrated with 60 mM Tris, pH 8.0. The flow-through solution, which contained the xylanolytic activity, was then adjusted to 1 M $(NH_4)_2SO_4$ and 50 mM sodium phosphate buffer, and pH 6.8 (butter B). This solution was then applied to a Phenyl Sepharose 6 Fast Flow column (Amersham Biosciences, Buckinghamshire, UK) that had been equilibrated with buffer B. After the column had been washed with buffer B, the protein concerned was eluted with 500 mM $(NH_4)_2SO_4$ and 50 mM sodium phosphate buffer, pH 6.8 at a flow rate of 1 ml/min.

Protein analysis Protein concentrations were determined using the Bradford method with bovine serum albumin as a standard (Bradford, 1976). Protein patterns were analyzed by SDS-PAGE (Laemmli, 1970), and the purity of the protein on SDS-PAGE was estimated by absorbance scanning using a Model GS-700 Imaging Densitometer equipped with Molecular Analyst Software (BioRad, Hercules, USA). The purified enzyme was blotted onto a PVDF membrane for N-terminal protein sequencing.

Quantitative assay for xylanolytic activity Xylanolytic activity was quantitatively determined using 3,5-dinitrosalicyclic acid (DNS). The assay was based on the enzymatic hydrolysis of xylan, and the reaction of the liberated reducing sugar with DNS (Bailey *et al.*, 1992). Xylose was used as a reference reducing sugar for preparing a standard curve. One unit of xylanase activity was defined as the quantity of enzyme that liberated reducing sugar at the rate of 1 µmole/min. To establish optimal pH and temperature profiles, the enzyme-substrate reaction was carried out at different pHís and temperatures as described in the appropriate figure legends. In order to investigate thermostability and pH stability, the enzyme solution was incubated at different temperatures with different time intervals, or stored in buffers with different pH's for 4 h before measuring activity under optimal conditions. To monitor the effects of different chemicals on enzyme activity, $CuSO_4$, $CoCl_2$, $ZnSO_4$, EDTA, $FeSO_4$, $CuCl_2$, NaCl, $MgSO_4$, $MgCl_2$, $MnSO_4$, sucrose, xylose, sorbitol, b-mercaptoethanol, acetic acid, or glycerol were added separately to the reaction solution, and enzymatic activities were measured under optimal conditions.

Substrate specificity The cellulase activity of the purified enzyme was assayed in a similar manner to its xylanase activity except that 1% (w/v) carboxymethylcellulose and glucose were used as the substrate and reference sugar, respectively. The activities of β-xyloxidase, α-L-arabinofuranosidase, and acetylxylan esterase were assayed for 10 min at 50ºC using p-nitrophenyl-β-D-xylopyranoside, p-nitrophenyl-α-L-arabinofuranoside, and p-nitrophenyl acetate (Sigma, St. Louis, USA), respectively. Following the addition of 2 volumes of 1 M sodium carbonate, the p-nitrophenol product was quantified at 405 nm using an absorbance coefficient of 18.5 ml · µmole^{-1} · cm^{-1} (Lin *et al*., 1999). Azure Cross Link substrates (AZCL-substrate) (Megazyme, Ireland) were used to determine its enzymatic activity on the β/α-glucosidic linkage. The method was based on the release of a colour labeling group, azure dye, after the hydrolysis of the AZCL-substrate. The AZCL-substrate (0.2% (w/v)) was incubated with an equal volume of the enzyme solution with appropriate dilution at the optimal temperature for 30 min. The reaction was stopped by heating at 95ºC for 20 min and the mixture centrifuged. The absorbance of the supernatant was measured at 620 nm. One unit of enzyme activity was defined as the amount of enzyme that produced 1 unit of absorbance of azure dye at 620 nm in one hour.

Cloning of the xylanase gene from *A.* cf. *niger* BCC14405 Total RNA was isolated from BCC14405 which had been grown for 3 days in a wheat bran liquid medium containing xylan (1.5% w/v) at

30ºC and Tri-reagent (MRC, USA), according to the manufacturer's instructions. First strand cDNAs, were synthesized from 1 mg of total RNA using ImProm-II™ reverse transcriptase (Promega, Madison, USA). PCR was performed using a primer based on the nucleotide sequences of endoxylanases B from *Aspergillus* species (5'-ATGCTTACCAAGAACCTTCTC-3') and an oligo(dT) flanked primer (5'-CCGGAATTCAAGCTTCTAGAGGATCCT-3'). The PCR product was cloned into the cloning vector, pGEM®-T Easy (Promega, Madison, USA), and the resulting plasmid was then introduced into *E. coli* strain DH5a. Transformants were selected on LB plates supplemented with ampicillin (100 mg/ml), and the gene was identified by DNA sequencing.

Results

Identification of strain BCC14405 To identify the fungi that exhibited thermoresistant characteristics, collected specimens were heated at 80ºC for 30 min and then isolated on potato dextrose agar (PDA). Strain BCC14405 collected from elephant dung, was able to grow on PDA after being incubated at 80ºC for 30 min. The xylan plate diffusion enzyme assay indicated that BCC14405 had high xylanolytic activity. From morphological observations, this strain had dark brown to black conidial heads, which is indicative of the *Aspergillus* section *Nigri*. To identify this strain at the molecular level, its internal transcribed spacer (ITS) sequence was amplified and analyzed. The alignment of this ITS fragment, 600 bp in length (AY551186), showed that it was identical to those of *A. niger* UWFP515 (AY213633), *A. phoenicis* (U65307), *A. tubingensis* (AJ280008), and *Gliocladium cibotii* (AF048739). From these results, BCC14405 was identified as *A.* cf. *niger*. For the toxicity assay, a mouse fibroblast-like cell line (L929), a baby hamster kidney cell line (BHK(21)C13), a human hepatoma cell line (HepG2), and a rat small intestine cell line (IEC6) were exposed to crude extracts of *A.* cf. *niger* BCC14405 for 24 h. Within the concentration range 6.25-200 µg/ml, this fungal extract was not toxic to BHK(21)C13, HepG2, or IEC6 cell lines. However, it was weakly toxic to L929 cells with an ID_{50} of 95±19 µg/ml.

Zymogram When crude proteins in BCC14405 culture medium were subjected to native-PAGE, xylanolytic activity was detected using AZCL-xylan as substrate; only a single protein band showed enzyme activity (Fig. 1A). This protein was excised and subsequently subjected to SDS-PAGE, which showed that the molecular mass of the xylanolytic enzyme was approximately 21 kDa (data not shown).

Purification and characterization of the xylanolytic enzyme from *A.* cf. *niger* BCC14405 The enzyme was further purified using DEAE Sepharose and Phenyl Sepharose 6 Fast Flows. The purification steps are summarized in Table 1, and resulted in a 5-fold purification with a yield of 20.7%. The purified xylanolytic enzyme had specific activity of 5870

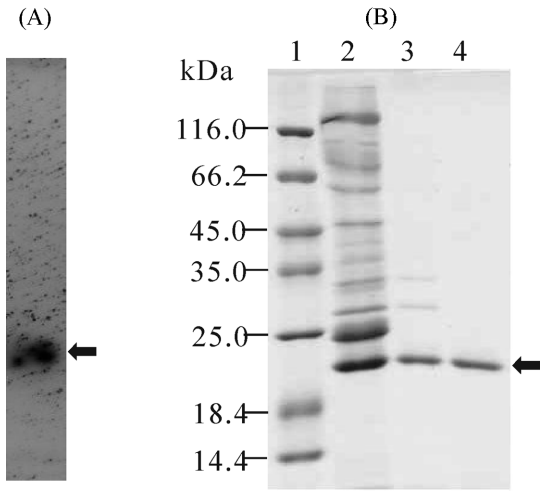

Fig. 1. (A) Zymogram showing the xylanolytic activity of *A.* cf. *niger* BCC14405 crude protein. Crude proteins were subjected to Native-PAGE and the gel was overlaid on AZCL-xylan agar. Xylanolytic activity, observed as azure dye release from AZCL-xylan, was detected as a color zone. (B) SDS-PAGE analysis of protein components in the crude and purified fractions of endo-1,4-β-xylanase from *A.* cf. *niger* BCC14405. Lane 1, Protein markers; lane 2, crude proteins; lane 3, partial purified fraction, flow-through fraction from a DEAE Sepharose Fast Flow column; lane 4, purified fraction, 500 mM M $(NH_4)_2SO_4$ eluted fraction from a Phenyl Sepharose 6 Fast Flow column. The arrows indicate endo-1,4-β-xylanase from *A.* cf. *niger* BCC14405.

U/mg protein, and its molecular mass by SDS-PAGE mobility was approximately 21 kDa (Fig. 1B), which agreed with the zymogram results. The purity of the purified protein, as estimated by SDS-PAGE and densitometry, was 81.1%. N-terminal protein sequencing revealed an amino acid sequence of STPSSTGENNGFYYSFWTDG, and a homology search for this sequence using BLAST indicated a similarity to endoxylanases from *Aspergillus* species. The purified endo-1,4-β-xylanase had maximal activity at 55ºC and pH 5.0 (Fig. 2A and 2B). It retained 60% of its activity after being treated for 30 min at 50ºC (Fig 3) and appeared to be stable at pHís greater than 5.0 (Fig. 2B). Interestingly, its thermostability was increased by 30-50% when 2M sorbitol was added to the enzyme solution (Fig. 3). At birchwood xylan concentrations ranging from 2.5 to 20 mg/ml, the enzyme reaction was found to follow Michaelis-Menten kinetics with K_m and V_{max} values of 8.9 mg/ml (n = 4), and 11,100 units/mg protein respectively, as determined by a Lineweaver-Burk plot. The activity of the purified endo-1,4-β-xylanase was measured in the presence of various additives, refer to Fig. 4 for details. Most of the inorganic salts tested showed no significant effect on enzyme activity. However, 10 mM $CuSO_4$, EDTA, or $FeSO_4$ lead to a 40-50% reduction in enzyme activity.

Substrate specificity Specific activity was measured using various substrates (Table 2). The results obtained showed that

Table 1. Steps used to purify the xylanolytic enzyme in *A.* cf. *niger* BCC14405

	Protein concentration (μg/ml)	Total volume (ml)	Total protein (μg protein)	Specific activity (U/mg protein)[a]	Total activity (U)[a]	Yield (%)	Purification fold
Crude proteins	21.6	60	1300	1180	1530	100.0	1.0
DEAE-Sepharose	4.05	89	360	3660	1320	86.3	3.1
Phenyl-Sepharose	2.25	24	54	5870	317	20.7	5.0

[a]One unit of xylanase activity was defined as the quantity of enzyme required to liberate reducing sugar at rate of 1 μmole/min.

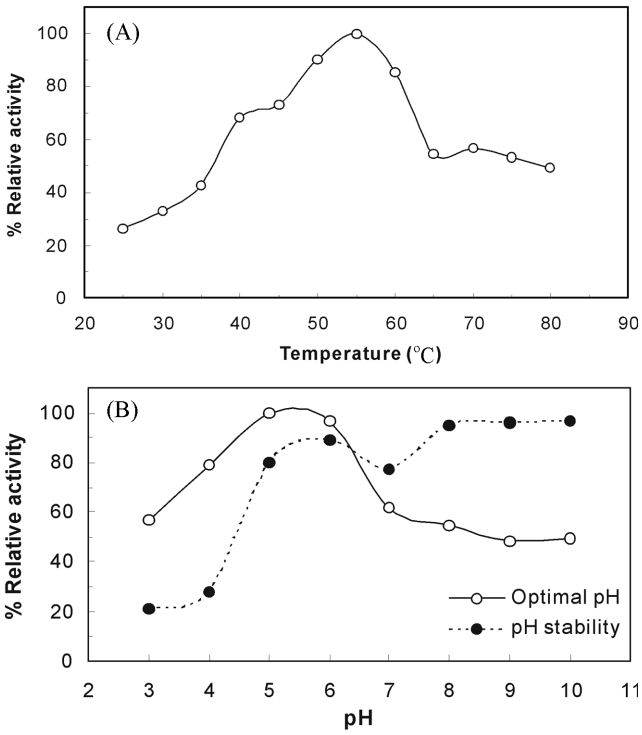

Fig. 2. Effects of temperature (A) and pH (B) on the activity of endo-1,4-β-xylanase from *A.* cf. *niger* BCC14405. The optimal temperature (A) was obtained by assaying the enzyme activity in 0.1 M sodium phosphate buffer at different temperatures as indicates. The optimal pH (B, opened circle) was obtained by examining the effects of different pH values. The buffers used were 0.1 M citrate buffer for pH 3.0-5.0, 0.1 M phosphate buffer for pH 6.0-7.0, and 0.1 M Tris-HCl buffer for pH 8.0-10.0. For pH stability testing (B, closed circle), the enzyme solutions were incubated at room temperature with different pH buffers for 4 h before standard activity assays were performed. The maximal activity as shown by each curve was defined as 100% relative activity.

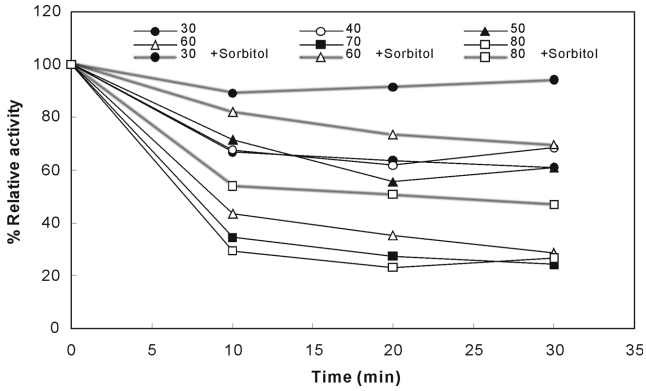

Fig. 3. Thermostability of endo-1,4-β-xylanase from *A.* cf. *niger* BCC14405. The enzyme solutions with (black lines) and without 2 M sorbitol (dashed lines) were incubated at different temperatures for the indicated times intervals before performing standard activity assays.

the purified xylanase hydrolyzed only xylan and was free of cellulase, β-xylosidase, α-L-arabinofuranosidase, acetylxylan esterase, amylase, and β-glucanase activity. Its ability to hydrolyze AZCL-xylan suggested that this enzyme is endo-acting and that it cleaves the b-glucosidic bond linkage.

Cloning of the endo-1,4-β-xylanase gene In order to identify and characterize the endo-1,4-β-xylanase gene, cDNAs were synthesized from total cellular RNA extract. The endo-1,4-β-xylanase gene was amplified using a primer designed on the basis of the nucleotide sequences of endoxylanases from *Aspergillus* species and an oligo (dT) flanked primer. Its 840-bp PCR product was cloned into pGEM®-T Easy vector, and the resulting recombinant plasmids were sequenced. The nucleotide sequence of this endo-1,4-β-xylanase gene, designated *xylB* (AY551187), revealed an open reading frame (ORF) of 678 bp and a GC content of 57.77%. The ORF was predicted to code for a polypeptide of 225 amino acids with a molecular mass of 24 kDa and pI of 1.71. This deduced amino acid sequence was found to be identical to that of endo-1,4-xylanase B from *A. niger* (AY126481-1). In addition, it showed 99, 94, and 74% identities to the endo-1,4-xylanases from *A. tubingensis* (A39368-1), *A. kawachii* (D38070-1) and *A. oryzae* (AB044941-1), respectively. The N-terminal protein sequence of this purified enzyme, STPSSTGENNGFYYSFW TDG, corresponded to the amino acid residue numbers 38 to 57 (bold letters in Fig. 5), suggesting that the first 37-animo acids constituted a signal peptide. The molecular mass of the 178 amino acid mature protein was predicted to be 20.1 kDa, which corresponded with its SDS-PAGE determined size (Fig. 1B).

Table 2. Substrate specificity of endo-1,4-β-xylanase from *A.* cf. *niger* BCC14405

Substrate	Enzyme	Activity (U/mg)
Xylan	Xylan hydrolytic enzymes	5870[a]
Carboxymethyl cellulose	Carboxymethylcellulase	ND[c]
p-Nitrophenyl-β-D-xylopyranoside	β-Xylosidase	ND[c]
p-Nitrophenyl-α-L-arabinofuranoside	α-L-Arabinofuranosidase	ND[c]
p-Nitrophenyl acetate	Acetyl xylan esterase	ND[c]
AZCL-Xylan	Endo 1,4 β-xylanase	5260[b]
AZCL-HE cellulose	Cellulase	ND[c]
AZCL-Amylose	Amylase	ND[c]
AZCL-β-Glucan	Glucanase	ND[c]

[a]One unit of enzyme activity was defined as the quantity of enzyme required to liberate reducing sugar at a rate of 1 μmole/min.
[b]One unit of enzyme activity was defined as the amount of enzyme required to produce 1 unit of absorbance of azure dye at 620 nm in one hour.
[c]ND, activity could not be detected.

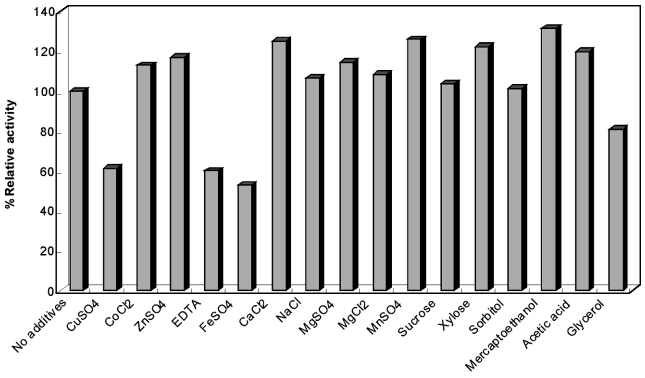

Fig. 4. Effects of additives on the activity of *A.* cf. *niger* BCC14405 endo-1,4-β-xylanase. The eEnzyme activities were assayed in the presence of 10 mM $CuSO_4$, 10 mM $CoCl_2$, 10 mM $ZnSO_4$, 10 mM EDTA, 10 mM $FeSO_4$, 10 mM $CuCl_2$, 10 mM NaCl, 10 mM $MgSO_4$, 10 mM $MgCl_2$, 10 mM $MnSO_4$, 10 mM sucrose, 10 mM xylose, 10% sorbitol, 0.1% mercaptoethanol, 0.1% acetic acid, and 10% glycerol. The enzyme activity in the absence of without any additives was defined as 100% relative activity.

Discussion

Fungi are among nature's most diverse organisms. It is estimated that there are approximately 1.5 million species of fungi (Hawksworth, 2001), approximately 12,000 of which have been found in Thailand (http://mycology.biotec.or.th/Current_Research/Diversity/MainDiversity.htm). Due to the biodiversity shown by fungi, they have long been recognized as a source of valuable enzymes with novel characteristics. The strain BCC14405 was one of a number of local strains isolated from elephant dung, collected in Khao Yai National Park. It proved to be highly thermoresistant and showed continuous growth on PDA after heat treatment at 80°C for 30 min. Of the 116 thermoresistant fungal strains tested in a previous study, BCC14405 also exhibited the highest xylanolytic activity. Thus, this strain represents a potential industrial source of xylanolytic enzyme.

Based on its phenotypic characteristics and a phylogenetic analysis of its ITS data, BCC14405 was identified as *A.* cf. *niger* belonging to Aspergillus section Nigri. Many fungal strains belonging to this group are of economical important since they are widely used in the fermentation industry to produce organic acids and hydrolytic enzymes. However, some of its closely related species such as *A. niger* and *A. carbonarius* have been found to produce ochratoxins that often contaminate food and feed (Varga *et al*., 2003). Thus, we examined the cytotoxicity of *A.* cf. *niger* BCC14405 but found no significant effect upon several standard cell lines. This finding indicates that *A.* cf. *niger* BCC14405 could be used for commercial enzyme production.

High levels of xylanolytic enzyme production were observed when *A.* cf. *niger* BCC14405 was cultivated in 5% wheat bran broth supplemented with 1.5% xylan under aerobic conditions (data not shown). In the present study, a chromatography protocol was developed for purifying the enzyme. The optimal enzyme activity of the purified enzyme was observed at 55°C and pH 5.0. However, the activity of the purified enzyme was less stable than that of the crude protein extract. After incubating the enzyme for 30 min at 60°C, its activity fell to 30%, whereas the crude protein extract fell to only 90%. These results suggest that some substances in the crude protein, such as chaperone proteins, may protect the enzyme from degradation at high temperatures. In addition, the purified enzyme was stable at pH values between 5.0 to 10.0 and was particularly thermostable after adding 2 M sorbitol. Therefore, to prevent enzyme degradation during transportation and enzyme-handling, a pH of 8.0 and sorbitol (2M) supplementation is suggested. This positive effect of sorbitol on enzyme thermostability was first reported by Georgae *et al.* (2001), they found that when 2 M sorbitol was added that *Thermomonospora* sp. xylanase residual activity increased by 30% after treatment 80°C for 30 min at 80°C of

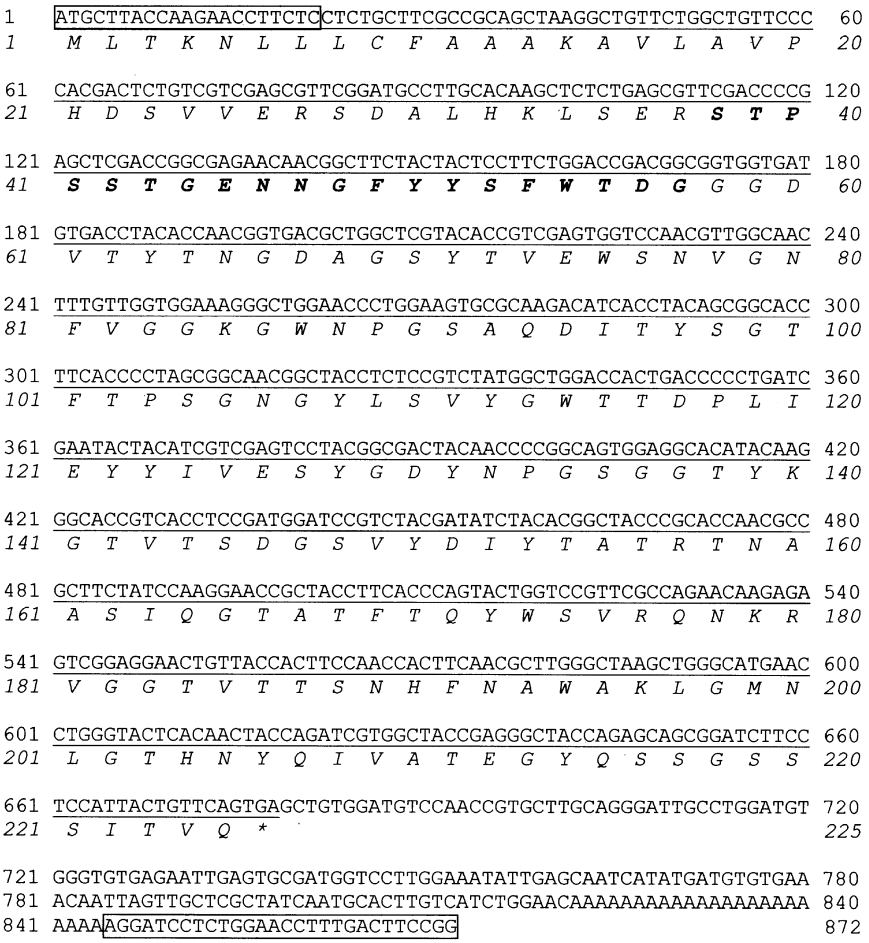

Fig. 5. Nucleotide sequence of the endo-1,4-β-xylanase gene (*xylB*) from *A.* cf. *niger* BCC14405 and its flanking sequence (AY551187). The coding region of the gene is underlined. Deduced amino acids are shown as italic letters under the nucleotide sequence. Asterisk indicated the stop codon. The N-terminal protein sequence of the mature enzyme is given in bold. Primer sequences are boxed.

xylanase from *Thermomonospora* sp. was increased by 30%. The sSorbitol, one of a polyols, was expected suggested to play a role in supporting the native conformation of the protein because of its due to its abilityy to maintain solvophobic interactions and its capability to form hydrogen bond. The V_{max} of the purified enzyme, 11,100 U/mg, indicated indicates that the enzyme was is capable of able to hydrolyzeing xylan faster than other those from other known organisms (Sunna and Antranikian, 1997). MoreoverIn addition, several chemicals and organic salts, and even its end product, the xylose, did not significantly influence the its enzyme enzymic activity (Fig. 4). Moreover, the lack of Non-inhibitoryion effect of by β-mercaptoethanol and or acetic anhydride indicated that disulfide bonds and positively charged amino groups are not critical required for the enzyme its activity (Taneja *et al*., 2002).

The purified enzyme was a cellulase-free xylanase, which showed only endo-1,4-β-xylanase activity, and no activities of enzymes of the xylanolytic group, i.e., xylosidase, arabinofuranosidase, and acetate esterase activities. The N-terminal protein sequence of the mature enzyme suggests that the first 37-amino acid sequence is a signal peptide. In addition, the hydrophobic residues (54%) of this peptide may allow penetration of the fungal membrane and subsequent release. These results corresponded to the homology analysis of its deduced amino acid sequence. Based on the relationships between the physiochemical properties of enzymes and their molecular weights and pIs, Wong *et al.* (1988) suggested that basic proteins with a low molecular weight belong to family 10 and that acidic proteins of high molecular weight belong to family 11. However, the endo-β-1,4-xylanase from *A.* cf. *niger* BCC14405, which has a low molecular weight and a low pI is an exception to this general pattern. Since the tertiary structure predicted for this protein, based on Burkhard Rostís sequence analysis and structure prediction server, showed that it belongs to family 11.

At least 5 xylanases have been found in *A. niger*, (John *et al.*, 1979; Berrin *et al.*, 2000). These differ in molecular mass (from 20 to 50 kDa) and amino acid sequence. Most of the detailed information on enzyme properties, function, and structure available was derived from study of endoxylanase type A (Gorbacheva and Rodionova, 1977; Berrin *et al.*, 2000;

Tahir *et al.*, 2002). Based on structural analysis, both endoxylanase A and B belong to family 11. However, they are classified as type A and B because their amino acid sequences share only 41% identity. Although, the amino acid sequences of endo-1,4-endoxylanases B from *A. niger* (AY126481-1), *A. tubingensis* (A39368-1), and *A. kawachii* (D38070-1) were available on the database, their properties have not been reported. In addition, de Graaff *et al.* (1994) showed that *A. tubingensis* NW756A produces endoxylanase B in low quantity and found that the enzyme is difficult to purify from culture broth (Patent number US 5,610,046).

The Thai isolate *A.* cf. *niger* BCC14405 produced endoxylanase B as the main xylanolytic enzyme (Fig. 1A). Here we report the purification, characterization, and the nucleotide sequence of the endoxylanase B from the wild-type fungus, *A.* cf. *niger*, for the first time. Because it has a high V_{max} and chemical tolerance, this endo-β-1,4-xylanase could be useful for several industrial applications, such as, xylan conversion in wastes from agricultural and food industries. In order to improve the production of this enzyme and its stability under extreme conditions, the *xylB* gene will be mutagenized and overexpressed in *E. coli*.

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