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THESIS

PURIFICATION, CHARACTERIZATION AND INDUCTION OF XYLANASE FROM *Thermomyces lanuginosus* ISOLATED IN THAILAND

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A Thesis Submitted in Partial Fulfilment of
the Requirements for the Degree of
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The characterizations on thermostability of the pure enzymes of low and high thermostable xylanase produced by new isolates of *T. lanuginosus* THKU-9 and THKU-49 were elucidated. Half-life at 70°C of the pure xylanases from *T. lanuginosus* THKU-9 and THKU-49, in 50 mM phosphate buffer (pH 6.0) was 178 and 336 min, respectively. These enzymes were unstable at pH 5.0 and completely lost their activity after incubation at 70°C for 30 min. The xylanase produced by THKU-9 retained 87% and 30% activity in 50 mM sodium phosphate buffer (pH 7.0) after 1080 min incubation at 60°C and 70°C, respectively whereas xylanase produced by THKU-49 retained full activity and 41% activity, respectively. The types and concentrations of buffer had the effect on thermostability of the pure enzymes. The enzymes in phosphate buffer were more stable than those in citrate buffer. When buffer concentration increased, the half-life of the enzymes decreased significantly. Amino acid sequence analysis of low thermostable *T. lanuginosus* THKU-9 xylanase and high thermostable *T. lanuginosus* THKU-49 xylanase showed that high thermostable xylanase had a single substitution (V96G), which is a small hydrophobic amino acid, of β sheet (B5) of the protein locating on the outer surface of the enzyme structure.

A central composite design (CCD) was performed in order to find the best conditions of pH and temperature for β-xylanase activity and to maintain its activity for prolonged periods of time of pure xylanase produced by *T. lanuginosus*THKU-49. The CCD used for the analysis of treatment combinations showed that a regression models of optimization of xylanase activity and xylanase stability were good agreements to experimental results with R^2 = 0.98 and 0.99, respectively. The maximum activity of xylanase was obtained at 66°C and pH 6.3. The maximum enzyme stability was 70°C and pH 7.3. Under this condition xylanase having half-life of 825 min indicated the highest thermostable xylanase.

The strains produced high xylanase either in the xylan or xylose medium having ratio of xylanase activity in a range of 1.1-1.5. Addition of xylose to the xylan medium did not decrease xylanase production by *T. lanuginosus* THKU-11, THKU-25 and ATCC 44008 that were members of this group. In contrast, there was another group that produced high xylanase only in the xylan medium. Addition of xylose to the xylan medium resulted decreasing of xylanase formation in *T. lanuginosus* TISTR 3465, THKU-85 and ATCC 46882 that were belonged to this group. Though, xylose caused xylanase induction by resting cell of tested strains in both groups but the low xylanase formation by growing cell in the xylose medium may cause by catabolic repression of xylose in the particular strains. In addition, RAPD analysis allowed us to distinguish between the high and low xylanase producing strains using xylose as a carbon source.

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Khwanchai Khucharoenphaisan

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INTRODUCTION

Over the last few decades, there has been a growing interest in lignocellulose bioconversion as a renewable energy source. Xylan is the major constituent of hemicellulose and has a high potential for degradation to useful end products. Microbial xylanases are the preferred catalysts for xylan hydrolysis due to their high specificity, mild reaction conditions, and negligible substrate loss and side product generation. Xylanases have found applications in the food, feed and pulp and paper industries (Wong and Saddler, 1993). Xylanases are special significance to the pulp and paper industry, where they can reduce the amount of chlorine and chlorine dioxide used for bleaching paper pulp. Xylanase pretreatment has been reported to lower bleaching chemical consumption and to result in greater final brightness (Kulkarni *et al.*, 1999).

The thermophilic fungus *Thermomyces lanuginosus* (formerly known as *Humicola lanuginosa*, Cooney and Emerson, 1964) is one of the best xylanase producers yet reported (Singh *et al.*, 2000a). It grows at temperatures up to 60^{o}C and it has capability of producing high titers of cellulase-free xylanase. Its xylanase shows not only remarkable thermostability but also is active over a wide pH range. A notable variability in the production of xylanase by *T. lanuginosus* strains has been observed (Anand *et al.*, 1990; Gomes *et al.*, 1993; Alam *et al.*, 1994; Hoq and Deckwer, 1995; Bennett *et al*., 1998; Puchart *et al.*, 1999). The xylanase production of *T. lanuginosus* and their phylogenetic had some relationships using molecular techniques as random amplified polymorphic DNA (RAPD) (Singh *et al*., 2000b). There is a wide variability in the levels of xylanase production of *T. lanuginosus.* Therefore *T. lanuginosus* produced other enzymes, which is used to biobleaching of pulp processes, such as mannanase and pectinase (Puchart *et al.*, 1999; Singh *et al*.,

2000c). Up to date, *T. lanuginosus* SSBP producing the highest half-life (232 minutes) at 70^{o}C of xylanase has been reported (Singh *et al*., 2000d). The lowest half-life (40 min) at 70^{o}C of xylanase was produced by *T. lanuginosus* DSM 10635 (Xiong *et al.*, 2004). The newly isolated *T. lanuginosus* CAU44 from soil in China and *T. lanuginosus* CSB 288.54 from Centraalbuteau voor Schimmelculturen, produced thermostable xylanase, which was stable at 65^{o}C for 30 min (Jiang *et al.*, 2005; Li *et al.*, 2005).

Since pulp-bleaching processes are carried out at high temperature and under alkaline conditions, thermostable and alkali-tolerant xylanases are well suited for such industrial processes. The potential benefits of using these enzymes for biotechnological processes has encouraged widespread research endeavors towards producing desirable xylanases through protein engineering using techniques such as site-directed mutagenesis (Wakarchuk *et al.*, 1994a; Georis *et al.*, 2000; Mesta *et al.*, 2001; Turunen *et al.*, 2001, 2002; Liu *et al.*, 2002; Fenel *et al.*, 2004), directed evolution (Arase *et al*., 1993; Chen *et al.*, 2001; Inami *et al.*, 2003; Palackal *et al.*, 2004) and DNA shuffling (Shibuya *et al.*, 2000; Ahsan *et al.*, 2001; Gibbs *et al.*, 2001). *T. lanuginosus* DSM 5826 produces a high level of cellulase-free, thermostable xylanase, which is catalytically active over a broad pH range (Singh *et al.*, 2003). This xynA was first cloned into *E. coli* as a LacZ-fusion protein (Schlacher *et al.*, 1996) and the protein was later crystallized to elucidate its enzyme structure and mechanism of catalysis (Gruber *et al.*, 1998). This served as the basis for further improvement of the enzyme on the genetic level. Directed evolution has been used to improve the existing properties of enzymes (Giver *et al.*, 1998). This revolutionary type of protein engineering technology mimics Darwinian evolution in nature and does not require extensive knowledge of the gene of interest. It consists steps of random mutagenesis, screening and recombination (Arnold and Volkov, 1999). However, evolution in nature may give rise to strains producing enzyme with different properties including their thermostability.

Many hydrolytic enzymes are inducible by certain low molecular weight substances structurally related to the natural substrates. Xylan, xylooligosaccharides, xylobiose, xylose and non-metabolized xylosides are common specific inducers (Purkarthofer and Steiner, 1995; Sachslehner *et al.*, 1998; Miyazaki *et al.*, 2005). Besides, we also found that xylanase produced by strain of *Humicola lanuginosa* (Griffon and Maublanc) Bunce (formerly known as *T. lanuginosus*) could be induced by oat spelt xylan, xylooligosaccharides and xylose as a carbon source with activity of 19.8, 1.10 and 0.37 U/ml (Koochareanpisan *et al.*, 2001). The effect of xylose on xylanase induction of *Humicola lanuginosa* (Griffon and Maublanc) Bunce was lower than that of oat spelt xylan with a 53.5 folds. This ratio of depression compared to 5.6-fold in *T. lanuginosus* DSM 5826 (Purkarthofer and Steiner, 1995) and 6.1-fold *T. lanuginosus* DSM 10635 (Xiong *et al.*, 2004). The induction and repression systems in *T. lanuginosus* may be difference.

T. lanuginosus producing high thermostable xylanase will be searched from natural sources to synthesize the enzyme. This work will be advantaged to application of this enzyme in industries. To determine thermostable factors of pH and temperature, some selected strains of the *T. lanuginosus* producing high thermostable xylanase and that of low thermostable were analysed in molecular level and characterized some properties of those xylanase. Moreover, the different mechanisms of *T. lanuginosus* xylanase induction will be determined using selectively isolated strains. Relationships between genetic variations of *T. lanuginosus* using RAPD technique on their xylanase production using xylan and xylose as a carbon source were investigated.

OBJECTIVES

1. To isolate and select *T. lanuginosus* which produced high/low thermo-stable xylanase.

2. To purify the xylanase produced by the selected strain of *T. lanuginosus* and characterize on their properties include their thermostability.

3. To study xylanase induction patterns by oat spelt xylan and xylose of selected strains of *T. lanuginosus.*

4. To study the relationship among the strains induced and repressed xylanase production using RAPD technique.

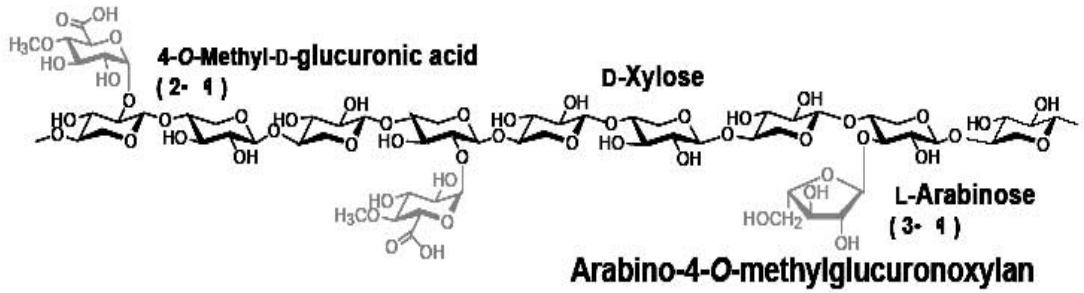
LITERATURE REVIEW

1. Structure composition of xylan in plants

Cellulose, hemicellulose and lignin constitute the major biopolymers found in wood. Four types of hemicelluloses were predominant in plants, namely xylan, mannan, galactan and arabinan (Whistler and Richards, 1970). Xylans were hemicelluloses and it was the second most abundant polysaccharide in nature and was surpassed only by cellulose in abundance (Whistler and Richards, 1970; Collins *et al*., 2005). These compounds were present in the cell wall and in the middle lamella of plant cells. They were classified according to the nature of the linkages joining the xylose residues. β-1,3-linked xylans were found only in marine algae, those xylans containing a mixture of β-1,3- and β-1,4-linkages only in seaweeds and β-1,4-linked xylans occur in hardwoods, softwoods and grasses (Barry and Dillon, 1940; Dekker and Richards, 1976; Kato and Nevins, 1984; Timell, 1965). Hetero-β-1,4-D-xylans constitute the major portion of the hemicellulose in terrestrial plants (Timell, 1965; Whistler and Richards, 1970). Native xylans were complex polymers containing vary amounts of arabinose, 4-O-methylglucuronic acid and acetic acid groups attached to the main xylose chain, depending on the botanical origin of the xylan (Johannson and Samuelson, 1977; Puls and Schuseil, 1993).

Xylan accounts for 10-35% of the dry weight of hardwoods (angiosperms) (Puls and Schuseil, 1993). The main hemicellulose in hardwood was O-acetyl-4-methyl-glucurono xylan (Puls and Schuseil, 1993). On average, the degree of polymerization (DP) was 200 with 10% of the backbone units substituted at C-2 with 4-O-methylglucuronic acid and 70% of the xylopyranosyl units acetylated at C-2 and/or C-3 as show in Fig.1A (Lindberg *et al*., 1973; Puls and Schuseil, 1993). Small amounts of rhamnose and galacturonic acid may also form part of the main chain (Coughlan and Hazlewood, 1993).

A



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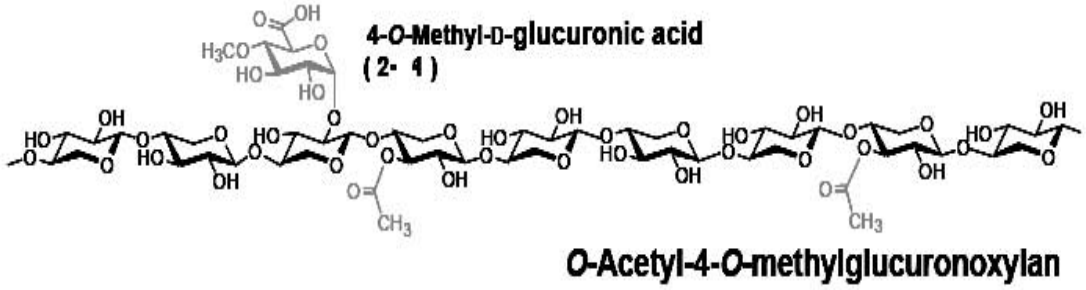

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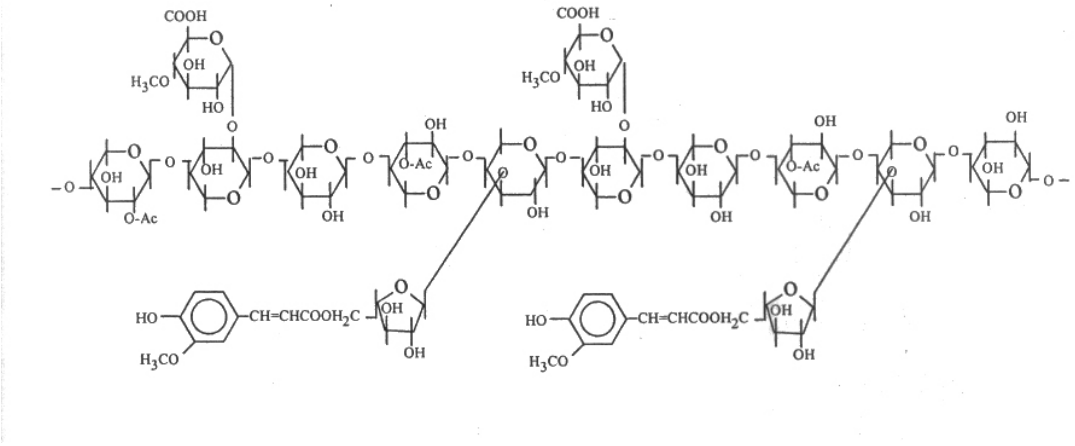

Figure 1 Structure of softwoods xylan (A), hardwoods xylan (B) grass xylan (C).

Source: Bastawade (1992); Coughlan and Hazlewood (1993)

Hetero-β-1,4-D-mannans (galactoglucomannan and glucomannan) comprise approximately two-thirds and arabino-4-O-methylglucuroxylan about one-third of the total hemicellulose found in softwoods (Johannson and Samuelson, 1977). Softwoods (gymnosperms) contain 10-15% arabino-4-O-methylglucuronoxylan in which was located mainly in the tertiary wall of pinewood (Puls and Schuseil, 1993). Softwood xylan was not acetylated and consists of a backbone containing β-1,4-linked xylose units with α-1,2-linked 4-O-methylglucuronic acid and α-1,3-linked L-arabinofuranoside substituents (Fig.1B) (Joseleau *et al*., 1992; Puls and Schuseil, 1993). The ration of arabinose to xylose was 1:8 and two out of ten xylose units were substituted with uronic acid (Joseleau *et al*., 1992; Puls and Schuseil, 1993). Softwood xylans contain less α-1,2-linked 4-O-methylglucuronic acid than hardwood xylans and the L-arabinofuranosyl side chains were linked to the main chain *via* C-2 and/or C-3 (Joseleau *et al*., 1992; Puls and Schuseil, 1993). Some of the arabinosyl side-chains were substituted at C-5 with feruloyl or ρ-coumaroyl residues (Joseleau *et al*., 1992; Meuller-Harvey *et al*., 1986; Puls and Schuseil, 1993). Grass arabinoxylans differ from species to species and from tissue to tissue within the same species, regarding the proportion and composition of the xylan present (Meuller-Harvey *et al*., 1986; Puls and Schuseil, 1993). It made up of D-glucuronic acid and/or its 4-O-methyl ether and arabinose (Fig. 1C).

2. Characteristics of *Thermomyces lanuginosus*

Thermomyces lanuginosus (formerly known as *Humicola lanuginose*) was a widely distributed thermophilic fungus commonly isolated from self-heating masses of organic debris (Emerson, 1968). It was first isolated in 1899 by Tsiklinskaya from a potato, which had been inoculated with garden soil and grown on white bread kept at 52-53^{o}C (Cooney and Emerson, 1964). This thermophilic fungus has been isolated in the British Isles, Denmark, Italy, USA, Canada, Nigeria, Ghana, South Africa, India, Indonesia, Brazil and Japan. These strains have been reported to occur in dry and waterlogged grassland, loamy garden soil and aquatic sediments but the fungus was more specifically associated with organic substrates, roots and leaves of grasses,

composts of various plant materials and the dung of various birds and mammals (Singh *et al.*, 2000a; Hoq and Deckwer, 1995; Hoq *et al.*, 1994). It has even been isolated from air in Indonesia and the British Isles (Hudson, 1992). It was a thermophilic fungus, as strains generally grow between a maximum temperature of 60^{o}C and a minimum of 30^{o}C with an optimum growth temperature of 50^{o}C. By comparison, thermotolerant fungi generally have a growth maximum temperature about 50^{o}C and a minimum temperature below 20^{o}C whereas the growth maximum temperature of mesophilic fungi was generally below 37^{o}C (Fig. 2). The optimum growth pH of most strains investigated was 6.5. *T. lanuginosus* was classified as a Deuteromycetes (imperfect fungus), that was unicellular or septate and reproduces asexually by forming aleurioconidia. On various media, colonies of fungus grow rapidly reaching 2.5 to over 5 cm in diameter at 45-50^{o}C within 2 days. Initially, the colonies appear white and felt-like and were less than 1 mm high, but soon turn grey or greenish-grey, starting from the center of the colony. Subsequently, the colony turns purplish brown and the agar substratum stains a deep pink or wine color, due to diffusible substances secreted by the colony. Mature colonies appear dull dark brown to black. Immature conidia with a diameter of 5.5-12 μm were colorless and smooth walled (Fig. 3). They turn dark brown and globe as they mature and form a thick outer conidial wall that was characteristically wrinkled. Aleuriospores were generally unbranched but occasionally they branch once or twice near the base and appear to cluster. Septations commonly occur in the aleuriospores but they were difficult to observe. The mycelium was partly found on the surface, and partly immersed and there were no stroma or hyphopodia. The aleuriospores were straight or curved, colorless or brown and smooth (Cooney and Emerson, 1964).

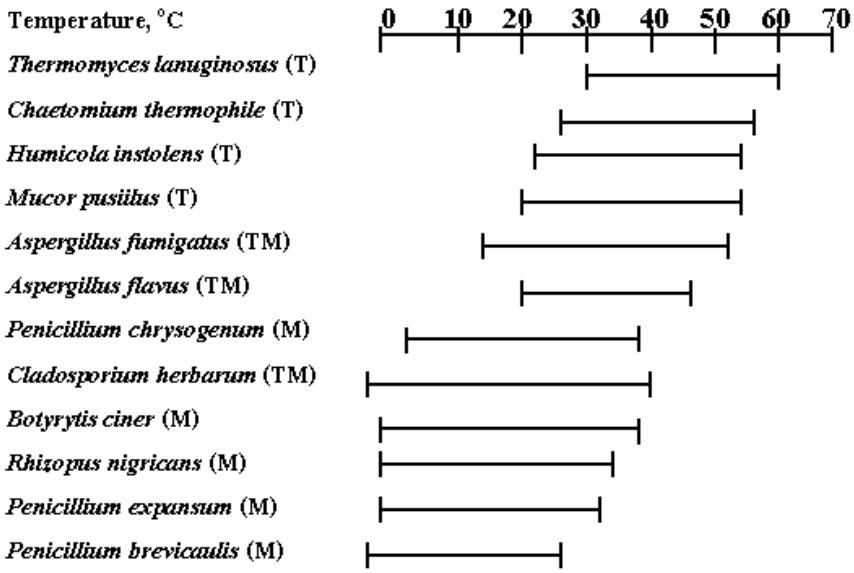

Figure 2 Approximate temperature growth ranges of some representative thermophilic (T), thermotolerant (TM) and mesophilic (M) fungi.

Source: Singh *et al*. (2003)

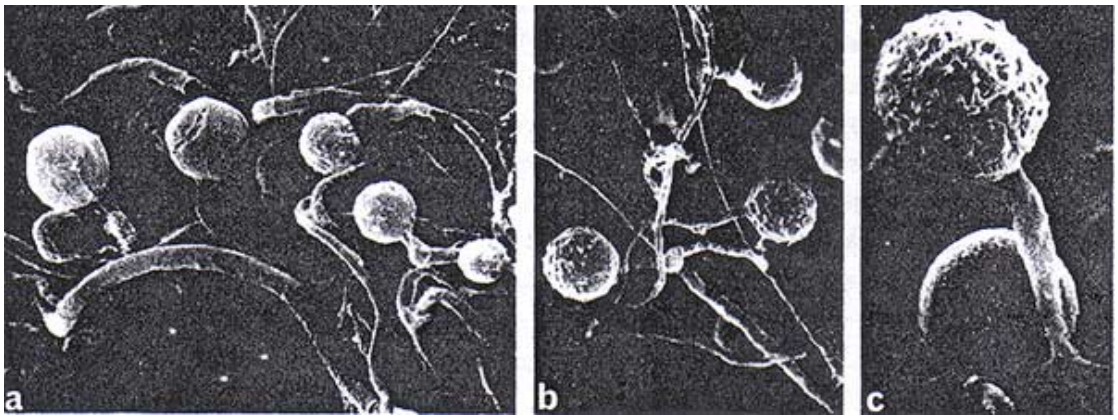

Figure 3 Single spore of *T. lanuginosus* produced as balloon-like swelling process at the tips of short hyphal branches; a, b (1000x) and c (2500x).

Source: Domsch *et al.* (1993)

The genetic diversity detected among *T. lanuginosus* strains obtained from various geographical locations was found to be low. PCR-based amplification of the nuclear ribosomal DNA and the subsequent sequencing of these fragments yielded identical size fragments and sequences and pointed to a high degree of conservation in the rDNA region of the genome of *T. lanuginosus*. The 5.8 S rDNA and the flanking ITS was conserved regions frequently used in phylogenetic studies for differentiation among species and populations within species (Mitchell *et al*., 1995). However, the study of Singh *et al.* (2000b) indicated that this ITS region was appropriation for phylogenetic comparisons within this species. A BLAST search (National Center for Biotechnology Information, USA) has shown that the ITS region and the 5.8 S rDNA sequences were also strongly conserved in others thermophilic fungi, suggesting a possible recent taxonomic divergence in this group of fungi.

The Hyphomycetes genus *Thermomyces* was close to *Humicola* and has been combined with it by several authors. However, it can be distinguished by aleurioconidia, which have an ornamented surface and were generally supported by distinct stalk cells. Aleurioconidia of *Thermomyces* mostly arising on 10-15 μm long lateral stalk cell, dark brown, think walled, with wrinkled surface, 6-10 μm. The genus of *Thermomyces* contains four species were *T. lanuginosus, T. verrucosus, T. ibadanensis* and *T. stellata. T. lanuginosus* has aleuriospores 6-12 μm diameter which globose and irregularly sculptured, which were typical characteristics for identification of the species. *T. verrucosus* was distinguished from *T. lanuginosus* in having verrucose aleurioconidia 10-17 μm diameter. In case of *T. ibadanensis* has smooth walled aleurioconidia 4-8 μm diameter. *T. stellatus* has aleurioconidia, which were singly on the tip of the aleuriophore, were dark brown and stellate with maturity, 5.3 μm diameter and 7.6 μm in length.

3. Xylanolytic enzymes

Due to the complex structure of hemicelluloses, several different enzymes were needed for their enzymatic degradation or modification. The two main glycosyl

hydrolases depolymerising the hemicellulose backbone were endo-xylanase and endo-mannanase (Suurnäkki *et al.*, 1997). Since xylan was a complex component of the hemicelluloses in wood, its complete hydrolysis requires the action of a complete enzyme system (Fig. 4), which was usually composed of xylanase, xylosidase and debranching enzymes such as α-arabinofuranosidase, α-glucuronidase, acetylxylan esterase, and hydroxycinnamic acid esterases that cleave side chain residues from the xylan backbone. All these enzymes act cooperatively to convert xylan to its constituents (Sunna and Antranikian, 1997).

Xylanases attack randomly the backbone of xylan to produce both substituted and non-substituted shorter chain oligomers, xylobiose and xylose (Eriksson *et al.*, 1990). Xylosidases were essential for the complete breakdown of xylan as they hydrolyze xylooligosaccharides to xylose (Poutanen and Puls, 1998). The enzymes of arabinofuranosidase, α-glucuronidase and acetylxylan esterase act in synergy with the xylanases and xylosidases by releasing the substituents on the xylan backbone to achieve a total hydrolysis of xylan to monosaccharide (Eriksson *et al.*, 1990).

4. Classification of xylanolytic enzyme

Classification of glycosyl hydrolase into several families on the basis of homologies in structural elements and hydrophobic clusters, were derived from the two dimensional representation of the amino acid sequence. Xylanases were found in two families in the families 10 (F) and 11 (G). The family 10 was endo-β-1,4-xylanase with higher molecular weigh than family 11 xylanases (>30 kDa), acidic p*I*s and presenting (α/β) barrel folds in three-dimensional (3D) structure (Dominguez *et al.*, 1995). Family 11 was endo-β-1,4-xylanase with low molecular weigh (<30 kDa) and basic p*I*s. (Henrissat and Bairoch, 1993). The 3D structures of family 11 xylanases had overall shape of a right hand as described by Torronen *et al.* (1994). It consists of two large β-pleated sheets and a single α-helix that forms a structure resembling a

partially-closed right hand (Torronen and Rouvinen, 1995; Torronen and Rouvinen, 1997).

Both families contain bacterial and fungal enzymes as shown in Table 1. It suggested that the acquisition of xylanase activity has involved at some stage of lateral gene transfer between fungi and bacteria (Gilbert and Hazlewood, 1993).

The xylanase produced by new species of *Scytalidium thermophilium* in which was isolated from Japanese soil was also in xylanase family G/11 using partial amino acid alignment technique (Boonlue *et al.*, 2003).

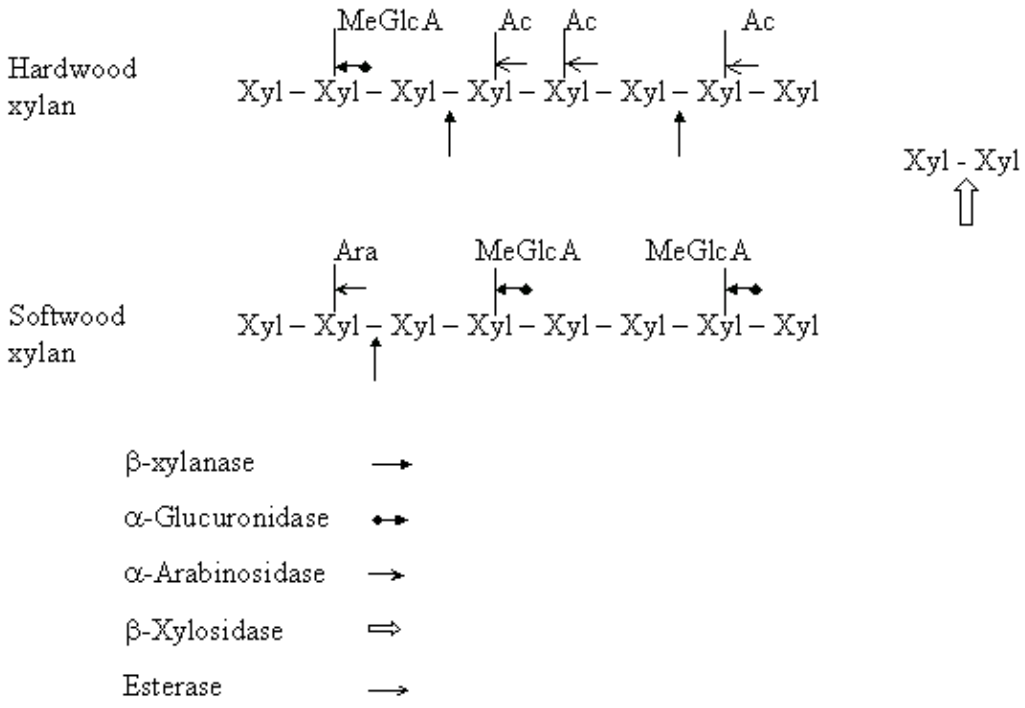

Figure 4 Xylanolytic enzymes involved in the degradation of hardwood and softwood xylan.

Ac, acetyl group; Ara, α-arabinofuranose; MeGlcA, α-4-O-methylglucuronic acid; Xyl, xylose.

Table 1 Classification of xylanase catalytic domains into structurally related families according to amino acid sequence similarities.

Family[a]	Organism[s]	Enzymes	Accession no.[c]
F	*Actinomadura* sp.	XynII	U08894
	Aspergillus niger (=kawachii)	XynA	D14847
	Bacillus sp. (C-25)	XynA	D00087
	Bacillus stearothermophilus 21	XynA	Z29080/D28121
	Bacteroides ovatus	Xyn	U04957
	Butyrivibrio fibrisolvens (H17c)	XynB	X61495
	Butyrivibrio fibrisolvens	XynA	A37755
	Caldocellum saccharolyticum	XynA	M34459
	Caldicellum stercararium (F-9)	XynB	D12504
	Clostridium thermocellum	XynX	M67438
	Clostridium thermocellum	XynY	X83269
	Clostridium thermocellum	XynZ	M22624
	Cryptococcus albidus	Xyn	X12596
	Filobasidium floriforme	Xyn	JS0734
	Fusarium oxysporum	Xyn	L29380
	Magnaporthe grisea	Xyn	L35730
	Neocallimastix partriciarum	Xy1Z	X76919/S71569
	Penicillium chrysogenum	Xy1	M98458
	Pseudomonas fluorescens	XynA	X15429
	Pseudomonas fluorescens	XynB	X54523
	Ruminococcus flavefaciens	XynA[f]	Z11127
	Streptomyces lividans	XynA	M64551
	Streptomyces thermoviolaceus	XynI	A43937
	Thermoanaerobacter	XynA	M97882
	Saccharolyticum	Xyn	S16922
	Thermoascus aurantiacus	XynA	L18965

Table 1 (Continued)

Family[a]	Organism[b]	Enzymes	Accession no.[c]
	Thermophillic bacterium	XynA	Z46264
	Thermotoga maritima	XynA	D32065
	Aeromonas caviae	Xyn	X78115
	Aspergillus awamori	XynA	A19535
	Aspergillus niger	XynC	D14848/45138
	Aspergillus kawachii	XynA	L26988
	Aspergillus tubigensis	XynA	U10298
G	*Aureobasidium pullulans*	XynS	X59058
	Bacillus sp.	XynY	X59059
	Bacillus circulans	Xyn	X07723
	Bacillus pumilus	XynA	X00660
	Bacillus stearothermophilus	XynA	U15985
	Bacillus subtilis	Xyn	M36648
	Celulomonas fimi	XynD	X76729
	Clostridium acetobutylicum	XynB	M31726
	Clostridium stercorarium	XynA	D13325
	Cochliobolus carbonum	Xyn1	L13596
	Fibrobacter succinogenes	XynC[e]	U01037
	Fibrobacter succinogenes	XynC[f]	U01037
	Humicola insolens	Xy11	X76047
	Magnaporthe grisea	Xyn22	L37529
	Neocallimastix frontalis	Xyn1	X82266
	Neocallimastix frontalis	Xyn2	X82439
	Neocallimasstic patriciarum	Xyn1A	X65526
	Neocallimasstic patriciarum	Xyn1A	X65526
	Nocardiopsis dassonvillei	XynII	PQ0202
	Ruminococcus flavefaciens	XynA[c]	Z11127
	Ruminococcus flavefaciens	XynB[c]	Z35226

Table 1 (Continued)

Family[a]	Organism[b]	Enzymes	Accession no.[c]
G	*Ruminococcus flavefaciens*	XynD[c]	S61204
	Schizophyllum commune	Xyn	A44597
	Streptomyces lividans	X1nB	M64552
	Streptomyces lividens	X1nC	M64553
	Streptomyces sp. (EC3)	Xyn	X81045
	Thermomonospora fusca	XynN	U01242
	Thermomyces lanuginosus	XynA	O43097
	Trichoderma harzianum	Xyn	A44593
	Trichoderma reesei	Xyn1	X69573/X51973
	Thrchodrma reesei	Xyn2	X65974/X51975
	Trichoderma viride	Xyn	A44594/A44595

Note: [a] Family are classified according to Gilkes *et al.* (1991)

[b] Strain designations are given in parentheses

[c] GenBank, SWISS-PROT, EMBL or PIR database accession numbers are given if available; literature references are cited in other cases.

[e] N-terminal catalytic domain.

[f] C-terminal catalytic domain.

Source: Tomme *et al.* (1995)

5. Production of β-xylanase by *T. lanuginosus*

T. lanuginosus strain SSBP has been reported to be the best producer of cellulase-free xylanase when grown on coarse corncobs with an activity of 3575 U/ml and a specific activity of 3005 U/mg (Singh *et al.*, 2000c) (Table 2).

Table 2 Effect of carbon sources on xylanase production by some strains of *T. lanuginosus.*

Strains	Xylanase activity (U/ml)		References
	Carbon sources		
	Corncobs	Birchwood xylan	
ATCC 16455	2600	1309	Puchart *et al.*, 1999
ATCC 28083	1270	780	Puchart *et al.*, 1999
ATCC 34626	1780	1277	Puchart *et al.*, 1999
ATCC 36350	1330	1089	Puchart *et al.*, 1999
ATCC 46882	2839	1613	Puchart *et al.*, 1999; Bennett *et al.*, 1998
CBS 218.34	1670	183	Puchart *et al.*, 1999
CBS 224.63	ND	371	Puchart *et al.*, 1999
CBS 288.54	2579	1197	Puchart *et al.*, 1999
CBS395.62	2260	590	Puchart *et al.*, 1999
DSM 5826	1630	616	Puchart *et al.*, 1999
DSM 5826	1438	15	Gomes *et al.*, 1993a
IMI 110803	1320	256	Puchart *et al.*, 1999
IMI 131010	320	326	Puchart *et al.*, 1999
IMI 140524	450	1016	Puchart *et al.*, 1999
IMI 158749	70	291	Puchart *et al.*, 1999
IMI 84400	2460	1404	Puchart *et al.*, 1999
IMI 96213	2200	717	Puchart *et al.*, 1999
MH 4	150	697	Hoq and Decwer, 1995
RM-B	2449	1452	Puchart *et al.*, 1999
RT-9	564	1570	Hoq and Decwer, 1995
RT-9	427	525	Singh *et al.*, 2000c
SSBP	3575	462	Singh *et al.*, 2000c

ND is not determined.

These levels were much higher than xylanase activity levels of 2172 and 2726 U/ml produced by *T. lanuginosus* strains DSM 5826 and ATCC 46882, respectively, grown on the same substrate in shake-flask cultures (Singh *et al.*, 2000c; Puchart *et al.*, 1999; Purkarthofer *et al.*, 1993; Bennett *et al.*, 1998). When *T. lanuginosus* was cultivated on various carbon sources, significant differences of xylanase production were occurred. Corncobs were found to be the most effective substrate for xylanase production among various lignocellulosic substrates evaluated such as corn leaf, wheat bran, wheat straw, barley husk and birchwood xylan (Singh *et al.*, 2000a; Singh *et al.*, 2000c; Gomes *et al.*, 1993a; Purkarthofer and Steiner, 1995; Bennett *et al.*, 1998). The high xylanase production on coarse corncobs may be due to their greater particle size (coarse corncobs, 2-7 mm; fine corncob 62 mm), which leads to slower solubilisation of reducing sugars and creates a support system for fungal growth and enzyme release (Purkarthofer and Steiner, 1995). The influence of agitation on xylanase production of *T. lanuginosus* DSM 5826 was investigated (Purkarthofer *et al.*, 1993). A shaking speed of 120 rpm provided the optimal conditions for enzyme formation. At a decreased shaking speed of 100 rpm, the fungus showed poor growth and enzyme production was reduced dramatically whereas at high shaking speeds of 150-250 rpm enzyme production was adversely affected. The low xylanase activity produced at the slow shaking speed was ascribed to poor oxygen transfer within the medium, whereas the low xylanase production at high shaking speeds was thought to be due to greater hypha branching, mycelium fragmentation and early sporulation (Purkarthofer *et al.*, 1993).

Random amplification of polymorphic DNA (RAPD) was a modification of the polymerase chain reaction (PCR) in which a single primer able to anneal and prime at multiple locations throughout the genome can produce a spectrum of amplified products that were characteristics of the template DNA (Welsh and McClelland, 1991; Williams *et al.*, 1990). The polymerase chain reaction-randomly amplified polymorphic DNAs (RAPD-PCR) has been used for genetic and molecular studies as it was a simple and rapid method for determining genetic diversity and

similarity in various organisms. It also has the advantage that no prior knowledge of the genome under research was necessary (Yoon and Kim, 2001).

In an attempt to distinguish between high and low xylanase-producing strains, Singh *et al.* (2000b) examined the phylogenetic properties of eight *T. lanuginosus* strains and found no differences in the sequence of the internal transcribed region of the 5.8S rDNA while random amplified polymorphic DNA (RAPD) analyses showed that a relationship between the RAPD pattern and levels of xylanase produced could be established using certain primer UBC 241, whose sequences were 5'-GCCCGACGCG-3'. Strains DSM 5826 and SSBP that produced xylanase of 32000 and 59600 nkat/ml, respectively, were apparently closely related while strains ATCC 28083 and ATCC 58160 that produced xylanase of 9000 and 6300 nkat/ml, respectively, also showed a close relationship. However, not all strains producing low levels of xylanase grouped together indicating that RAPD analysis with primer UBC 241 has result in an ambiguous separation of strains based on their ability to produce xylanase. This observation would assist in attempts to find other high xylanase producing strains.

6. Regulation of xylanase synthesis

Despite the increase in knowledge of microbial xylanolytic systems in the past years, studies on induction and secretion of xylanases were necessary to develop efficient xylanase producers for possibility of commercial applications. Xylanase production from various bacteria and fungi has been shown to be inducible but rare examples of constitutive xylanase expression have also been reported. In general, the xylanase induction was a complex phenomenon and the level of response to an individual inducer varied with the organisms. An inducer giving maximum xylanase activity in one species may be the inhibitor of activity in other species. The substrate derivatives and the enzymatic end products might often play a key positive role in the induction of xylanases. However, they could also act as the end-product inhibitors, possibly at much high concentrations (Kulkarni *et al.*, 1999).

6.1 Induction

Efficient production of xylanolytic enzymes was dependent upon the choice of an appropriately induced substrate and the medium composition (Kulkarni *et al.*, 1999). Generally, xylanases were induced in most microorganisms during growth on substrates containing xylan (Purkarthofer and Steiner, 1995). Xylan, being a high molecular mass polymer, could not penetrate the cell wall and apparently low molecular mass fragments of xylan play a key role in the regulation of xylanase biosynthesis. These fragments include xylose, xylobiose, xylooligosaccharides, and heterodisaccharides containing xylose that were liberated from xylan by the action of low levels of constitutively produced enzymes (Kulkarni *et al.*, 1999) as show in Fig. 5. Xylanase produced by *T. lanuginosus* was shown to correspond to an induction or repression mechanism. A low level of xylanase was constitutively formed without the presence of an inducing substance. Xylanase production was induced in *T. lanuginosus* DSM 5826 with D-xylose having the strongest effect (1225 nkat/ml) indicating that D-xylose was the natural inducer (Purkarthofer and Steiner, 1995). The highest xylanase activity (7100 nkat/ml) of *T. lanuginosus* DSM 5826 was found in xylan-grown culture whereas very low activity (3.5 nkat/ml) was found in glucose-grown culture (Purkarthofer and Steiner, 1995).

In the presence of easily metabolisable substances such as glucose, fructose or lactose, xylanase was also formed, although, the activity in the presence of these repressors was similar to basal levels (Purkarthofer and Steiner, 1995). Xylan had the most pronounced effect on xylanase production by this fungus as the level of induction. D-xylose, D-arabinose, D-ribose and L-arabinose does not occur to the same degree as xylan. During the initial induction period, *T. lanuginosus* DSM 5826 only formed constitutive levels of xylanase activity, which led to slow liberation of xylooligosaccharides from xylan. These fragments induced xylanase production leading to a highly final level of enzyme activity. The reason was believed to be the long ailability finducing molecules that were slowly liberated resulting in a

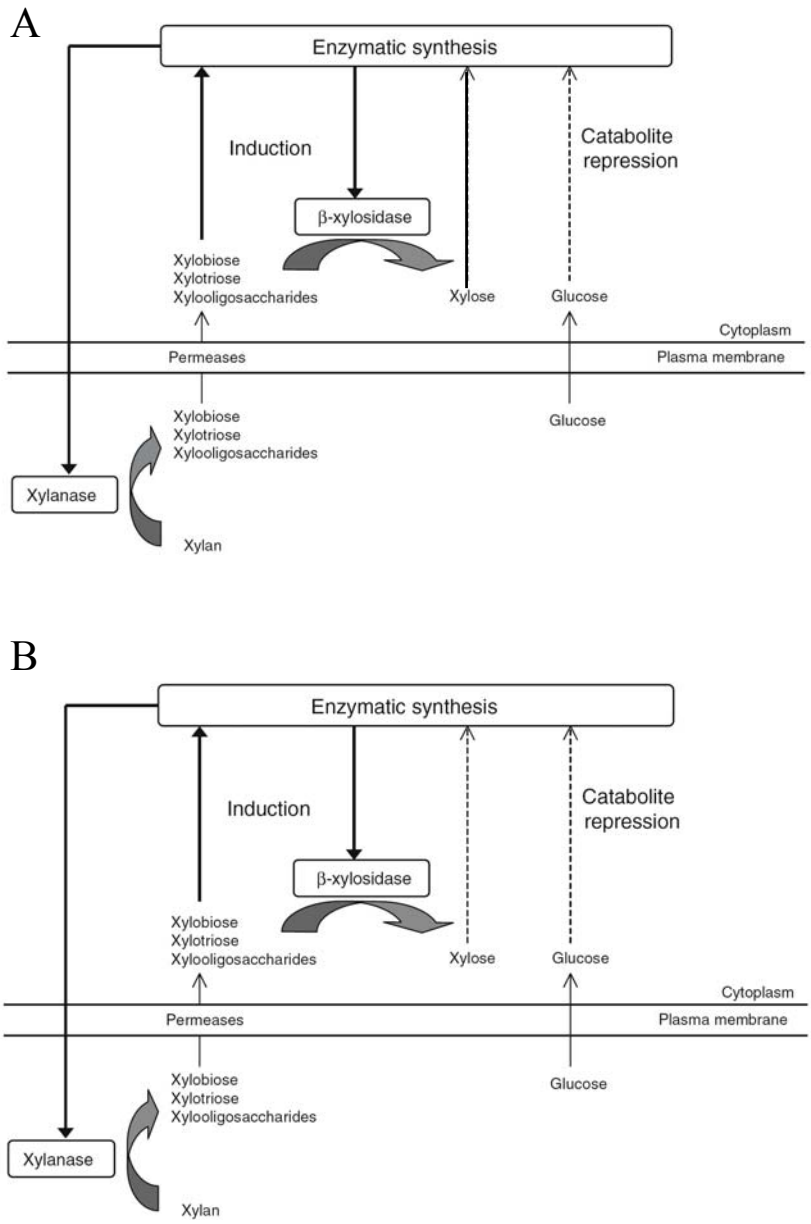

Figure 5 Hypothetical scheme of the regulation of the xylanolytic complex involving endo-xylanase and β-xylosidase in *Aspergillus sydowii* MG49 (A) and *Penicillium canescens* (B).

Source: Ghosh and Nanda (1994); Vavilova and Vinetskii (2003)

delayed exhaustion of an inducer. A similar mechanism was also suggested by Biely and Petrakova (1984) to explain the induction of xylanase in the yeast

Cryptococcus albidus by xylobiose. Ghosh and Nanda (1994) also explained the induction mechanism of xylanase from *Aspergillus sydowii* MG49 by xylose, xylobiose and β-D-methyl xyloside. With the sequential addition of xylose, xylanase formation was delayed but lasted longer. The kinetics of xylanase secretion showed a dependence on the concentrations of the inducer. Therefore, the availability of an inducer at low levels and over extended period was thought to lead to hyper-production of enzyme in *T. lanuginosus* DSM 5826 (Purkarthofer and Steiner, 1995). In some case, xylose inhibited xylanase production in fungi such as xylanase 1 and xylanase 2 produced by *Trichoderma reesei*. Xylanase 1 was induced by the presence of xylose only but not with xylobiose whereas xylanase 2 was induced by xylobiose only (Zeilinger *et al.*, 1996).

Hoq *et al.* (1994) reported that 10g/l birch wood xylan and 30g/l corncob induced xylanase synthesis in cultured growth of *T. lanuginosus* RT9 in which isolated in Bangladesh with activities of 8,725 and 7,110 nkat/ml, respectively. In contrast, Xylose (5g/l) repressed xylanase synthesis of this fungus with activity of 19 nkat/ml. Moreover, the xylanase formation using 5g/l glucose and non carbon source having 7 and 12 nkat/ml, respectively, were similar to xylanase level using xylose as a carbon source.

Xiong *et al.* (2004) reported that 15g/l of substrates such as xylan and xylose stimulated xylanase formation of *T. lanuginosus* DSM 10635 with activities of 497 and 83.2 U/ml, respectively, in growing cell condition for 4 days. In contrast of those, glucose and non-carbon source repressed xylanase formation with activities of 0.31 and 0.95 u/ml, respectively.

Liu *et al.* (1999) reported that extracellular xylanase synthesis in the yeast *Trichosporon cutaneum* SL409 was inducible. Active synthesis of xylanase took place during growth on separate 1% xylan and 1% xylose at levels of 947.5 and 225.8 nkat/mg dry weight, respectively. The negligible xylanase activities resulted when glucose, mannose, lactose, cellobiose, sucrose, xylitol, xylulose and maltose were

employed as carbon sources. The enzyme could be induced in washed glucose-grown cells using xylan or xylose. However, methyl D-xylopyranoside, a synthetic analogue of xylobiose was not an inducer of xylanase in *T. cutaneum* SL409. Xylanase induction was subject to glucose repression.

Vavilova and Vinetskii (2003) also studied induction of xylanase in the fungus *Penicillium canescens*. They reported that the fungus could be induced xylanase synthesis with activities of 0.9 and 2.1U/ml using 10 mM arabitol and 10 mM arabinose as an inducer of washed glycerol-grown mycelium, respectively. In contrast, xylose, xylitol, fructose and glucose could not induced in washed glycerol-grown mycelium after 24-h incubation.

Rizzatti *et al.* (2008) also induced xylanase synthesis of fungus *Aspergillus phoenicis.* The 72-h old cultures were harvested from vogel minimal liquid medium (VML medium), rinsed and resuspended in fresh VML medium supplemented with different carbon sources, and incubated for an additional 48 h. Significantly enzymatic levels were detected in the cultures supplemented with xylan, xylose and beta-methylxyloside with specificity of 171, 152 and 152 U/mg protein, respectively. Addition of glucose to media supplemented with these inducers strongly repressed the xylanolytic activity. Ribose, xylitol, galactose and fructose, did not induce the xylanase activity, and some induction was observed in the presence of arabinose.

7. Characterization of *T. lanuginosus* xylanase

Xylanase of *T. lanuginosus* has been purified from a number of strains. The molecular mass of the enzyme was found to be in the range of 22.5-29.0 kDa depending on the method of determination. The pI value of the xylanase from various strains was reported to be between 3.8 and 4.1 (Bennett *et al.*, 1998; Anand *et al.*, 1990; Cesar and Mrsa, 1996, Lin *et al.*, 1999; Kitpreechavanich *et al.*, 1984b; Bakalova *et al.*, 2002; Xiong *et al.*, 2004). Xylanase from strains ATCC 46882 and SSBP liberated mainly xylose and xylobiose from beechwood O-acetyl-4-O-methyl-D-glucuronoxylan (Bennett *et al.*, 1998; Lin *et al.*, 1999). Similarly xylanase from

strain ATCC 46882 released xylose and xylobiose from beechwood 4-O-methyl-D-glucuronoxylan and in addition also released an acidic xylooligosaccharide from 4-O-methyl-D-glucuronoxylan. Furthermore, Schlacher *et al.* (1996) found that the amino acid sequence of the xylanase from strain DSM 5826 was highly homology to other family 11 xylanases. The optimum temperature and pH of purified xylanase from various strains have been reported to be in the range of 60-75^{o}C and 6.0-7.0 respectively (Table 3). These values were similar to those observed in crude extracts of xylanase (Lischnig *et al.*, 1993; Singh *et al.*, 2000a; Singh *et al.*, 2000c; Gomes *et al.*, 1993a; Gomes *et al.*, 1993b; Alam *et al.*, 1994). The temperature stability of the purified xylanase from various strains differed somewhat depending on the experimental conditions (Table 3). Overall, the crude enzyme of *T. lanuginosus* strain was apparently more thermostable than the purified xylanase. Lin *et al.* (1999) suggested that some unknown factors might be present in the extract that stabilizes the protein. The kinetic properties of purified xylanases from *T. lanuginosus* have been investigated (Table 3).

T. lanuginosus has been reported by a number of laboratories to produce a highly thermostable xylanase based on the half-life of the enzyme (Lischnig *et al.*, 1993; Singh *et al.*, 2000a,d; Gomes *et al.*, 1993a; Gomes *et al.*, 1993b). The xylanase of *T. lanuginosus* strain SSBP was reported to be the most stable (half-life = 337 min at 70^{o}C), whereas that of the DSM 5826 strain (half-life = 201 min at 70^{o}C), and other strains showed lesser degrees of stability (half-life = 126 min or less at 70^{o}C). The xylanase of *T. lanuginosus* strain SSBP retained its full activity at temperatures up to 65^{o}C and 45% of its activity after 30 min at 100^{o}C and also retained its total activity after 14 days at 60^{o}C (Singh *et al.*, 2000d). The xylanases from other strains demonstrated a lower degree of enzyme activity and the xylanase produced by the strain ATCC 16455 was the least stable with only 10% activity remaining after 30 min at 100^{o}C. In contrast, the xylanase of *T. lanuginosus* strain RT 9 had previously been reported to retain its full activity at temperatures of up to 80^{o}C after 30 min and 68% of its activity after 30 min at 100^{o}C (Alam *et al.*, 1994). However, a 48% loss was observed for the storage stability tests at 55^{o}C after 21 days.

Table 3 Biochemical properties of β-xylanases produced from *T. lanuginosus*.

Strains	MW (kDa)	p*I*	Optimal pH	Optimal Temp. (^oC)	Half-life at 70^oC (minutes)	K_m (mg/ml)	Source of xylan	Hydrolysis product	Reference
(Griffon and Maublanc) Bunce	21.0	4.1	6.0	65	ND	7.3	BW	Xyl1, Xyl2	Kitpreechavanich *et al.*, 1984
(Griffon and Maublanc) Bunce	22.5	ND	6.0	65	ND	0.91	LW	ND	Anand *et al.*, 1990
DSM 5826	25.5	4.1	6.5	60-70	201 (pH 6.5)	ND	LW	ND	Cesar *et al.*, 1996 Bennett
ATCC 46882	25.7	3.7	6-6.5	75	30 (pH 6.0)	ND	BW	Xyl1, Xyl2	*et al.*, 1998
SSBP	23.6	3.8	6.5	70-75	ND	ND	BW	Xyl1, Xyl2	Lin *et al.*, 1999
SSBP	24.7	3.8	6.5	70	232 (pH 6.5)	3.26	BW	ND	Singh *et al*., 2000a,b
ATCC 44008	22.0	5.8	7.0	60	ND	5.14	BW	ND	Bakalova *et al.*, 2002
DSM 10635	25.5	3.7	6.5	70	40 (pH 6.5)	3.85	BW	ND	Xiong *et al.*, 2004
CAU 44	25.6	ND	6.2	75	ND	ND	WE-AX, WU-AX	Xyl1, Xyl2	Jiang *et al.*, 2005

ND, not determined

BW, birchwood xylan

LW, larchwood xylan

WE-AX, water-extractable arabinoxylan

WU-AX, water-unextractable arabinoxylan

Xyl1, xylose

Xyl2, xylobiose

Gruber *et al.* (1998) was found that the structures of the xylanase from *T. lanuginosus* closely resemble structures of other family 11 xylanases. The two active-site glutamates were consistent with Glu117 acting as the nucleophile and Glu209 acting as the acid-base catalyst. The fully conserved residue of Arg122 stabilized the negative charge on Glu117. Modeling studies of an enzyme-xyloheptaose complex indicated that only the three central sugar units were rigidly bound. The thermostability of this xylanase was due to the presence of an extra disulfide bridge not observed in most mesophilic variants, as well as to an increase in the number of ion-pair interactions.

There was one disulfide bridge in the *T. lanuginosus* enzyme which does not exist in the majority of other family 11 xylanases. It connects the C-terminus of the β strand B9 (residue 110) with the N-terminus of the α-helix (residue 154). While a similar disulfide bridge probably also exists in the *Schizophyllum commune* xylanase (Oku *et al.,* 1993) and other xylanases in which were known crystal structure having a hydrogen bond at the corresponding position.

Shibuya *et al.* (2000) reported that the thermostability of *Streptomyces lividans* xylanase B (SlxB-cat) was significantly increased by the replacement of its N-terminal region with the corresponding region from *Thermomonospora fusca* xylanase A (TfxA-cat) without observing a decrease in enzyme activity. In spite of the significant similarity between the amino acid sequences of the two xylanases, their thermostabilities were quite different. To facilitate an understanding of the contribution of structure to the thermostability observed, chimeric enzymes were constructed by random gene shuffing and the thermostable chimeric enzymes were selected. A comparative study of the chimeric and parental enzymes indicated that the N-terminus of TfxA-cat contributed to the observed thermostability. However, too many substitutions decreased both the thermostability and the activity of the enzyme. The mutants with the most desirable characteristics, Stx15 and Stx18, exhibited significant thermostabilities at 70^{o}C with optimum temperatures which were 20^{o}C

higher than that of SlxB-cat and equal to that of TfxA-cat. The ability of these two chimeric enzymes to produce reducing sugars from xylan was enhanced in comparison with the parental enzymes. These results suggest that these chimeric enzymes inherit both their thermostability from TfxA-cat and their increased reactivity from SlxB-cat. This demonstrates that random shuffing between a mesophilic enzyme and its thermophilic counterpart represents a facile approach for the improvement of the thermostability of a mesophilic enzyme.

Turunen *et al.* (2001) showed mutation experiment of three positions was introduced to the XYNII mutant containing a disulfide bridge (S110C–N154C) in the α-helix. The disulfide bridge increased the half-life of XYNII from less than 1 min to 14 min at 65^{o}C. An additional mutation at the C-terminus of the α-helix (Q162H or Q162Y) increased the half-life to 63 min. Mutations of Q162H and Q162Y alone had a stabilizing effect at 55^{o}C but not at 65^{o}C. The mutations N11D and N38E increased the half-life to about 100 min. However, there was no essential difference between the specific activities of the mutants and the wild-type XYNII.

Sriprang *et al.* (2006) improved the thermostability of the xylanase from *Aspergillus niger* BCC14405 by site directed mutagenesis using overlap extension PCR method. Two mutants were ST4 and ST5 have been gotten from the process, which substituted serine and threonine to arginine on the Ser/Thr surface of xylanase. The amino acid sequences of xylanase from the mutants enhanced the thermostability differed in 4 amino acids containing T25R, T65R, S67R, and S184R for mutant ST4 and differed in 5 amino acids containing T25R, S33R, T65R, S67R and S184R for mutant ST5. In the present of 1% birchwood xylan, the half-life in 100 mM sodium phosphate buffer pH 5.8 at 50^{o}C of modified xylanases produced by ST4 and ST5 mutants was increased for18 and 20 times from 14 to 257 min and 14 to 285 min, respectively. The both engineered xylanases were not stable in the absence of the 1% birchwood xylan. The modified xylanase produced by ST4 mutant had half-life for 45

min that was similar to that of wild-type xylanase. The modified xylanase produced by ST5 mutant was only 17 min lower than that of wild-type xylanase.

Stephens *et al.* (2007) improved the thermostability of the xylanase from *T. lanuginosus* DSM 5826 by directed evolution using error-prone PCR. The amino acid sequences of xylanase from the mutants that enhanced the thermostability differed in 3 amino acids for mutant 2B7-6 and had single mutation for mutants 2B11-16 and 2B7-10. Only one amino acid substitution (D72G) of xylanase from mutant 2B11-16 and substitution (Y58F) of xylanase from mutant 2B7-10 resulted in increase 2-time increasing half-life at 70^{o}C from 89 min to 168 min and 2.5-time increasing half-life at 70^{o}C from 89 min to 215 min, respectively. The single amino substitutions of xylanase in mutant 2B7-10 were occurred on the β-sheet, which was the hydrophilic at the outer surface of the enzyme structure. However, Stephens *et al.* (2007) reported that the most of amino substitution for the mutants producing high thermostable xylanases occurred within the β-sheet of enzyme in which forms the hydrophobic region of the enzyme.

Wang and Xia (2008) improved the pH stability of the xylanase from *Thermobifida fusca* by two round of random mutagenesis using DNA shuffling. The mutant of 2TfxA98 containing 5 substitutions of T21A, G25A, V87P, I91T and G217L was obtained. Among these, substitution of V87P, I91T and G217L were located in the surrounding area of the catalytic site. The mutant 2TfxA98 xylanase showed about 80% of the initial activity after 180 min of incubation in the presence of 50 mM Tris/HCl buffer (pH 9.0) at 50^{o}C, while the recombinant TfxA almost completely lost its activity under the same conditions. Both recombinant TfxA and mutant 2TfxA98 were stable at 70^{o}C (pH 7.0) during 30 min incubation but it seem to be that the mutant was slightly less stable than the parent at 70^{o}C after 30 min of incubation.

8. Molecular and structural biology of *T. lanuginosus* xylanase

8.1 Xylanase gene (*xynA*) of *T. lanuginosus*

In the genomic xylanase sequence, the sequence was interrupted by 106 bp of intron. This intron showed perfect 5' (AGBGTANGT) and 3' (ACAGB) intron splice site with the internal consensus sequence 'CAGCTAAC' (Schlacher *et al.*, 1996). The 5' of the complementary DNA (cDNA) corresponded to position 35 of the genomic DNA sequence. The conserved sequences TATAAA and CCAAT motif, which were common presence in eukaryotic promoters (Unlikes, 1992) were found 89 bp and 202 bp, respectively on upstream of the ATG codon. cDNA of *T. lanuginosus* xylanase was firstly sequenced by Schlacher *et al.* (1996) as shown in Fig. 6. It was contained 989 bp and included open reading frame (ORF) 615 bp. An ATG codon in which probably serves as the translation-starting site was identified on 36 bp downstream of the 5' of the cDNA. The ORF (615 bp) of xylanase gene of *T. lanuginosus* encodes a polypeptide of 225 amino. The N-terminal of 31 amino acids represented a signal sequence (Schlacher *et al.*, 1996; Gruber *et al.*, 1998). In addition, the region around amino acid residue 32 reassembled a KEX-like protease cleavage site resulting in a processed polypeptide starting at the amino acid glutamine. A similar situation was found with the xylanase of *Trichoderma viride* and *Trichoderma reesei.* Two conserved glutamate residues of the xylanase correspond to residue E-117 and E-209 in the *T. lanuginosus* xylanase (Schlacher *et al.*, 1996; Gruber *et al.*, 1998; Ko *et al.,* 1992; Miao *et al.,* 1994; Wakarchuk *et al.,* 1994a). The region containing E-117 was highly conserved in family G xylanase, but the surrounding sequence of E-209 shows somewhat higher variability. Torronen *et al*. (1994) suggested that variations of this region were responsible for different pH stability values found with the enzyme.

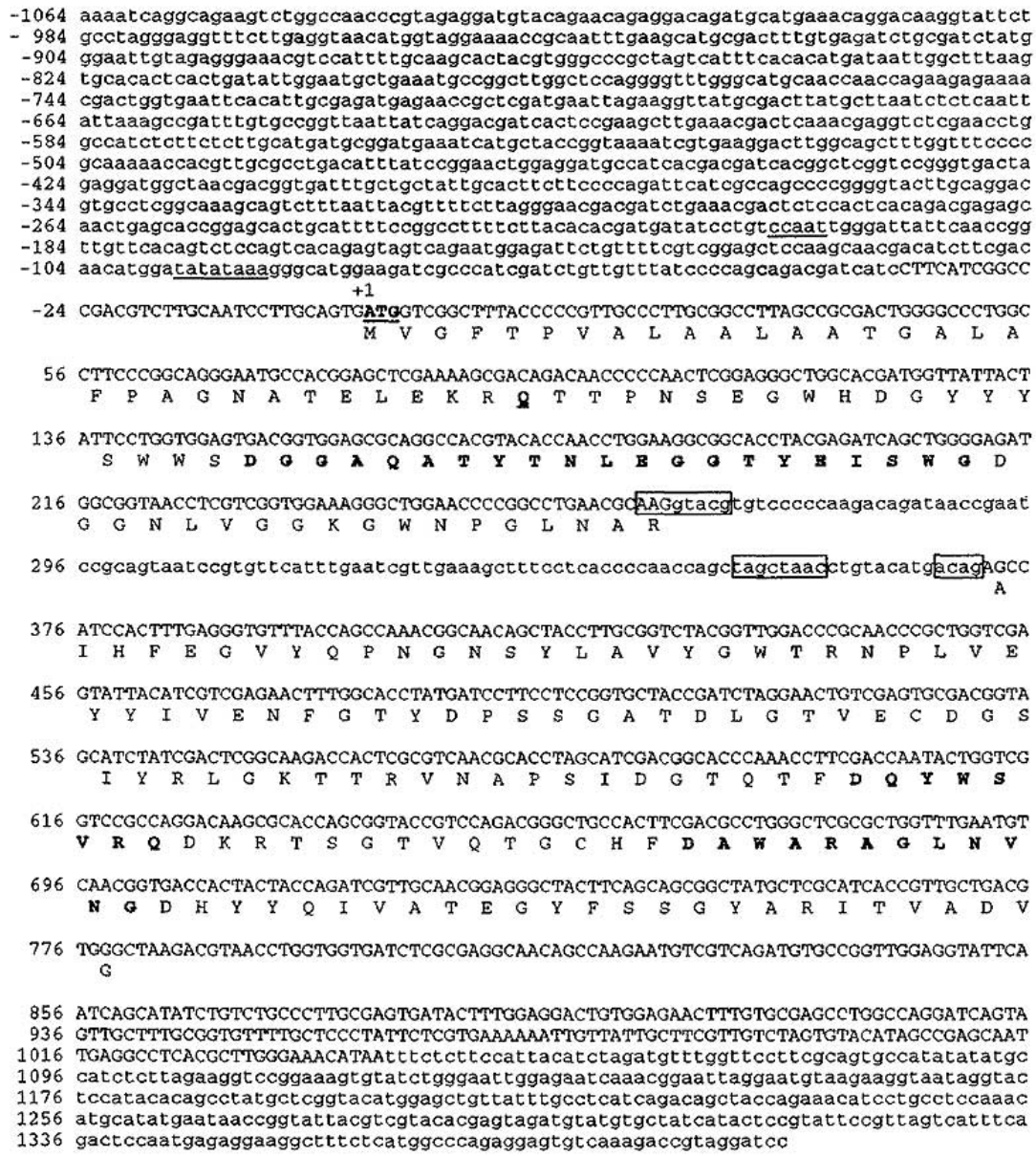

Figure 6 DNA sequence of *xyn A* gene (GenBank accession number U35436) and internal intron splice site consensus sequences are box.

Source: Schlacher *et al.* (1996)

8.2 Structure of xylanase from *T. lanuginosus*

Xylanase produced by *T. lanuginosus* strain was folded into a single ellipsoidal domain comprising two β sheets (A and B) and a single three turn α-helix (Fig. 7). The overall structures of xylanase were similar and have been described as a partially closed right hand. The hydrophobic faces of the two β sheets pack together to form a sandwich, which was described as fingers. The twisted parts of the β-sheets formed a cleft in one side of the molecule, which together with the helix were described as a palm. The active site was located at the concave side of the cleft. A long loop between the β8 and β7 strands was described as a thumb. However, it did not make any hydrogen bonds with other parts of the molecule (Torronen *et al.,* 1994). Two conserved glutamate residues were catalytically active residues and were located on either side of the cleft. According to mutagenesis and mechanism-based inhibitors these residues have been identified as a nucleophile and an acid:base catalyst (E86 and E177) in the *T. lanuginosus* xylanase structure, respectively (Gruber *et al.*, 1998).

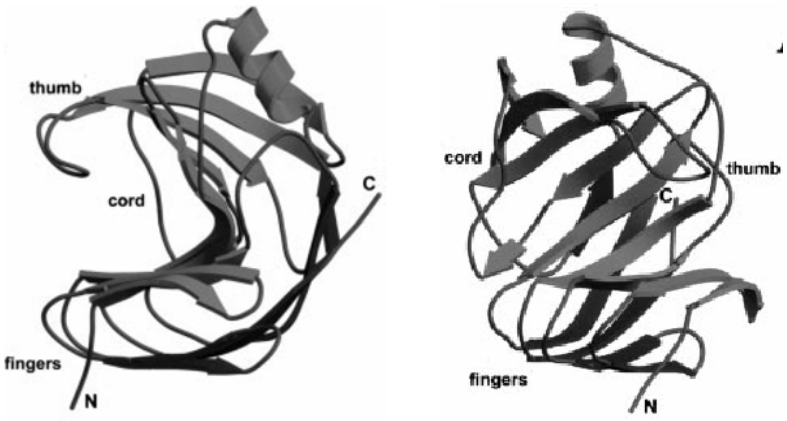

Figure 7 Two perpendicular views of the structure of the xylanase from *T. lanuginosus* generated with programs MOLSCRIPT and Raster 3D.

Source: Gruber *et al.* (1998)

9. Other hemicellulose degrading enzymes produced *T. lanuginosus*

Production of other hemicellulases such as mannanase and mannosidase were not detected in *T. lanuginosus* strain DSM 5826 after growth on corncobs whereas both enzymes were produced at low levels in *T. lanuginosus* SSBP (62 U/ml) (Singh *et al.*, 2000c). However, growing the fungus on galactomannan (locust bean gum) did not result in the induction of higher levels of mannan-degrading enzymes than other xylan-containing substrates (Singh *et al.*, 2000c). Furthermore, Puchart *et al.* (1999) reported that 17 strains of *T. lanuginosus* that were ATCC 46882, CBS 288.34, ATCC 44008, IMI 11003, CBS 224.63, IMI 140524, IMI 158749, CBS 395.62, CBS 218.34, ATCC 22070, IMI 96213, IMI 131010, ATCC 34626, ATCC 36350, ATCC 28083, ATCC 16455 and DSM 5826 were able to produce β-xylanases. Only three strains of IMI 158749, CBS 218.34 and IMI 13101 produced mananase and three strains of IMI 158749, ATCC 34626 and ATCC 36350 produced pectinase. The strains producing high β-xylanases secreted low amounts of xylan-debranching enzymes and did not produce mannan and arabinan-degrading enzyme systems. Only the strains showing low xylanase production exhibited a higher degree of xylan utilization and also the ability to produce a mannanolytic enzyme system.

10. Biological application of xylanases

Commercial xylanases were industrially produced in Japan, Finland, Germany, Republic of Ireland, Denmark, Canada and the USA. The microorganisms used to obtain these enzymes were *Aspergillus niger*, *Trichoderma sp*. and *Humicola insolens*. Nevertheless, commercial xylanases could also be obtained from bacteria. Xylanase began to be used in the 1980s for preparation of animal feed and later in the food, textile and paper industries. Currently, xylanase and cellulase and pectinases accounted for 20% of the world enzyme market.

10.1 Xylanases in animal feed

Xylanases were used in animal feed along with glucanases, pectinases, cellulases, proteases, amylases, phytase, galactosidases and lipases. These enzymes break down arabinoxylans in the ingredients of the feed reducing the viscosity of the raw material (Twomey *et al*., 2003). The arabinoxylan found in the cell walls of grains has an anti-nutrient effect on poultry. When components were presented in soluble form, they might raise the viscosity of the ingested feed interfering with the mobility and absorption of other components. If xylanase was added to feed containing maize and sorghum, both of those were low viscosity foods, it might improve the digestion of nutrients in the initial part of the digestive tract resulting in a better use of energy. The joint action of the rest of the enzymes produced a more digestible food mixture. Young fowl and swine produced endogenous enzymes in smaller quantities than adults, so that food supplements containing exogenous enzymes should improve their performance as livestock. Moreover, this kind of diet was found to reduce unwanted residues in the excreta (phosphorus, nitrogen, copper and zinc), an effect that could have a role in reducing environmental contamination.

10.2 Manufacture of bread, food and drinks

Xylanases might be employed in bread-making, together with α-amylase, malting amylase, glucose oxidase and proteases. The xylanases broke down the hemicellulose in wheat-flour helping in the redistribution of water and leaving the dough softer and easier to knead. During the bread-baking process, they delayed crumb formation allowing the dough to grow. Using of xylanases, there has been an increased in bread volumes, greater absorption of water and improved resistance to fermentation (Maat *et al*., 1992; Harbak and Thygesen, 2002; Camacho and Aguilar, 2003). Amount of arabino-xylooligosaccharides in bread would be beneficial to health. In biscuit-making, xylanase was recommended for making cream crackers lighter and improving the texture, palatability and uniformity of the wafers. The juice and wine industries make up a good part of the enzyme market. The production of fruit and vegetable juices required methods of extraction, clearing and stabilization.

Nowadays, xylanases in conjunction with cellulases, amylases and pectinases, leaded to an improved yield of juice by liquefaction of fruit and vegetables, stabilization of the fruit pulp, increased recovery of aromas such as essential oils, vitamins, mineral salts, edible dyes, pigments etc. This enzyme was also used in coffee-bean mucilage (Wong *et al.*, 1988; Wong and Saddler, 1993). The mainly desirable properties of xylanases for use in the food industry were high stability and optimum activity at an acid pH.

10.3 Pharmaceutical and chemical applications

Xylanase and xylan were little used in the pharmaceutical industry. Sometimes, xylanases were added in combination with a complex of enzymes (hemicellulases, proteases and others) as a dietary supplement. Hydrolytic products of xylan such as β-D-xylopyranosyl residues could be converted into combustible ethanol, solvents and artificial low-calorie sweeteners. The first steps were the delignification of hemicellulose material rich in xylan following by hydrolysis by xylanases and hemicellulases to produce sugars such as β-D-xylopyranosyl units. Next, the products were fermented by yeasts (*Pichia stipitis* and *Candida shehatae*) as outlined in Fig. 8 in order to produce xylitol or ethanol (Screenath and Jeffries, 2000).

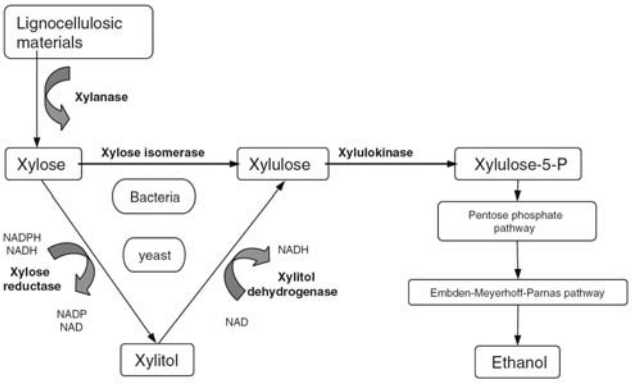

Figure 8 Simplified scheme of xylitol and ethanol production by bacteria and yeasts from lignocellulosic materials.

Source: Polizeli *et al.* (2005)

Xylitol was a polyalcohol with a sweetening power comparing to sucrose (Parajó *et al*., 1998). The development of a more appropriate technology for xylitol production has generated great hope of its wider use in the food, pharmaceutical and odontological industries.

10.4 Textiles

The xylanolytic complex could be used in the textile industry to process plant fibers such as linen. For this purpose, the xylanase should be free of cellulolytic enzymes. One process consisted of incubating dried ramie (China grass) stems with xylanase to liberate the long cellulose fibers intact. After using this method, there was no need to use the strong bleaching step due to lignin did not undergo oxidation, which would lead to darkening of the fibers (Prade, 1995; Brühlmann *et al*., 2000; Csiszar *et al.*, 2001). Relatively little research has been done on the enzymatic preparation of textile fibers. This appears to be a promising market demanding the development of new techniques.

10.5 Cellulose pulp and paper

In pulp bleaching, xylanases selectively degraded the accessible hemicellulose fraction of woods and it has been found to enhance the extractability of lignin (Viikari *et al.*, 1994). Xylanases from several *T. lanuginosus* strains have exhibited promising results when they were applied as a bleaching agent to kraft and sulfite pulp produced from sugar cane bagasse, eucalyptus and beech. Significant reduction of chemicals required to attain the desired kappa number was found while increased brightness and viscosity was achieved (Gubitz *et al.*, 1997; Haarhoff *et al.*, 1999; Madlala *et al.*, 2001). Commercial xylanases were typically produced by mesophilic filamentous fungi such as *Trichoderma reesei* and *Aspergillus niger*, which were excellent protein secretors. However, these xylanases might not be sufficiently thermostable for processes where enzymes active at high temperatures. Therefore the thermostable xylanase of *T. lanuginosus* might be suitable for high temperature processes.

There were two hypotheses about the role of xylanases in cellulose pulp bleaching. In the first, the xylanases would act on the xylan precipitated on the lignin (Viikari *et al*., 1994). This xylan was precipitated due to lowering of the pH at the end of the cooking stage. Its removal by the action of xylanases would leave the lignin more exposed to the compounds employed in the bleaching of cellulose pulp. The second hypothesis was based on the ability of lignin to form complexes with polysaccharides such as xylan, and the fact that some of the bonds were alkali resistant and might not have been hydrolyzed during the Kraft process (Buchert *et al*., 1992). The xylanases acted by cleaving the remaining bridges between the lignin and xylan, opening the structure of the cellulose pulp and leading to the fragmentation of xylan and subsequent extractions of the fragments (Paice *et al.*, 1992).

MATERIALS AND METHODS

1. Isolation and identification of *Thermomyces lanuginosus* producing xylanase

1.1 Isolation

The samples; soil, compost and dung, were collected from differently ecological areas. All samples were determined moisture content, organic matter and pH as soon as they arrive. Then, samples were dried at temperature of 50^{o}C. The samples were suspended in sterize-distilled water and then spreaded on YpSs agar plus 0.08% streptomycin. The spreaded plates were incubated at 50^{o}C until fungal mycelium formed as colony. The number of thermophilic fungi and *T. lanuginosus* were count. The expected colony of thermophilic fungus *T. lanuginosus* were selectively picked up based on morphology under the microscope. The expected isolates were identified according to the manual of fungal taxonomy (Cooney and Emerson, 1964; Domsch *et al.*, 1993; Salar *et al.*, 2007).

1.2 Molecular identification

Extracted DNAs were used for amplify internal transcribed spacer region (ITS) rDNA by PCR. PCR product was cloned to pGEM-T easy vector cloning kit (Promega®). Quantum Prep® Plasmid Miniprep Kit (Bio-Rad, US) was used for extracted plasmid from transformant. The samples were prepared for sequence PCR using 2 pmol M13 forward (5'GTAAAACGACGGCCAGT-3') or M13 reverse primer (5'-CAGGAAACAGCTATGAC-3'), approximately 0.1-0.2 pmol DNA and added Themo Sequenase^{TM} Primer Cycle Sequencing reaction kit (Amesham Biosciences). Thermocycler was used for PCR with the following parameters: initial denaturation for 1 min at 95^{o}C followed by 25 cycles of denaturation for 30 s at 95^{o}C, annealing for 30 s at 55^{o}C, elongation for 1 min at 72^{o}C. The products were sequenced using a 5500-5 DNA sequencer (Hitashi). Data from the forward and the

reverse sequences were analyzed using the Genetyx version 5.0 program and compared with reference sequences obtained from the database of DDBJ web site.

1.3 Phylogenetic analysis

Multiple-sequence alignments of the nucleotide sequences of the strains of *T. lanuginosus* and other fungi such as *Aspergillus niger, Chaetomium thermophillum, Humicola grisea, Humicola insolens, Humicola fuscoatra, Phytophthora capsici, Scytalidium thermophilum, Schizophyllum commune, Talaromyces emersonii, Thermoascus aurantiacus, Thermomyces lanuginosus, Trichoderma harzianum, Trichoderma viride* and *Trichoderma reesei* using random selection were made using the Genetyx5 in order to investigate the genetic variation of *T. lanuginosus* strains that isolated in Thailand. Then, phylogenetic trees were constructed from the evolutionary distance data by applying the sequences obtained to the algorithm of the neighbor-joining (NJ) and kimura 2-parameter method (Serra *et al.*, 2006). To evaluate the robustness of branches in the inferred tree, the bootstrap resampling method of Felsenstein with 1000 replicates was used. MEGA4 software was used to construct the phylogenetic trees for comparison.

1.4 Production of xylanase, pectinase and mannanase

An actively growing 3-day old colony of each *T. lanuginosus* (1 or 2 agar block) grown on yeast glucose medium was inoculated into synthetic medium (15 ml) consisted of (per liter of water) 2 g of KH_2PO_4, 0.3 g of $CaCl_2$, 0.3 g of $MgSO_4$, 5 g of peptone, 3 g of yeast extract, 3 g of malt extract and 10 g of respective substrates (oat spelt xylan, pectin and mannan) in 50 ml Erlenmeyer flasks. Duplicate flasks were shaken at 120 rpm and incubated at 45^{o}C for 5 days. The clear filtrated supernatants obtained from cultured broth through Whatman No. 1 filter paper (Whatman International Ltd., USA) were used assays the enzyme activities. The cultured supernatants from the medium having separate oat spelt xylan, pectin and mannan as a carbon source was used to determine β-xylanase and β-xylosidase, pectinase, and mannanase activity, respectively.

1.5 Thermostability determination

Thermostability of each crude xylanase was determined. Each micro tube (1.5 ml) containing 0.5 ml of each sample was incubated at 70^{o}C. The residual xylanase activity was measured at intervals from 0 to 1080 min.

2. Purification and characterization

2.1 Xylanase production

T. lanuginosus THKU-9 representative of low thermal stable xylanase producing strain and THKU-49 representative of high thermal stable xylanase producing strain were used in this experiment. They were preserved at Bangkok MIRCEN with codes of TISTR 3590, TISTR 3597, respectively. They were cultivated for 1-7 days using oat spelt xylan as a carbon source following method of cultivation in topic 1.3. The clear supernatant was obtained from filtration of cultured broth through Whatman No.1 filter paper (Whatman International Ltd., USA) was used for assay of β-1,4-xylanase activity using DNS method, extra-cellular protein using Lowry's method and pH.

2.2 Purification of xylanase

The cultured supernatants of *T. lanuginosus* THKU-9 and THKU-49 were purified using same protocol. Two litres of cultured supernatant were used and subjected to 80% $(NH_4)_2SO_4$ saturation. The precipitated protein was collected using centrifugation at 10,000 rpm for 5 min and dissolved in 50 mM sodium phosphate buffer (pH 6.0). Dialysis bag having cut off 10,000 Dalton was used to remove small proteins and salt from sample. The dialyzed enzymes were further purified using an anion-exchange column chromatograph as DEAE Sepharose fast flow. The column of ion-exchange chromatograph (3x10cm) was previously equilibrated with 50 mM phosphate buffer (pH 6.0) for 3-bed volume before the dialyzed enzyme solution was subjected. Each enzyme was gradual eluted from the column with 500 ml of the

buffer contained 0-0.5 M NaCl at the flow rate 60 ml/h. Each fraction (5 ml) was collected. Each fraction was determined xylanase activity and then combined the activity fraction.

Xylanase fractions from above step were pooled and further purified using hydroxyapatite column chromatograph with column size was 3x10cm. Equilibrate and washing buffer was 10 mM phosphate buffer (pH 6.0). The bound xylanase was eluted using 20-500 mM sodium phosphate buffer (pH 6.0) gradient. The active fractions were pooled and concentrated using ammonium sulfate precipitation.

The concentrated enzymes were further purified with Sephadex G-100 gel filtration column chromatograph (2x120 cm). The column was equilibrated with 2-3 bed volume of 3 mM phosphate buffer (pH 6.0). The same buffer was used as elution buffer at the flow rate of 60 ml/h. Each fraction was determined xylanase activity and then combined the activity fraction. Pooled fraction was determined the xylanase specificity and purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.3 Characteristics of the pure xylanase

2.3.1 Molecular weight determination

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Molecular weight of the pure enzyme was determined on SDS-PAGE. The molecular weight was estimated compared to standard proteins; (SM0431 Fermentas, USA) which were β-galactosidase (116,000), bovine serum albumin (66,200), ovalbumin (45,000), lactate dehydrogenase (35,000), Rease Bsp98I (25,000), β-lactoglobulin (18,400), and lysozyme (14,000) (Appendix Figure B1).

2.3.2 Substrate specificities and K_m

The reaction mixtures containing each pure enzyme and 0.5% each of substrate (final conc.) in 50 mM sodium phosphate buffer (pH 6.0) are incubated at

50°C for 10 min. Reducing sugars liberated are determined using DNS method. The substrates used were oat spelt xylan, soluble oat spelt xylan, insoluble oat spelt xylan, rice straw hemicellulose, alkali treated corncob, cassava starch, carboxymethylcellilose, avicel, filter paper, locust bean gum and p-Nitrophenyl β-D-xylopyranoside.

To calculate K_m value on the hydrolysis of oat spelt xylan, soluble oat spelt xylan and insoluble oat spelt xylan, 2-10 g/l of each substrate in 50 mM sodium phosphate buffer (pH 6.0) was incubated at 50^{o}C for 2-20 min. The K_m value was calculated from the initial rate based on a Lineweaver–Burk reciprocal plot as shown in Appendix Figure A1 and Appendix Figure A2 (Lineweaver and Burk, 1934).

2.3.3 Effect of metal ions and chemicals on the activity

The effect of various additives on enzyme activity of pure xylanase was analyzed following method of Heck *et al.* (2006). The xylanase activity was tested by incubating pure xylanase in the present of 1 mM solution of K^+, Li^+, Na^+, Sn^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+}, EDTA and β-mercaptoethanol for 15 min at 50^{o}C before the reaction with substrate. The xylanase activity assayed in the absence of additive was taken as control.

2.3.4 Combination effect of temperature and pH on the activity of the enzyme

Central composite design (CCD) was used to generate 13-treatment combinations, with pH and temperature as independent variables using software as Minitab version 14. The experimental matrix corresponded to design with coded variables containing star points (α = 1.414) and five replications of the central point. The data of experiments were analyzed using software as SPSS version 10.

2.3.5 Effect of buffer concentration on xylanase stability

The effect of buffer concentration on thermostability of the pure enzymes was performed at 70^{o}C in 10, 50, 100, 200 and 400 mM of acetate buffer (pH 5.0), citrate buffer (pH 6.0) and sodium phosphate buffer (pH 6.0 and 7.0). The residual xylanase activities at intervals were measured. Half-life at 70^{o}C of the pure enzymes of each buffer was determined. Half-life of enzyme was the time that the activity remained 50% of its original activity.

2.3.6 Thermostability of xylanase at various temperatures

Thermostability of each pure enzyme was also determined in 50 mM phosphate buffer (pH 7.0) in which was incubated at 50, 60, 70, 80, 90 and 100^{o}C. The residual xylanase activities were measured at intervals from 0 to 1080 min.

2.3.7 Combination effect of temperature and pH on xylanase stability

Each microtube (1.5 ml) containing 0.5 ml of pure enzyme (~ 120 U/ml) was incubated at various temperatures and pHs at same above-mentioned condition in topic of 2.2.4, 13-treatment generated combinations. The half-life of xylanases was measured at intervals from 0 to 5 days. The data of experiments were analyzed by software of SPSS version 10.

2.3.8 Mode of action of pure xylanase on oat spelt xylan and xylobiose

To determine mode of action of xylanase, 10 mg of oat spelt xylan, and xylobiose was separately incubated at 50^{o}C for 24 h with 3 units of pure xylanase in reaction volume of 1.0 ml containing 50 mM sodium phosphate buffer pH 6.0. The aliquots were analyzed at differently time intervals for hydrolysis products on TLC plates. Reducing sugars were detected using aniline-hydrogen phthalate reagent (Li *et al.*, 2005).

2.3.9 Binding ability on insoluble substrates

Each pure xylanase in 50 mM sodium phosphate buffer (pH 6.0) were incubated in polycarbonate tube (10ml, Ø 16.1 x 81.1 mm) with different concentrations of various insoluble substrates; insoluble oat spelt xylan, alkali treated corncob carboxymethyl cellulose and cassava starch at 4^{o}C for 24 h. Separation of particle of insoluble substrates and supernatant were done using centrifuge at 7,000 rpm for 10 min at 4^{o}C and then determined the residual xylanase activity in the supernatant (unbound enzyme). To confirm binding, the particle was washed using distilled water for 4 times (Ponpium *et al.*, 2000) The xylanase activity was also determined from enzyme-bound particle.

2.3.10 Binding ability on insoluble xylan at different concentration of NaCl

Each pure xylanase in polycarbonate tube (10 ml) with 50 mM sodium phosphate buffer (pH 6.0) were incubated with different concentrations insoluble oat spelt xylan supplemented with various NaCl concentration at 4^{o}C for 24 h. Separation of particle of insoluble substrates and supernatant were done using centrifuge at 7,000 rpm for 10 min at 4^{o}C and then determined the residual xylanase activity in the supernatant (unbound enzyme). To confirm binding, the particle was washed using distilled water for 4 times and then xylanase activity was determined from enzyme-bounded particle.

2.4 Sequencing of the xylanase gene of selected strains

Complementary DNAs (cDNA) of *T. lanuginosus* THKU-9 xylanase and *T. lanuginosus* THKU-49 xylanase were synthesized using RNA as template. cDNA was cloned to pGEM-T easy vector cloning kit (Promega®). Quantum Prep® Plasmid Miniprep Kit (Bio-Rad, US) was used for extracted plasmid from

transformant. The samples were prepared for sequence PCR using 2 pmol M13 forward (5'GTAAAACGACGGCCAGT-3') or M13 reverse primer (5'-CAGGAA ACAGCTATGAC-3'), approximately 0.1-0.2 pmol DNA and added Themo Sequenase^{TM} Primer Cycle Sequencing reaction kit (Amesham Biosciences). Thermocycler was used for PCR with the following parameters: initial denaturation for 1 min at 95^{o}C followed by 25 cycles of denaturation for 30 s at 95^{o}C, annealing for 30 s at 55^{o}C, elongation for 1 min at 72^{o}C. The products were sequenced using a 5500-5 DNA sequencer (Hitashi). Data from the forward and the reverse sequences were analyzed using the Genetyx version 5.0 program and compared with reference sequences obtained from the database of DDBJ web site.

2.5 Phylogenetic analysis

The phylogenetic tree was constructed from the evolutionary distance data by applying the sequences obtained to the algorithm of the neighbor-joining (NJ) and *p*-distance method (Kulkarni *et al.*, 1999). To evaluate the robustness of branches in the inferred tree, the bootstrap resampling method of Felsenstein with 1000 replicates was used. MEGA4 software was used to construct the phylogenetic tree for comparison.

3. Xylanase induction

3.1 Growth experiments

3.1.1 Xylanase production

For evaluation of xylanase production of during growth on separate oat spelt xylan and xylose as a carbon source at various concentration of 0.1%, 0.5% and 1%, an actively growing 3-day old colony of each *T. lanuginosus* strain (1 or 2 agar block) grown on yeast glucose medium was inoculated into synthetic medium (15 ml) consisting of (per liter of water) 2 g of KH_2PO_4, 0.3 g of $CaCl_2$, 0.3 g of

$MgSO_4$, 5 g of peptone, 3 g of yeast extract, 3 g of malt extract and 10 g of substrate in 50 ml Erlenmeyer flasks. The initial pH was adjusted to a value of 6.0. The flasks were shaken at 120 rpm and incubated at 45^{o}C for 5 days. The clear supernatant obtained from filtration of cultured broth through Whatman No. 1 filter paper (Whatman International Ltd., USA) was used for determination of xylanase activity.

3.1.2 Addition of xylose and glucose on xylanase production medium

Selected strains of *T. lanuginosus* in which were TISTR 3465, THKU-11, THKU-25, THKU-85, ATCC 44008 and ATCC 46882 were grown in the medium containing 1% xylan as a carbon source following above method. Then, separately each syrup of xylose and glucose was added to the 3rd day xylan grown culture to make up 10 g/l and further shaken at 120 rpm and incubated at 45^{o}C for 5 days. The supernatants of each filtrated culture were periodically sampled for determination of xylanase activity.

Additions of various xylose concentrations were also investigated. These selected strains of *T. lanuginosus* were grown in the medium containing 1% xylan as a carbon source. Then, syrup of xylose was added to the 3rd day xylan grown culture to make up 1, 2.5, 5, 10, 20 g/l and further shaken at 120 rpm and incubated at 45^{o}C for 5 days. The supernatants of each filtrated culture were sampled for determination of xylanase activity.

3.2 Induction experiments

The freshly washed mycelium were suspended in 50 mM sodium phosphate buffer (pH 6.0) to give a mycelium suspension. Aliquots of this suspension (15 ml) containing 0.11-0.15 g dry mycelium in 125 ml Erlenmeyer flasks were rapidly mixed with various compounds tested as inducers in which were oat spelt xylan and xylose (final concentrations are 10, 100, 500, 1000 mg/l). Moreover, one of them did not add any compound, which was used as a control. Oat spelt xylan and xylose were dissolved at first under sterilization in a small volume of 50 mM sodium

phosphate buffer (pH 6.0) and then mixed with the mycelium suspensions. The mixtures were incubated continuously on 120-rpm reciprocal shaker (JEIO TECH, model BS-30) at 50^{o}C. At various time intervals of 10 h, aliquots were taken and filtrated. The filtrated supernatants were analysed for xylanase activities.

3.3 Random amplified polymorphic DNA (RAPD) analysis

Genomic DNAs of 14-selected strains of *T. lanuginosus* which were THKU-2, THKU-4, THKU-9, THKU-11, THKU-25, THKU-30, THKU-33, THKU-49, THKU-56, THKU-77, THKU-85, THKU-86, ATCC 44008 and ATCC 46882 were used as templates for analysis. Three primers used in this study were UBC 235, 241 and 280 whose sequences were 5'-CTGAGGCAAA-3', 5'-GCCCGACGCG-3', 5'-CTGGGAGTGG-3', respectively. A standard PCR procedure was used to amplify genomic DNA (Williams *et al*., 1990). The PCR reaction volume of 10 μl contained 0.3 μM primer, 0.2 mM each of dATP, dCTP, dTTP and dGTP, 50 ng of genomic DNA, separate 0.2 U of Blend Taq Plus DNA polymerase (Life Science Department, Toyobo Co., LTD, Japan) and recombinant Taq DNA polymerase (Life Science Department, Toyobo Co., LTD, Japan) and 3.0-3.5 mM $MgCl_2$ in 1X PCR buffer. Reaction mixtures were performed using an automated thermocycler (Takara PCR Thermal Cycler Dice). PCR was performed with initial denaturation for 2 min at 94^{o}C followed by 35 cycles of denaturation for 30s at 94^{o}C, annealing for 30s at 34^{o}C, elongation for 2 min at 68^{o}C (for Blend Taq Plus DNA polymerase) or 72^{o}C (for recombinant Taq DNA polymerase). A final elongation step of 5 min at 72^{o}C was included. PCR products were analyzed by electrophoresis in 2% agarose gels consisting 0.0005 mg/ml of ethidium bromide, in 0.5X Tris-Borate-EDTA (TBE) and banded patterns were analyzed using the Gel Documentation 2000 System (Bio-Rad, US).

3.4 Phylogenetic tree construction

Polymorphic bands were identified using electrophoresis imaging. The bands were scored as present (1) or absent (0). The pair-wise evolutionary distances are given by generated the distance matrix. Choose and cluster the pair of samples with the minimum distance between two samples. The branching point is positioned at a distance of number of distance/ No. of sample = distance of branch, then join these two sample as one new node. Calculate distance from these node to other node by using the equation (Opperdoes, 1997).

$$d(c,k) = \frac{d(i,k)+d(j,k)}{2} ; c = \{i,j\}$$

Following the first clustering i and j are considered as a single cluster and calculate the new distance matrix as mention above. Erase column and line in the distance matrix that contains first 2 samples then repeat the calculation last one row and column. The distance between a simple (i,j) and a composite sample is the average. Then a new distance matrix is recalculated using the newly calculated distances and the whole cycle is being repeated.

4. Analysis methods

4.1 Determination of moisture content

Soil moisture was measured for the whole set of soil samples. The procedures carried out by weighing the fresh samples and then drying them in an oven, at 110^{o}C until there are constant. Moisture content was obtained by the difference between the fresh weight and the dry one.

4.2 Determination of organic matter

Soil samples were subjected to determination the organic matter. The suspension of soil was transferred to a flask 250 ml and titrated with a solution of

ferrous ammonium sulfate. The ferrous ammonium sulfate solution was slowly added to the soil suspension causing the ferrous to be oxidised instantaneously by the remainder of the dichromate until the potential shift at the electrode indicates when all dichromate has been taken out by the ferrous. The organic matter content may be calculated then from the difference of the originally added quantity of dichromate and the remainder, which was determined by the quantity of the added ferrous until titration endpoint. The organic matter of soils was calculated as follows:

% Organic carbon = ((ml of $K_2Cr_2O_7$ - ml of $(NH_4)_2FeSO_4$) x 0.003 x 100 x 1.33)/ dry weigh of soil sample

% Organic matter = %organic carbon x 1.72

4.3 Determination of soil pH

Dry soil samples mixed with distilled water in ration of 1:1. Then, the soil/water solution was used for pH measurements using the pH meter.

4.4 DNA extraction.

The flesh mycelium of 15 representative strains consisted of THKU-2, THKU-3, THKU-9, THKU-11, THKU-16, THKU-17, THKU-25, THKU-30, THKU-33, THKU-43, THKU-49, THKU-55, THKU-79, THKU-80 and THKU-81 were selected from low and high themostable xylanase producing strains collected from liquid medium consisted of (per liter of water) 2 g of KH_2PO_4, 0.3 g of $CaCl_2$, 0.3 g of $MgSO_4$, 5 g of peptone, 3 g of yeast extract, 3 g of malt extract and 10 g of oat spelt xylan, after 72-h cultivation by centrifugation at 8,000 rpm at 4°C for 5 min. The mycelium was powdered in mortar and pestle with liquid nitrogen. Mycelium powder was transferred to a micro centrifuge tube and then 500-600 µl of DNA extraction buffer added. Equal volume of phenol/chloroform was added and mixed well. Aqueous phase was separated by centrifugation at 13,000 rpm for 5 min at room

temperature. The aqueous phase (upper phase) was transferred to fresh micro centrifuge tube and an equal volume of chloroform/isoamylalcohol was added and mixed well. Aqueous phase was separated by centrifugation at 13,000 rpm for 5 min at room temperature. The aqueous phase (upper phase) was transferred to fresh micro centrifuge tube and an equal volume of isopropanol was added and mixed by inversion to precipitate DNA. DNA pellet was collected by centrifuge at 13,000 rpm for 5 min at 4^{o}C. The supernatant was poured off and 500 µl of 70% ethanol added then mixed by inversion. DNA pellet was collected by centrifuge at 13,000 rpm for 2 min at 4^{o}C. DNA was collected by centrifugation. The samples were dried under a vacuum for 15 min at room temperature and then re-suspended in 50 µl of TE buffer. The DNA solution was kept at 4^{o}C (Sambrook *et al.*, 1989).

4.5 PCR amplification of internal transcribed spacer region (ITS) rDNA

DNA dissolved in TE buffer was used as the template in amplification. Fungal ITS rDNA were PCR amplified from the genomic DNA with the universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTA TTGATATGC-3') as forward and reverse sequencing primers, respectively. PCRs were conducted in 100 µl reaction volume. Each reaction tube contained approximately 50-100 ng of DNA template, 10 µl of 10x PCR buffer, 10 µl of 2 mM dNTPs, 2 µl of 25 pM/µl each primers, and 0.5 µl of 250 unit Hot Star Taq DNA polymerase (QIAGEN GmbH, Germany). PCR was performed using an automated thermocycler (Takara PCR Thermal Cycler Dice). PCR was performed with initial denaturation for 15 min at 95^{o}C followed by 25 cycles of denaturation for 30s at 94^{o}C, annealing for 30s at 50^{o}C, elongation for 1 min at 72^{o}C. A final elongation step of 10 min at 72^{o}C was included. PCR products were checked on 1% agarose gels (Sambrook *et al.*, 1989). Then, PCR products were purified using QiAquick® gel DNA extraction kit (Qiagen, Japan). The quantity of purified PCR products was determined by electrophoresis.

4.6 Enzyme assays

4.6.1 Xylanase activity assay (Singh *et al.*, 2000a, b, c, d)

The reaction mixture of 0.2 ml enzyme solution with an equal volume of 1% soluble oat spelt xylan in 50 mM phosphate buffer pH 6.0 was incubated at 50^{o}C for 10 min. The hydrolytic reaction was stopped by addition of 0.6 ml 3,5-dinitrosalicylic acid reagents (DNS) solution and was mixed well with vortex mixture. The reaction was incubated in boiling water for 5 min, then, rapidly cooled down in ice bath. After that, 6 ml of distilled water was added to the reaction mixture and mixed well with vortex mixture. The adsorption of reaction mixture was measured at 550 nm and calculated with standard curve of reducing sugar (xylose) as shown in Appendix Figure B2A. One unit of endo-β-1,4-xylanase was defined as the amount of enzyme releasing 1 μmole equivalents of D-xylose per min.

4.6.2 Mannanase activity assay (Großwindhager *et al.*, 1999)

The reaction mixture of 0.2 ml enzyme solution with an equal volume of 1% soluble locus bean gum in 50 mM phosphate buffer pH 6.0 was incubated at 50^{o}C for 30 min. The hydrolytic reaction was stopped by addition of 0.6 ml DNS solution and was mixed well with vortex mixture. The reaction was incubated in boiling water for 5 min, then, rapidly cooled down in ice bath. After that, 6 ml of distilled water was added to the reaction mixture and mixed well with vortex mixture. The adsorption of reaction mixture was measured at 540 nm and calculated with standard curve of reducing sugar (mannose) as shown in Appendix Figure B2B. One unit of endo-β-1,4-mannanase activity was defined as the amount of enzyme releasing 1 μmol equivalents of D-mannose per min.

4.6.3 Pectinase activity assay (Debing *et al.*, 2006)

The reaction mixture of 0.2 ml enzyme solution with an equal volume of 1% citrus pectin in 50 mM phosphate buffer pH 6.0 was incubated at 50^{o}C

for 30 min. The hydrolytic reaction was stopped by addition of 0.6 ml DNS solution and was mixed well with vortex mixture. The reaction was incubated in boiling water for 5 min, then, rapidly cooled down in ice bath. After that, 6 ml of distilled water was added to the reaction mixture and mixed well with vortex mixture. The adsorption of reaction mixture was measured at 540 nm and calculated with standard curve of galacturonic acid as shown in Appendix Figure B2C. One unit of pectinase activity was defined as the amount of enzyme releasing 1 μg of reducing sugars calculated as galacturonic acid per min.

4.7 SDS-PAGE (Laemmli, 1970)

SDS slab gel electrophoresis was carried out with 10% of running gel and 5% of stacking gel. Running buffer for SDS-PAGE contained 15 ml of 2x SDS-running buffer, 5 ml of water and 10 ml of 30% acrylamide-bis, then, this mixture was evacuated to degas. At the time ready to make running gel, 120 μl of 0.1875 g/ml ammonium persulfate and 45 μl of TEMED were added. The mixture was gently mixed and poured into the chamber between the 2 glass plates. A small amount of water was applied onto the surface of the running gel solution and the solution was left standing until polymerized. Stacking gel of the SDS-PAGE contained 7.5 ml of 2x SDS-stacking buffer, 5 ml of water and 2.5 ml of 30% acrylamide-bis and the mixture was evacuated to degas. At the time ready to make stacking gel, 60 μl of 0.1875 g/ml ammonium persulfate and 22.5 μl TEMED were added. The mixture was gently mixed and poured onto the surface of the polymerized running gel. The wells in which the samples were loaded, were made in the stacking gel by a template comb. The stacking gel was left standing to be polymerized.

Twenty micro litre of cultural supernatant supplemented with 20% SDS-SAB and boiled for 2 min were applied on the gel. Electric currents that used to run the samples in the stacking gel and the running gel were 20 and 40 mA, respectively. Electrophoresed gel was stained with Coomassie Brilliant Blue R-250 for 2 h, then, twice destain were done with the Destain I solution for 1 h and several time repeat

destained or second destained overnight until the protein band could be distinguished from the clear background. The gel was transferred to the Destain II for 1 h.

4.8 Zymogram analysis (Ratanakhanokchai *et al.*, 1999)

The cultural supernatant in the sample application buffer was boiled for 2 min and subjected to electrophoresis on an SDS-10% polyacrylamide gel as described above. After electrophoresis, the gel was soaked in 25% (vol/vol) isopropanol with gentle shaking to remove the SDS and renature the proteins in the gel. The gel was then washed four times for 30 min at 4°C in 0.5 M phosphate buffer (pH 6.0). After that, the gels were incubated with 1% soluble oat spelt xylan in 50 mM phosphate buffer pH 6 at 50^{o}C for 30 min. After further incubation, the gel was soaked in 0.1% Congo red solution for 30 min at room temperature and washed with 1 M NaCl until excess dye was removed from the active band. After the gel was submerged in 0.5% acetic acid, the background turned dark blue and the activity bands were observed as clear colorless areas.

4.9 Protein determination

Protein content was quantitatively determined using Lowry's method (Lowry *et al*., 1951) and Bovine serum albumin was used as standard. Eluted protein content in the column was measured by the absorbency at 280 nm.

4.9.1 Protein assay with absorbency at 280 nm: protein content in fraction obtained from each of purification step was directly determined using spectrophotometer and quart cuvette with absorbency at 280 nm.

4.9.2 Protein assay with Lowry method: 0.5 ml of sample or standard bovine serum albumin of various concentrations was added into a test tube. Then 2.5 ml of solution E was added and mixed well. The reaction mixture was incubated at room temperature for 10 min. Then 0.25 ml of solution F was added and mixed very rabidly. The reaction mixture was further incubated at room temperature for 30 min to

let the colour developed in stable. The protein was measured at 750 nm. Standard concentrations of bovine serum albumin (0.05-0.3 mg/ml) were used as standard. Standard curve was shown in Appendix Figure B3.

4.10 Preparation of insoluble substrates

4.10.1 Insoluble oat spelt xylan

Oat spelt xylan was purchased from Sigma Co, USA. Insoluble oat spelt xylan was prepared by suspended 10 g of xylan in 1 liter of water. The suspension was boiled for 10 min and then was centrifuged at 10,000 rpm for 5 min. The xylan pellet was collected and washed four times with distilled water. The pellet was dried using lyophilize method.

4.10.2 Alkali treated corncob

Dry corncob was grinded until 5-mesh particle using grinder and then soaked in 1% NaOH solution for 24 h. The corncob particles were washed using distilled water until pH of particle becomes neutral and then dry up at 50^{o}C.

4.10.3 Cassava starch

Cassava starch powder was soaked in distilled water for 24 h and then filtrated using filter paper No 4. The particles were dry up at 50^{o}C.

4.10.4 Carboxymethyl cellulose

It was used directly as in soluble substrate with out any treatment.

4.11 RNA extraction

Mycelium samples were placed to the liquid nitrogen and grind with mortar and pestle. Powders were transferred to the micro-centrifuge tube and weighed, 50-100 mg. Next step followed protocol in Rneasy® Plant Mini protocol (Qiagen, Japan) for isolation of total RNA from plant cell and tissues and fungi.

To check total RNA, four microliters of total RNA sample and 1 μl of loading buffer consisting 2 μl saturated aqueous bromophenol blue solution, 10 μl of 0.5 M EDTA pH 8.0, 90 μl of 37% formaldehyde 250 μl of glycerol, 385.5 μl of formamide and 500 μl of 10X formaldehyde agarose (FA) gel buffer were mixed and incubated for 5 min at 65^{o}C and transferred to ice. Five micoliters of sample reaction were loaded to 1.2% FA gel. Gel running condition was run at 50 Voltage in 1X FA gel running buffer.

4.12 Complementary DNAs (cDNA) synthesis

cDNA was synthesized using Qiagen® One Step RT-PCR Kit. Reactions were mixed which one reaction containing 1 pg-2 μg of total RNA that calculated at absorbent 260 nm, 10 μl of RT-PCR buffer, 2 μl of dNTP mix, 10 μl of 5X Q-solution, 30 pmol of each primer; primer xyn-F (5'-AACATGGATATATAAAGGGC-3') and primer xyn-R (5'-ATTGAATACCTCCAACCGGC-3'), and 2 μl of RT-PCR enzyme mix. These reactions were subjected to one step RT-PCR which contain reverse transcription for 30 min at 50^{o}C having initial PCR activation step was 15 min at 95^{o}C. In step of denature, annealing and elongation for 45 s at 94^{o}C, 30 s at 50^{o}C and 1 min at 72^{o}C, respectively, were used for 30 cycles. The final extension was 10 min at 72^{o}C to complete elongation. The amplified fragment was detected on 1% agarose gel electrophoresis.

4.13 Preparation of washed mycelium for induction experiment

To obtain mycelium using in induction experiment, the active mycelium of selected strains were inoculated to synthetic medium. The synthetic medium (100 ml) consisted (per liter of water) 2 g of KH_2PO_4, 0.3 g of $CaCl_2$, 0.3 g of $MgSO_4$, 5 g of peptone, 3 g of yeast extract, 3 g of malt extract and 1 g of glutamic acid in 500 ml Erlenmeyer flasks. After sterilization and inoculation, the medium were incubated as previously described. Mycelium were harvested after 72 h at the lately exponential phase, then separated by filtration with suction pump and washed three times with sterile 50 mM sodium phosphate buffer (pH 6.0). The washed mycelium were dried at 105^{o}C to constant weight in incubator.

RESULTS AND DISCUSSION

1. Isolation and enumeration of thermophilic fungi and *T. lanuginosus*

1.1 Isolation of thermophilic fungi and *T. lanuginosus*

Thermophilic fungi were isolated and enumerated from samples; soils, compost, dung and bark, as shown in Table 4 and Appendix Table A1. Compost had the highest number of thermophilic fungi and *T. lanuginosus*. High numbers of thermophilic fungi were isolated from dung and near seashore but *T. lanuginosus* was not found. Thermophilic fungi and *T. lanuginosus* could not be isolated from cave, road side, ancient remains and hot spring. The samples had pH in range between 5.0-7.0. High organic matter was found from compost. However, it seems to be that it has no relation between content of organic matter, and pH to the number of thermophilic fungi and *T. lanuginosus*. A total of 90 isolates of expected *T. lanuginosus* was picked from samples as shown in Table 4.

1.2 Identification

1.2.1 Morphological identification of thermophilic fungus *T. lanuginosus.*

All expected *T. lanuginosus* excepted THKU-36, THKU-37 and THKU-38 grew at 50^{o}C on YG medium and produced black, gray or cream color (Fig. 9A). The hyphae have a colorless and septate arising at right angles to the filaments. Aleuriospores having size 6-10 μm in dimeter, colorless and smooth wall when young and becoming dark brown and sculptured with age were produced on each aleuriophore in which were globose and irregularly sculptured (Fig. 9B). These characteristics had highly importance to identify the thermophilic fungi as *T. lanuginosus* (Cooney and Emerson, 1964; Domsch *et al.*, 1993; Salar *et al.*, 2007).

Table 4 Comparison of number of thermophilic fungi and *T. lanuginosus* from samples collected at differently geographical location in Thailand.

Sources	No. of samples	pH	Organic matter (%)	Number (CFU/g)		Number of selected *T. lanuginosus* (isolates)
				Thermophilic fungi	*T. lanuginosus*	
Soils						
Orchard and Plantation	26	6.0	6.5-12.4	0-220,000	0-21,500	20
Cave	4	7.0	4.1-5.7	0	0	0
Near seashore	20	6.0	0.7-13.7	0-6,150	0	0
Road Side	38	5.0 - 6.0	3.4-13.7	0	0	0
Ancient Remains	7	5.0 - 6.0	3.2-14.1	0	0	0
National Park	10	6.0 - 7.0	6.3-19.2	1,440-11,340	300-2,160	10
Botanical Garden	8	6.0 - 7.0	5.9-34.3	0-7,200	0-2,340	7
Hot Sping	7	6.0	6.9-7.5	0	0	0
Domestic area	10	6.0	6.7-14.1	0-12,480	0-2,160	3
Embankment	3	6.0	5.1-15.5	300-840	0	0
Compost	7	6.0 - 7.0	23.1-41.2	9,300-131,300	660-11,110	60
Dung	3	6.0	12.4-13.7	13,237-32,320	0	0
Bark	3	6.0	2.6-13.5	550-1,430	90-210	1

A

B

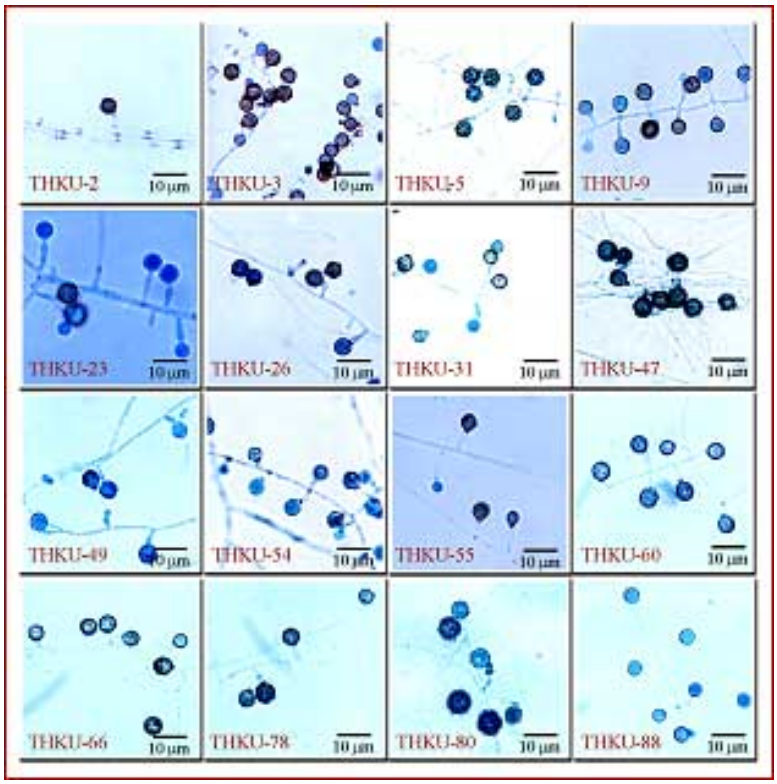

Figure 9 Colony (A) and aleuriospores (B) of some strains of isolated *T. lanuginosus.*

1.2.2 Identification using ITS sequence

To confirm morphological identification, genomic DNAs of the selected strains were extracted and showed only one band about 23,000 bp (Fig. 10A). Then, their genomic DNAs were used as template for ITS amplification.

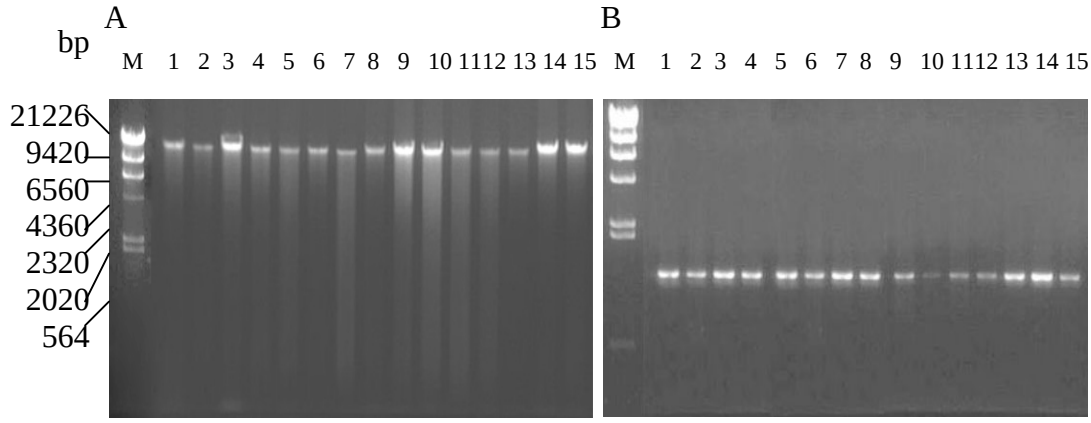

Figure 10 Ethidium bromide-stained agarose gel containing genomic DNA (A) and PCR amplified ITS rDNA (B) of thermophilic fungi.
lane M, lambda phage DNA digested with *Hind*III. Lanes 1-15, PCR product from THKU-2, THKU-3, THKU-9, THKU-11, THKU-16, THKU-17, THKU-25, THKU-30, THKU-33, THKU-43, THKU-49, THKU-55, THKU-79, THKU-80, THKU-81, respectively. Each sample was separated in 1% agarose.

Amplifications of internal transcribed spacer region (ITS) of the ribosomal gene of those isolates were performed using universal primers ITS1 and ITS4. The obtained PCR products were showed in Fig. 10B. The single band of 650 bp corresponding to the size of ITS region of nuclear ribosomal DNA was clearly seen. The ITS rDNA region of those strains were sequenced to identify to species level. The nucleotide sequences of ITS rDNA were obtained with the sequencing reaction using universal primers ITS5 and ITS4. The sequences were aligned with reported nucleotide sequence in DDBJ database using BLAST version 2.2.15 from the DDBJ website. The result showed ITS rDNA sequences of 11 strains; THKU-2,

THKU-3, THKU-9, THKU-11, THKU-30, THKU-43, THKU-49, THKU-55, THKU-79, THKU-80 and THKU-81, share 100% identities with ITS rDNA sequences of *T. lanuginosus* while other 4 strains; THKU-16, THKU-17, THKU-25 and THKU-33 showed identity to the fungus of 99.3, 99.6, 99.8 and 99.5%, respectively. This indicated that all selected strains were *T. lanuginosus*. These results corresponded with the previous identification with its morphological characteristics. The results of phylogenetic tree also classified our strains were *T. lanuginosus* with supported by 99% bootstrap (Fig. 11).

1.3 Production of xylanases, pectinase and mananase by *T. lanuginosus* strains

To determine ability to produce degrading enzymes; xylanases, pectinase and mananase, 85 strains of *T. lanuginosus* were cultivated in 15 ml of the liquid medium in 50 ml flask using 1% oat spelt xylanase as a carbon source. They produced different levels of xylanase activity as shown in Table 5. The result showed that 16 strains produced β-xylanase activity in range of 100-134 U/ml. Among these strains, THKU-86 produced the highest xylanase (Appendix Table A2).

Moreover, ability to produce mannan-degrading enzyme of those strains were also investigated in the liquid medium containing 1% locus bean gum as the carbon source. All isolates could grow well in the media but only THKU-23 was found to produce mannan depolymerizing enzyme, 0.7 U/ml, and showed ability to decrease the viscosity of the mannan in cultivated medium. Besides, ability to produce pectin-degrading enzymes was also determined. Though the fungi could grow in the pectin medium, but no pectin-degrading enzyme was detected.

Production of β-xylanase, mannanase and pectinase from strains of *Thermomyces lanuginosus* were previously reported. Puchart *et al.* (1999) reported that 17 strains of *T. lanuginosus* that were ATCC 46882, CBS 288.34, ATCC 44008, IMI 11003, CBS 224.63, IMI 140524, IMI 158749, CBS 395.62, CBS 218.34,

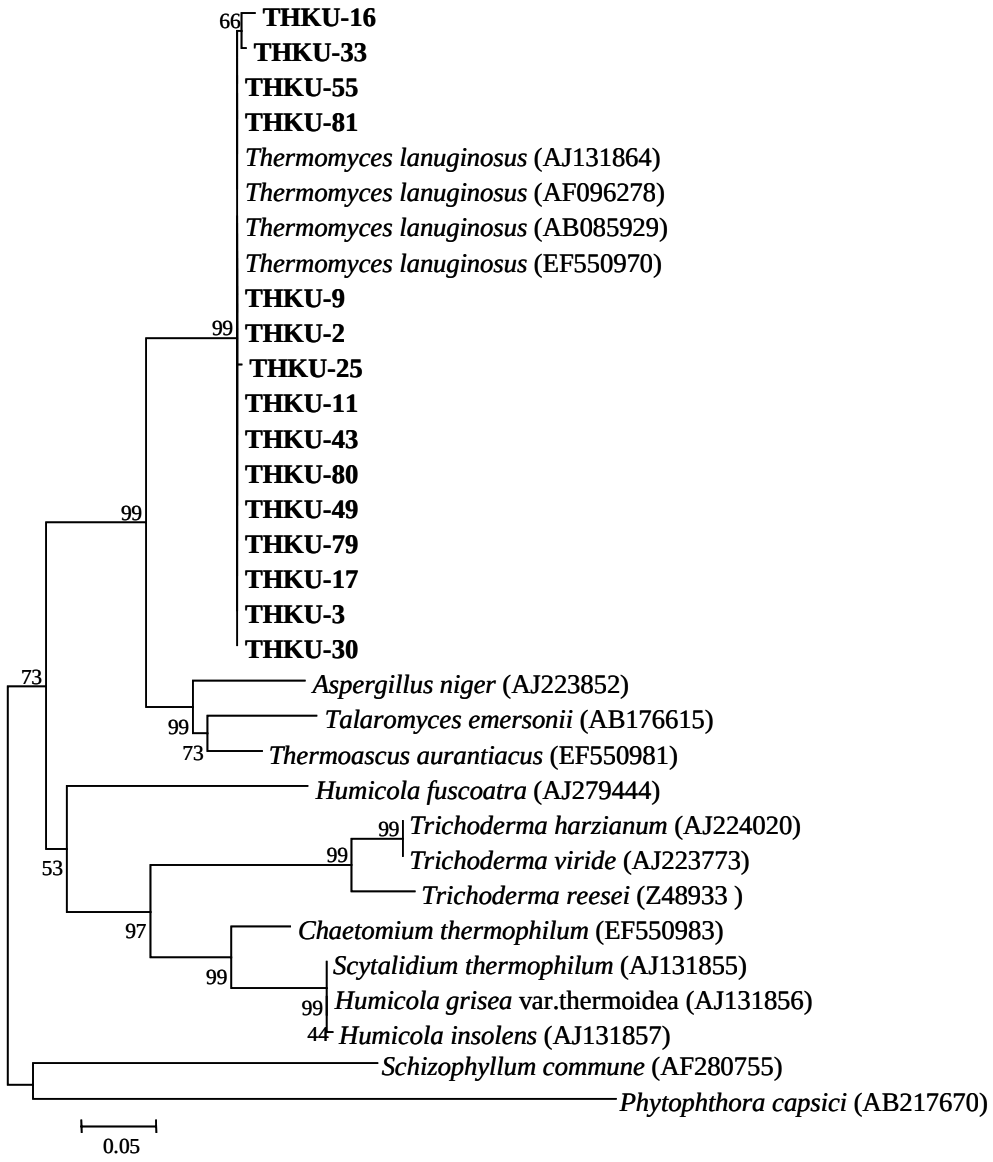

Figure 11 Phylogenetic tree of nucleotide sequence analysis of rDNA-ITS of thermophile, mesophile and thermophilic fungi contructed by Neighbor-joining method from MEGA4 program.

The tree is rooted by the nuecleotide sequence of *Phytophthora capsici* strain. Scale bar shown distance values under the tree means 0.05 substitutions per nucleotide position. Bootstrap analyses were performed with 1000 re-samplings and percent values are shown at the branching points. All isolated strains in this study are bold letter.

ATCC 22070, IMI 96213, IMI 131010, ATCC 34626, ATCC 36350, ATCC 28083, ATCC 16455 and DSM 5826 were able to produce β-xylanases. Only three strains of IMI 158749, CBS 218.34 and IMI 13101 produced mananase and three strains of IMI 158749, ATCC 34626 and ATCC 36350 produced pectinase. The strains producing high β-xylanases secreted low amounts of xylan-debranching enzymes and did not produce mannan and arabinan-degrading enzyme systems. Only the strains showing low xylanase production exhibited a higher ability to produce a mannanolytic enzyme. In contrast, production of mannanase was not detected in *T. lanuginosus* strain DSM 5826 after growth on corncobs whereas the enzyme was produced at low level in *T. lanuginosus* SSBP (62 U/ml). However, growing the fungus on galactomannan (locust bean gum) did not result in the induction of mannan-degrading enzymes than other xylan-containing substrates (Singh *et al.*, 2000c).

1.4 SDS-PAGE profiles and xylanase activities stained from crude enzyme produced by *T. lanuginosus*

Crude enzymes produced by all strains of *T. lanuginosus* growing on xylan medium were evaluated by SDS-PAGE. A similar main protein band corresponding to a molecular weight of 24.9 kDa were observed in most strains excepted crude enzymes of strains of THKU-36, THKU-37 and THKU-38 (Appendix Figure A3)**.** Crude enzymes were used to confirm that main protein was xylanase. Activity staining on active-PAGE gels of selected crude enzymes also revealed the presence of β-xylanase with molecular weight corresponding to main protein on SDS-PAGE as shown in Appendix Figure A4. Main xylanase activity was coincided with the main protein band in the crude enzyme. The molecular weight was within the range of values in which were reported for crude and purified xylanases from *T. lanuginosus* strains (Kitpreechavanich *et al*., 1984b; Bennett *et al*., 1998; Singh *et al.*, 2000b,c).

1.5 Thermostability of xylanase produced by *T. lanuginosus*

Crude xylanase produced by strains of *T. lanuginosus* in this study were investigated for their thermal stability. Half-lives of β-xylanases produced by each isolates were determined after keeping the enzyme at 70°C. It was found that half-live of crude enzyme were variable and forty strains had half-life lower than 40 minute as shown in Table 5. Only four *T. lanuginosus* strains, THKU-49, THKU-46, THKU-48, and THKU-55 produced high thermostable β-xylanase having half-life of 266, 254, 231 and 207 minutes, respectively. These enzymes were thermostable but low level of xylanase activities having 57, 57, 35 and 47 units/ml were detected, respectively. A strain of *T. lanuginosus* THKU-49 produced β-xylanase for 57 unit/ml but it showed the highest stability with half-life of 266 minutes. In contrast, β-xylanase from *T. lanuginosus* THKU-86 produced the highest xylanase (134 unit/ml) but its half-life of enzyme at 70°C was only 15 minutes. In contrast to those, *T. lanuginosus* THKU-9 produced the low xylanase (51 unit/ml) and its half-life of enzyme at 70°C was also low only 20 minutes. Thus, the enzymes of strains THKU-9 and THKU49 were used for purification and characterization in next experiment.

Table 5 Number of *T. lanuginosus* produced xylanases with different thermostability.

Range of half-life at 70°C (min)	Number of isolates
3-40	40
41-80	22
81-120	6
121-160	10
161-200	6
201-266	4

2. Purification and characterization

2.1 Production of xylanase

The xylanase productions of *T. lanuginosus* THKU-9 and THKU-49 started at early exponential phase in 2 days. They were rapidly increased during 4-day cultivation and 5-day cultivation for THKU-9 and THKU-49, yielded 51 and 57 U/ml, respectively. They increased to some extents of 57, 63 U/ml after 7-day cultivation as shown in Figs. 12A, B. The pHs of cultural supernatants were increased up to 8.1 at 4-day cultivation after that they were slowly decreased. Soluble proteins in the cultivations increased slightly.

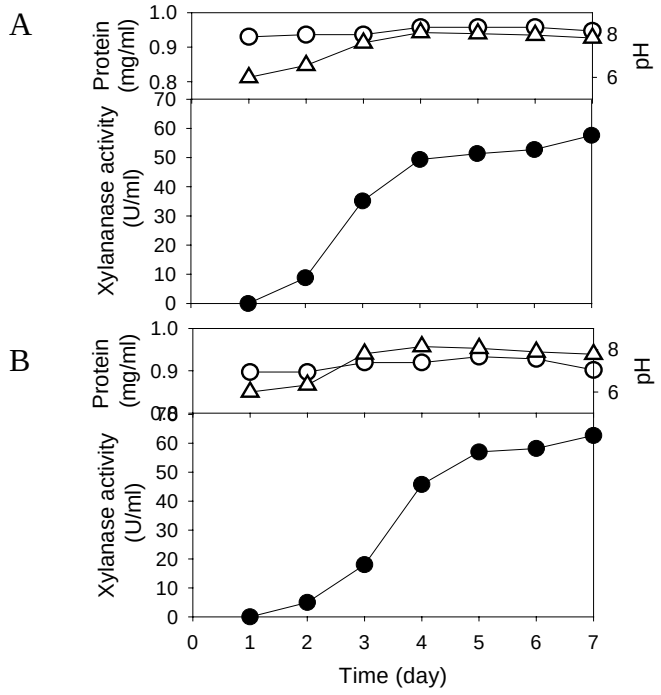

Figure 12 Time course of xylanase production by *T. lanuginosus* THKU-9 (A) and THKU-49 (B) cultivated in medium containing oat spelt xylan as a carbon source at 45°C.
xylanase activity (-●-), soluble protein (-○-), pH (-△-)

2.2 Purification of low and high thermostable xylanase produced by strains of *T. lanuginosus*

Crude enzyme solutions with the specific activities of 53 and 68 U/mg protein from cell-free suspension of *T. lanuginosus* THKU-9 and THKU-49, respectively, in which were cultivated in the liquid medium having oat spelt xylan as a carbon source were concentrated by salting out using 80% ammonium sulfate precipitation. The concentrated enzymes were further purified by DEAE sepharose, hydroxyapatile and Sephadex G-100. The result showed that xylanases of THKU-9 and THKU-49 could be purified to 5.7 and 7.7 folds, respectively, and their specific activities were 304 and 522 U/mg protein. The xylanase yields of THKU-9 and THKU-49 were 32% and 19%, respectively, were recovered (Table 6). The samples were proved to be a single protein band on SDS-PAGE (Fig. 13).

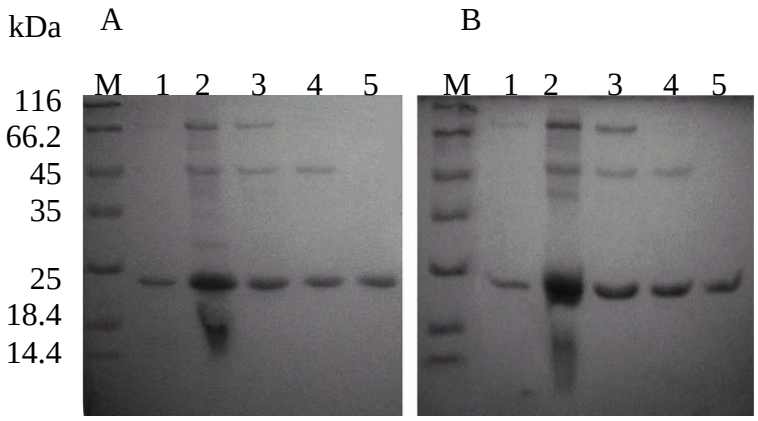

Figure 13 Protein patterns of *T. lanuginosus* THKU-9 (A), THKU-49 (B) from purification steps on 10% SDS-PAGE.
Lane M molecular weight standard, lane 1 crude enzyme, lane 2 precipitated with 80% ammonium sulfate and dialysis, lane 3 DEAE-Sepharose fast flow, lane 4 Hydroxyapatite, lane 5 Sephadex G-100.

Table 6 Summary of the purification of xylanases from *T. lanuginosus* THKU-9 and THKU-49.

Strains	Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (folds)
THKU-9							
	Crude	1300	1183	62842	53	100	1.0
	Precipitate	44	512	49284	96	78	1.8
	DEAE sepharose	196	153	24326	159	39	3.0
	Hydroxylapatite	200	112	23298	208	37	3.9
	Sephadex G-100	177	65	19911	304	32	5.7
THKU-49							
	Crude	1400	1180	80220	68	100	1.0
	Precipitate	20	211	70974	336	88	4.9
	DEAE sepharose	85	78	37400	478	47	7.0
	Hydroxylapatite	106	49	25090	517	31	7.6
	Sephadex G-100	123	29	14895	522	19	7.7

2.3 Characteristics of the pure xylanase from *T. lanuginosus*

2.3.1 Molecular weight of the purified enzyme

Molecular weight (MW) of the purified xylanase from *T. lanuginosus* strains of THKU-9, and THKU-49 were determined on SDS-PAGE (Fig. 13). The MW of the pure xylanases was 24.9 kDa with only 1 subunit. These pure enzymes had same MW as 24.7 kDa for *T. lanuginosus* SSBP (Singh *et al.*, 2000), 25.5 kDa for *T. lanuginosus* DSM 10635 (Xiong *et al.*, 2004), 25.7 kDa for *T. lanuginosus* ATCC 46882 (Bennett *et al.*, 1998), 21.5 kDa for *Humicola lanuginosa* (Kitpreechavanich *et al.*, 1984b) and 25.5 kDa for *T. lanuginosus* DSM 5826 (Cesar and Mrsa, 1996).

2.3.2 Substrate specificities and K_m of the pure xylanases

The reactions of the pure xylanases on different substrates were determined at 50°C. All reactions were performed in 50 mM phosphate buffer (pH 6.0) containing 0.5% substrates at final concentration. As shown in Table 7, pure xylanases were able to hydrolyze various kinds of substrates. The highest xylanase activities that are 1.20 ± 0.010 U/ml (112.1% relative activity) and 1.06 ± 0.009 U/ml (112.9% relative activity) with the soluble oat spelt xylan were observed from THKU-9 and THKU-49, respectively. However, low activities of these enzymes toward insoluble oat spelt xylan (27.8%-35.5%), rice straw hemicellulose (74.6%-77.3%) and alkali treated corncob (3.4-3.9%) as shown in Table 7. This indicated that pure enzymes have less specificity with insoluble substrate. Moreover, pure xylanases did not act towards carboxymethylcellulose, avicel, filter paper and locust bean gum because of no specificity, which a typically property of family 11 xylanases (Gruber *et al.*, 1998). Moreover, these pure xylanases also did not act towards cassava starch, and *p*-Nitrophenyl β-D-xylopyranoside because of differently linking types (α-linkage) and differently substrate-binding site, respectively.

Table 7 Substrate specificities of the pure enzymes of *T. lanuginosus* THKU-9 and THKU-49.

Sources of enzyme	Substrates	Final Conc. (%)	Xylanase activity [a] (U/ml)	Relative activity (%)
THKU-9				
	Soluble oat spelt xylan	0.4	1.20 ± 0.010	112.2
	Oat spelt xylan	0.5	1.07 ± 0.007	100.0
	Insoluble oat spelt xylan	0.5	0.38 ± 0.006	35.5
	Rice straw hemicellulose	0.5	0.80 ± 0.020	74.6
	Alkali treated corncob	0.5	0.04 ± 0.006	3.4
	Carboxymethylcellulose	0.5	0.00	0.0
	Cassava starch	0.5	0.00	0.0
	Avicel	0.5	0.00	0.0
	Filter paper	0.5	0.00	0.0
	Locust bean gum	0.5	0.00	0.0
	p-Nitrophenyl β-D-xylopyranoside	0.5	0.00	0.0
THKU-49				
	Soluble oat spelt xylan	0.4	1.06 ± 0.009	112.9
	Oat spelt xylan	0.5	0.94 ± 0.030	100.0
	Insoluble oat spelt xylan	0.5	0.26 ± 0.030	27.8
	Rice straw hemicellulose	0.5	0.72 ± 0.004	77.3
	Alkali treated corncob	0.5	0.04 ± 0.007	3.9
	Carboxymethylcellulose	0.5	0.00	0.0
	Cassava starch	0.5	0.00	0.0
	Avicel	0.5	0.00	0.0
	Filter paper	0.5	0.00	0.0
	Locust bean gum	0.5	0.00	0.0
	p-Nitrophenyl β-D-xylopyranoside	0.5	0.00	0.0

[a] Data are presented as mean of four replication with standard deviations.

Many xylanases from other microorganisms had both xylanase and cellulase activities. However, xylanase obtained from our strains were similar to xylanases from other *T. lanuginosus* strains (Singh *et al.*, 2003). It hydrolyzed only xylan and it was free from other enzymatic activities examined including carboxymethylcellulase, amylase, cellulase, cellobiosidase, mannosidase and

β-xylosidase. This substrate specificity was also a desirable property for pulps biobleaching.

Studies on K_m were important to understand the enzyme. K_m was a constant value showing the enzyme-substrate specificity. Moreover, it was a parameter that used to distinguish the difference between enzymes. The K_m value of pure xylanase of *T. lanuginosus* THKU-49 towards oat spelt xylan, soluble oat spelt xylan and insoluble oat spelt xylan were 8.4 ± 1.300, 7.3 ± 0.236 and 60.2 ± 6.788 mg/ml, respectively. The K_m of pure xylanase of strain THKU-49 towards soluble oat spelt xylan and oat spelt xylan lower than K_m values of THKU-9 was shown in Table 8. This indicated that pure enzyme of THKU-49 had higher specificity to soluble oat spelt xylan and oat spelt xylan than enzymes of THKU-9. Besides, K_m (60.2 ± 6.788) of pure xylanase of THKU-49 towards insoluble oat spelt xylan had similar levels of K_m from THKU-9 that are 52.5 ± 10.606 mg/ml. This might imply that xylanases of these strains did not have xylan-binding domain that was required for digestion of insoluble substrates. However, these xylanase could digest insoluble oat spelt xylan might cause of normal absorption between enzyme and substrate.

Table 8 K_m values of the pure xylanases of *T. lanuginosus* THKU-9 and THKU-49.

Substrates	K_m value (mg/ml)[a]	
	THKU-9	THKU-49
Soluble oat spelt xylan	10.3 ± 0.861	7.3 ± 0.236
Oat spelt xylan	12.4 ± 0.902	8.4 ± 1.300
Insoluble oat spelt xylan	52.5 ± 10.606	60.2 ± 6.788

[a] Data are presented as mean of four replication with standard deviations.

Different K_m values of 3.85, 3.26, 4.0±0.14, 7.30 mg/ml to birchwood xylan and K_m value of 4.7±0.4 mg/ml to birchwood xylan and K_m value of 0.9 mg/ml to larchwood xylan were found in xylanase produced by *T. lanuginosus* strains DSM 10635, SSBP, CBS 288.54, (Griffon and Maublanc) Bunce, CBS 288.54, (Griffon and Maublanc) Bunce, respectively (Xiong *et al.,* 2004; Lin *et al.,* 1999; Li *et al.,* 2005;

Kitpreechavanish *et al.*, 1984b; Anand *et al.*, 1990). Li *et al.* (2005) also used soluble and insoluble oat spelt xylan for determination of K_m of xylanase of *T. lanuginosus* CBS 288.54 having its values as 2.0±0.1 and 23.4±3.1 mg/ml, respectively.

2.3.3 Effect of metal ions and chemicals on the activity of the pure xylanase

Activities of the pure xylanase of *T. lanuginosus* THKU-9 and THKU-49 were measured in the presence of various metal ions and chemicals (Table 9). The considerably decreasing β-xylanase activity of THKU-49 was observed in the presence of Mn^{2+}, Sn^{2+} and EDTA at the concentration of 1 mM. The relative activities with Mn^{2+}, Sn^{2+} and EDTA were 85.5%, 83.2% and 74.6%, respectively. All added metal ions and chemicals had no effect on its activity of THKU-9. These effects might preclude the use of this xylanase in industrial processes where these chemicals were presented in relevant concentration. β-mercaptoethanol could not reduce activities because these xylanases had no S-S linkage of cystein residues at active site.

Most inorganic salts except Pb^{2+} and Hg^{2+}, EDTA, and β-mercaptoethanol showed no significant effect on *T. lanuginosus* SSBP xylanase activity (Lin *et al*., 1999). However, xylanase produced by *T. lanuginosus* DSM 5826 was stimulated activity by 1 mM of Mn^{2+}, $Fe^{2+}$and Co^{2+} and inhibition of the enzyme activity in the present of Hg^{2+} (Cesar *et al.*, 1996).

Table 9 Effect of metal ions and chemicals on the activity of the pure xylanase from *T. lanuginosus* THKU-9 and THKU-49.

Xylanase	Metal ions/Chemicals (1 mM)	Xylanase activity (U/ml)[a]	Relative activity (%)
THKU-9			
	None	1.18 ± 0.009	100.0
	K^+	1.19 ± 0.029	100.9
	Li^+	1.17 ± 0.006	99.0
	Na^+	1.19 ± 0.019	100.6
	Ca^{2+}	1.24 ± 0.010	104.5
	Co^{2+}	1.17 ± 0.031	98.7
	Mn^{2+}	1.11 ± 0.038	94.2
	Sn^{2+}	1.13 ± 0.031	95.9
	ME	1.20 ± 0.017	101.6
	EDTA	1.10 ± 0.018	93.0
THKU-49			
	None	1.08 ± 0.039	100.0
	K^+	0.99 ± 0.002	91.4
	Li^+	1.08 ± 0.059	99.7
	Na^+	1.06 ± 0.015	98.1
	Ca^{2+}	1.05 ± 0.053	97.0
	Co^{2+}	1.09 ± 0.050	100.1
	Mn^{2+}	0.93 ± 0.011	85.5
	Sn^{2+}	0.90 ± 0.015	83.2
	ME	1.03 ± 0.021	94.9
	EDTA	0.83 ± 0.106	76.4

[a] Data are presented as mean of four replication with standard deviations.

2.3.4 Effect of temperature and pH on the xylanase activity

Temperature and pH were factors affecting on xylanase activity. Treating them separately might not reflect their real influence on the xylanase activity such as optimum temperature changes along with pH. Therefore, central composite design (CCD) was used to generate 13-treatment combinations, pH and temperature, as independent variables using Minitab version 14 in order to obtain the best conditions for xylanase activity of *T. lanuginosus*. The result of experimental design

for enzyme activities was shown in Table 10. The significance of coefficients determined using *P*-values was shown in Table 11. High significance of temperature^2 and pH^2 indicate that temperature and pH could act as limiting factors and small variations in their values would alter xylanase activity to a considerable extent. The model clearly revealed significant interactions between temperature and pH. The equation models of xylanase activity of *T. lanuginosus* THKU-9 and THKU-49 were obtained:

Xylanase activity (U/mg protein) of THKU-9

= -2870.307+79.56*temp+238.948*pH+2.075*temp*pH-
0.701*temp*temp-30.046*pH*pH

Xylanase activity (U/mg protein) of THKU-49

= -5192.942+149.238*temp+312.187*pH +4.192*temp*pH-
1.332*temp*temp-46.99*pH*pH

These xylanases-equation models of *T. lanuginosus* THKU-9 and THLU-49 indicate that a remarkably high correlation between observation and prediction with R^2 value of 0.97 and 0.98, respectively. To confirm the applicability of the model, xylanase activities were determined at 66^{o}C (pH 6.3) which was values of the highest activities of the model suggested for activities of THKU-9 and THKU-49.

Table 10 Results of the experimental design for xylanases activity used in CCD, showing the 13-treatment combinations.

Treat	Code setting levels		Actual levels		Xylanase activity (U/mg protein)			
	Temp	pH	Temp	pH	Actual[a]		Predicted	
	(^{o}C)		(^{o}C)		THKU-9	THKU-49	THKU-9	THKU-49
1	-1.414	0	56	7.0	422	545	400	513
2	0	0	70	7.0	471	659	481	664
3	-1	1	60	8.0	351	451	364	468
4	0	0	70	7.0	482	663	481	664
5	0	0	70	7.0	483	660	481	664
6	1.414	0	84	7.0	278	276	287	292
7	-1	-1	60	6.0	459	623	479	657
8	0	0	70	7.0	485	668	481	664
9	1	1	80	8.0	332	401	325	394
10	0	0	70	7.0	484	663	481	664
11	0	-1.414	70	5.6	484	664	473	645
12	1	-1	80	6.0	358	413	356	394
13	0	1.414	70	8.4	373	495	371	499

Table 11 Coefficient of xylanases activity estimated by the regression model.

Independent variables	Coefficient (β)		Standard error (β)		Significant value (P-value)	
	THKU-9	THKU-49	THKU-9	THKU-49	THKU-9	THKU-49
Intercept	-2870.307	-5192.942	378.143	503.413	0.000	0.000
Temperature	79.56	149.238	6.526	8.820	0.000	0.000
pH	238.948	312.187	65.265	87.213	0.002	0.002
Temperature*pH	2.075	4.192	0.507	0.838	0.001	0.000
Temperature^2	-0.701	-1.332	0.039	0.056	0.000	0.000
pH^2	-30.046	-46.990	3.903	5.614	0.000	0.000

[a] Data are average values of four replicates analysis

In this case, the equation models predict enzyme activities of 503 and 669 U/mg protein in confidential level of 95% whereas experimental activity, 510 and 712 U/mg protein were obtained, respectively.

However, the highest xylanase activities gotten from the models and slightly lower than its activities did not have significantly different levels analyzing from response surface and contour plots. The response surface and contour shape of activities were shown in Fig 14. Thus, the optimum enzyme activities of *T. lanuginosus* THKU-9 and THKU-49 should be in the range of temperatures and pHs of 64-68^{o}C (pHs 6.0-6.5) and 62-70^{o}C (pHs 5.6-6.9), respectively. It could be observed that the activity of enzymes was notably dropped at temperature of 80^{o}C and pH 8.0. The optimum temperature and pH of these xylanases were closed to other xylanase produced by strains of CBS 288.54, DSM 10635, DSM 5826, ATCC 46882 and SSBP that were temperatures of 60-75^{o}C and pHs 6.0-6.5 (Li *et al*., 2005; Jiang *et al*., 2005; Xiong *et al*., 2004; Singh *et al*., 2000a; Bennett *et al*., 1998; Cesar *et al*., 1996; Alam *et al*., 1994).

2.3.5 Stability of the pure xylanases of *T. lanuginosus*

Stability was a very important aspect of industrial enzymatic bioreactor. Two principal factors, pH and temperature, affecting enzymatic stability were analyzed in order to determine the condition to maintain xylanase activity for reaction to prolonged time. Effects of type, concentration and pH of buffers on the stability of pure xylanases at 70^{o}C were also investigated. When buffer concentration increased, the half-lives of xylanases from *T. lanuginosus* THKU-9 and THKU-49 were significantly decreased (Table 12). Stability curve steeply decreased at high buffer concentrations. We hypothesized that an elevated buffer concentrations caused aggregation of enzymatic molecule, allowing for formation of dimers and trimers of the large molecule, which irreversibly inactivated the xylanase. Synergistic effect of high temperature and high concentration of buffer solutions might decrease solubility of the xylanase with the denaturation causing the decreasing in thermostability.

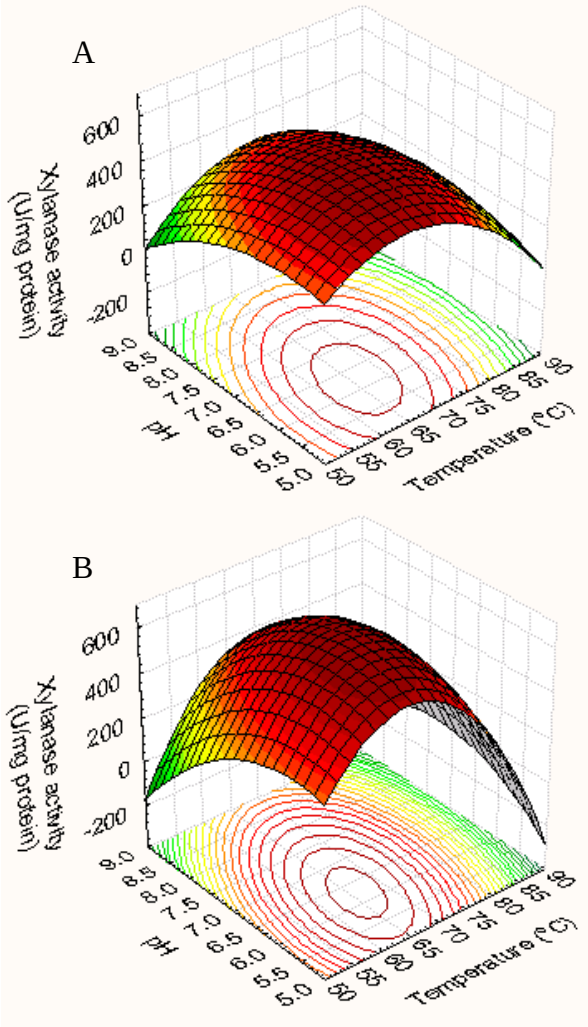

Figure 14 Response surface and contour plot for the effects of pHs and temperatures on β-xylanase activities of *T. lanuginosus* THKU-9 (A) and THKU-49 (B).

Table 12 Effect of buffer concentration and pH on thermostability of pure xylanase of *T. lanuginosus* THKU-9 and THKU-49.

Conc. Buffer (mM)	Half-lives (minute)					
	Citrate buffer (pH 6.0)		Phosphate buffer (pH 6.0)		Phosphate buffer (pH 7.0)	
	THKU-9	THKU-49	THKU-9	THKU-49	THKU-9	THKU-49
10	427	484	493	1251	1171	1012
50	83	84	178	336	523	674
100	30	45	144	96	292	455
200	18	33	79	86	173	254
400	10	10	48	69	65	158

Besides, half-lives of the pure xylanases of these strains in phosphate buffer was longer than in citrate buffer and the longest when the enzymes were kept in the buffer pH 7.0 (Table 12). The xylanases from these strains were very unstable at pH 5.0 and their activities were completely lost after incubation at 70^{o}C for 30 min. The thermal activity of pure xylanases of these strains was greatly dependent on pH, type and buffer concentrations. Because the p*I* of *T. lanuginosus* xylanase was very low (3.7), differences in the total ionization state of protein between pH 5.0-7.0 might cause the differences in stability of the enzymes.

The result of table 12 showed that pure xylanase of THKU-49 more stable than that of THKU-9 was presented in each buffer concentration except conditions of 10 mM phosphate buffer (pH 7.0), 100 mM phosphate buffer (pH 6.0) and 400 mM citrate buffer (pH 6.0). To obtain more details, the stability of each enzyme was determined at various temperatures in 50 mM phosphate buffer (pH 7.0). The pure enzymes of *T. lanuginosus* THKU-49 were fully stable to 1080 min up to 60^{o}C whereas the pure xylanase of *T. lanuginosus* THKU-9 was stable up to 50^{o}C and retained 87% at 60^{o}C (Figs. 15 A, B). At 70^{o}C, residual activities of xylanase of *T. lanuginosus* THKU-9 and THKU-49 were 30% and 41%, respectively, after keeping for 1080 min (Fig. 15C). The results confirmed that xylanases of *T. lanuginosus* THKU-49 were more stable than that of *T. lanuginosus* THKU-9. However, all enzymes were rapidly inactivated starting from 80-100^{o}C (Figs. 15D, E, F). The xylanases retained some of its activities even during incubation at 100^{o}C, as also observed in other *T. lanuginosus* xylanases (Singh *et al.*, 2000d; Xiong *et al.*, 2004; Singh *et al.*, 2003).

Central Composite Design (CCD) used to design experiment and Response Surface Methodology (RSM) were also used to determine the optimum condition in order to maintain xylanase activity of *T. lanuginosus* THKU-9 and THKU-49 for reaction to prolonged times. Based on the previous studies, temperature and pH were factors affecting on xylanases activities of *T. lanuginosus*. Treating them separately may not reflect their real influence on the enzymes stability. Therefore, CCD was used to generate 13-treatment combinations, with pH and temperature as

independent variables using Minitab version 14 in order to obtain the best conditions for the reaction of xylanases. The result of experimental design for enzyme stability as half-life was shown in Table 13. The significance of coefficients was determined using *P*-values as shown in Table 14. The equation models of their stabilities were obtained from 10 treatments without the treatments 1,3, and 7.

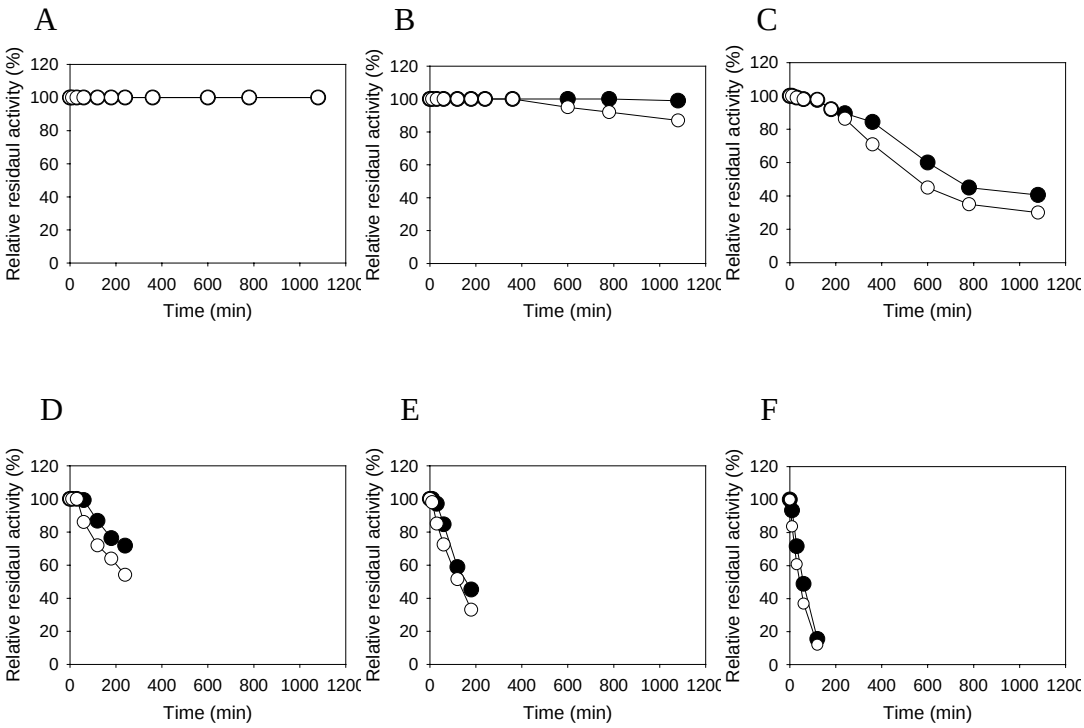

Figure 15 Relative activity of the pure xylanases produced by *T. lanuginsus* THKU-9 (-○-) and THKU-49 (-●-) in 50 mM sodium phosphate buffer (pH 7.0) at various temperatures.
50^{o}C (A), 60^{o}C (B), 70^{o}C (C), 80^{o}C (D), 90^{o}C (E), 100^{o}C (F)

Half-life of xylanase (min) of THKU-9

=-44090.4+909.273*temp+2979.357*pH-10.057*temp*pH-5.613*temp*temp-151.378*pH*pH

Half-life of xylanase (min) of THKU-49

= -50648.2+917.694*temp+5018.124*pH -16.711*temp*pH-5.49*temp*temp -261.906*pH*pH

Table 13 Results of the experimental design for stability used in CCD, showing the 13-treatment combinations.

Treat	Code setting levels		Actual levels		Half life (min)			
	Temp	pH	Temp	pH	Actual[a]		Predicted	
	(^{o}C)		(^{o}C)		THKU-9	THKU-49	THKU-9	THKU-49
1	-1.414	0	56	7.0	UD	UD	UD	UD
2	0	0	70	7.0	564	839	565	794
3	-1	1	60	8.0	UD	UD	UD	UD
4	0	0	70	7.0	569	768	565	794
5	0	0	70	7.0	554	783	565	794
6	1.414	0	84	7.0	205	171	208	168
7	-1	-1	60	6.0	UD	UD	ND	ND
8	0	0	70	7.0	569	783	565	794
9	1	1	80	8.0	436	322	438	319
10	0	0	70	7.0	560	809	565	794
11	0	-1.414	70	5.6	48	29	50	27
12	1	-1	80	6.0	325	292	327	290
13	0	1.414	70	8.4	485	537	487	535

[a] Data are average values of four replicates analysis

UD means undetectable

Table 14 Coefficient of stability estimated by the regression model.

Independent variables	Coefficient (β)		Standard error (β)		Significant value (P-value)	
	THKU-9	THKU-49	THKU-9	THKU-49	THKU-9	THKU-49
Intercept	-44090.4	-50648.20	1074.092	4748.911	0.000	0.000
Temperature	909.273	917.69	26.312	116.336	0.000	0.001
pH	2979.357	5018.12	55.814	246.772	0.000	0.000
Temperature*pH	-10.057	-16.71	0.554	2.451	0.000	0.002
Temperature^2	-5.613	-5.49	0.171	0.756	0.000	0.002
pH^2	-151.378	-261.91	2.723	12.040	0.000	0.000

This equation model in range of temperatures 70-84^{o}C and pHs 5.6-8.4 indicates that a remarkably high correlation between observation and prediction with R^2 values of 0.99. The thermostable equation of THKU-9 was also confirmed at 70^{o}C (pH 7.5) that provided the highest stability of enzyme from equation, and 70^{o}C (7.0, 6.0) from previous experiment. The half-lives obtained from equation at 70^{o}C (pH 7.5, pH 6.0, pH 7.0) were 605, 258 and 565 min in confident level of 95% while the half-lives of enzyme from experiment were 574, 178 and 523 min, respectively.

To confirm the model of THKU-49, the half-lives of xylanase were also determined. The condition obtained from equation that provided the highest stability of enzyme was 70^{o}C (pH 7.3) having half-life of 825 min while experimental value was 812 min. The experimental results of previous study revealed that half-lives of the enzyme at 70^{o}C (pH 6.0, 7.0) was 360 and 796 min, respectively, whereas the equation model predicted half-lives of 351 and 796 min, respectively, in confident level of 95%.

These thermostable results indicated that stabilities of these enzymes decreased when the pH of enzyme decreased. The half-lives of the enzymes were higher than half-life of xylanase from *T. lanuginosus* SSBP, DSM 5826, DSM 10635 and ATCC 46882 having 232 min (pH 6.5), 201 min (pH 6.5), 40 min (pH 6.5) and 30 min (pH 6.0), respectively (Singh *et al*., 2000a; Xiong *et al*., 2004). While the half-lives of the enzyme from *T. lanuginosus* THKU-9 at 70^{o}C between pH 6.0-7.3 were

similar level of xylanase of *T. lanuginosus* SSBP and DSM 5826. This result showed that pure xylanase of *T. lanuginosus* THKU-49 was the higher stability than xylanase produced by other strains of *T. lanuginosus*.

2.3.6 Action of pure xylanase on oat spelt xylan, xylooligosaccharides and xylobiose.

To investigate mode of action of the pure xylanases of *T. lanuginosus* THKU-9 and THKU-49, oat spelt xylan and xylobiose were used as the substrates. The results showed that xylanase of *T. lanuginosus* THKU-9 and THKU-49 digested oat spelt xylan and resulted the products of xylose, xylobiose, xylotriose and oligosaccharides within 5 min. TLC chromatograms indicated that xylobiose and xylose were the main products after the hydrolysis of oat spelt xylan, especially, after long time incubation for 24 h as shown in Fig. 16. The result indicated that these xylanases might have practical utility in production of xylobiose and xylose. These enzymes also could not hydrolyze xylobiose. This might implies that a sequence of oligomer at least three xylose residues was required for actively binding site of the enzyme. Moreover, the result suggested that these xylanases could be categorized as an endo-xylanase. Our result was similar to previous reports of Kitpreechavanich *et al.* (1984b) and Li *et al.*, (2005). Xylanase produced by *T. lanuginosus* strains ATCC 46882 and SSBP mainly liberated xylose and xylobiose from beechwood xylan (Bennett *et al.,* 1998; Puchart *et al.,* 1999), however, the major products of the hydrolysis of beechwood xylan and oat spelt xylan by pure xylanase from *T. lanuginosus* ATCC 44008 were found to be xylobiose and xylotriose (Bakalova *et al.*, 2002).

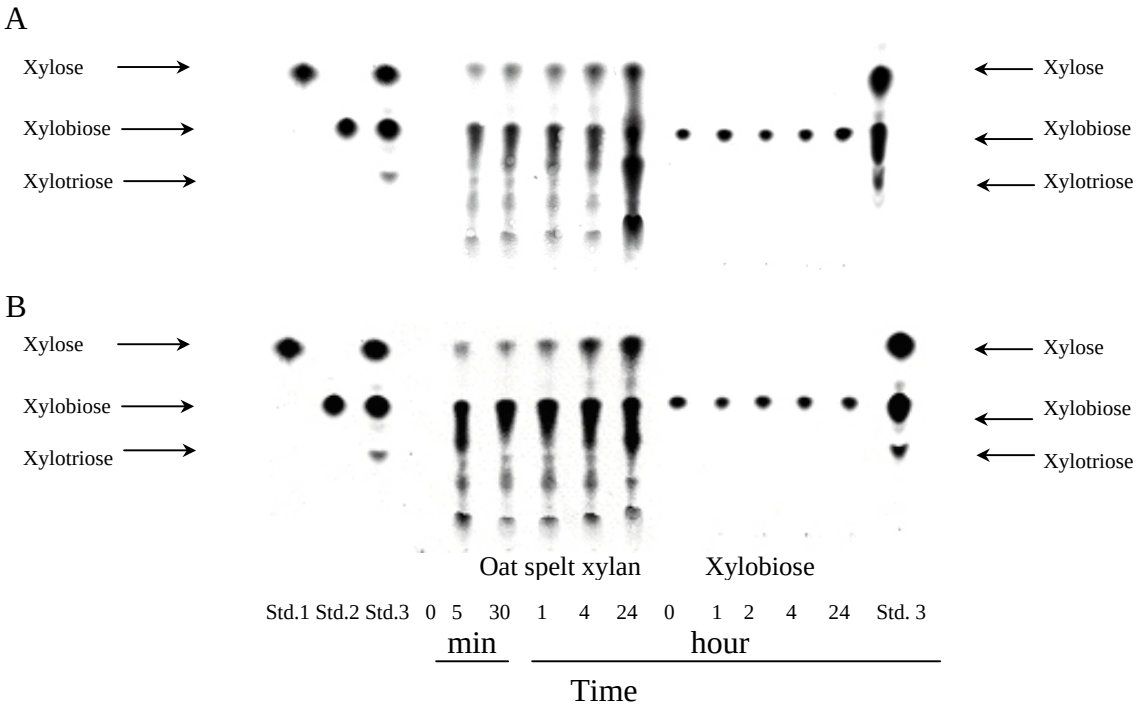

Figure 16 TLC analysis of hydrolyzates of oat spelt xylan and xylobiose with the pure xylanases of *T. lanuginosus* THKU-9 (A) and THKU-49 (B). Reaction mixture (100 μl) containing 1 mg of each substrate with 3 units of enzyme in 50 mM sodium phosphate buffer (pH 6.0) was incubated at 50^{o}C at different incubation in time.

2.3.7 Binding of pure xylanases

The ability of pure xylanases of THKU-9 and THKU-49 to bind insoluble substrates was studied by incubating these xylanases with different concentration of insoluble substrates. The results showed that these enzymes bound to the insoluble oat spelt xylan and alkali treated corncob in differently relative adsorption values, whereas these xylanases were unable to adhere to carboxymethyl cellulose and cassava starch (Figs. 17 A-C). Moreover, Fig. 17 showed that insoluble substrates at 25 mg/ml of each enzyme had highly affected on binding property. The difference in the binding affinity was also probably due to the differences in the composition of the substrates. In the case of oat spelt xylan, it had only pure xylan but alkali treated corncob containing about 40% of xylan. Thus, these xylanases could

bind to insoluble xylan better than those of alkali treated corncob. The xylanase activity also found from substrate-bound enzyme with 20 % of original activity.

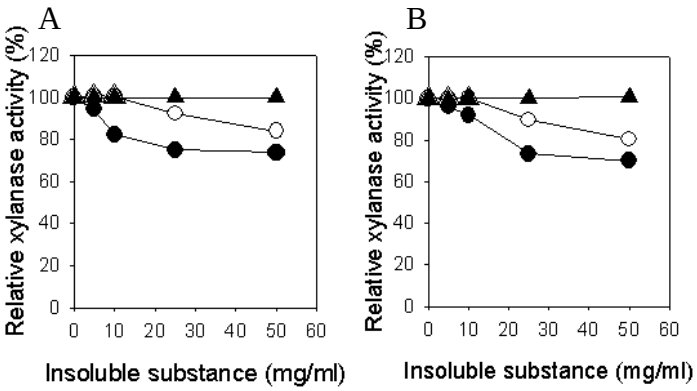

Figure 17 Binding ability of the pure xylanases of *T. lanuginosus* THKU-9 (A) and THKU-49 (B) to insoluble substrate.
The enzymes were incubated with 5-50 mg/ml insoluble substance in 50 mM sodium phosphate buffer (pH 6.0) at 4^{o}C.
insoluble oat spelt xylan (-●-) alkali treated corncob (-○-)
carboxymethyl cellulose (-△-) cassava starch (-▲-)

Most enzymes that have ability to bind to insoluble substrate could absorb to insoluble substrates using hydrophobic interaction between aromatic amino acid such as tyrosine and tryptophan, in polysaccharide-binding domain and sugars ring of substrates (Tormo *et al.*, 1996; Ponyi *et al.*, 2000). Xylanase of *T. lanuginosus* been in family 11 had only one domain of enzyme without xylan-binding domain (Nath and Rao, 2001; Irwin *et al.*, 1994). However, previous binding experiment showed that pure xylanases of *T. lanuginosus* THKU-9 and THKU-49 were able to absorb to insoluble oat spelt xylan and alkali treated corncob. Thus, addition of 49-330 mM of sodium chloride (NaCl) to the binding solution that contains enzyme and insoluble substrates to increase hydrophobicity of reaction in order to increase absorption ability was investigated. The result showed that addition of NaCl to the reaction did not increase absorption level between enzymes and each insoluble-substrate concentration (Fig. 18). This indicated that hydrophobicity was not a factor for increasing of absorption ability of our xylanases.

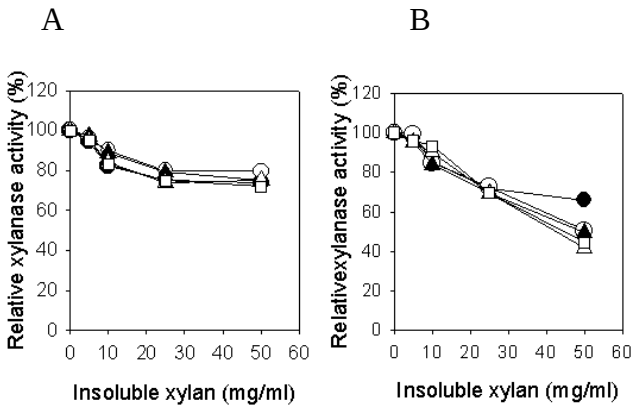

Figure 18 Binding ability of the pure xylanases of *T. lanuginosus* THKU-9 (A) and THKU-49 (B) to insoluble oat spelt xylan at different concentration of NaCl.

The enzymes were incubated with 5-50 mg/ml insoluble oat spelt xylan in 50 mM sodium phosphate buffer (pH 6.0) at 4^{o}C.

no added(-●-), 49 mM NaCl (-○-), 95 mM NaCl (-▲-),

180 mM NaCl (-△-), 330 mM NaCl (-□-)

2.4 Sequencing of the xylanase gene from selected strains

Previous experiment showed that xylanase of the strains of THKU-49 had high stability and xylanase obtained from THKU-9 showed low stability. Thus both strains were selected to determine differentiation of xylanase sequences.

Total RNAs of those strains were isolated using Rneasy® Plant Mini Kit protocol (Qiagen, Japan). At least two bands of RNA were obtained in each sample (Appendix Figure A5). Then, complementary DNAs were synthesized. The amplified fragments were detected on agarose gel electrophoresis as shown in Appendix Figure A3. The PCR products were sequenced. Data from the forward and reverse sequences were aligned to make a combined sequence and then translated to amino acid using the Genetyx version 5.0 program. The translated amino acid sequences were aligned with sequence of xylanase from *T. lanuginosus* DSM 5826 which was previously sequenced by Schlacher *et al.* (1996) (accession no. U35436) (Fig. 19).

The sequencing alignments revealed that the active site of xylanase of *T. lanuginosus* strains were at the same position of E117 and E209 (Gruber *et al.*, 1998). Amino acid sequences of xylanase from THKU-9 was similar to that of *T. lanuginosus* DSM 5826 whereas xylanase sequences of THKU-49 was difference, only one amino acid at hydrophobic region at position of V96G of the β-sheet (A5), respectively (Fig. 20).

```
DSM 5826   1:MVGFTPVALAALAATGALAFPAGNATELEKRQTTPNSEGWHDGYYYSWWSDGGAQATYTN 60
THKU-49    1:MVGFTPVALAALAATGALAFPAGNATELEKRQTTPNSEGWHDGYYYSWWSDGGAQATYTN 60
THKU-9     1:MVGFTPVALAALAATGALAFPAGNATELEKRQTTPNSEGWHDGYYYSWWSDGGAQATYTN 60
             ************************************************************

DSM 5826  61:LEGGTYEISWGDGGNLVGGKGWNPGLNARAIHFEGVYQPNGNSYLAVYGWTRNPLVEYYI 120
THKU-49   61:LEGGTYEISWGDGGNLVGGKGWNPGLNARAIHFEGGYQPNGNSYLAVYGWTRNPLVEYYI 120
THKU-9    61:LEGGTYEISWGDGGNLVGGKGWNPGLNARAIHFEGVYQPNGNSYLAVYGWTRNPLVEYYI 120
             ************************************.***********************

DSM 5826 121:VENFGTYDPSSGATDLGTVECDGSIYRLGKTTRVNAPSIDGTQTFDQYWSVRQDKRTSGT 180
THKU-49  121:VENFGTYDPSSGATDLGTVECDGSIYRLGKTTRVNAPSIDGTQTFDQYWSVRQDKRTSGT 180
THKU-9   121:VENFGTYDPSSGATDLGTVECDGSIYRLGKTTRVNAPSIDGTQTFDQYWSVRQDKRTSGT 180
             ************************************************************

DSM 5826 181:VQTGCHFDAWARAGLNVNGDHYYQIVATEGYFSSGYARITVADVG                225
THKU-49  181:VQTGCHFDAWARAGLNVNGDHYYQIVATEGYFSSGYARITVADVG                225
THKU-9   181:VQTGCHFDAWARAGLNVNGDHYYQIVATEGYFSSGYARITVADVG                225
             *********************************************
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Figure 19 Alignment of amino acid sequence of xylanase genes from different strains of *T. lanuginosus*.

The alignment was done by using the genetyx (version 5.0) alignment program. Alignment characters are indicated as follows: '*' indicates position with a conserved amino acid residue; '.' indicates position with a different amino acid residue.

Stephens *et al.* (2007) improved the thermostability of the xylanase from *T. lanuginosus* DSM 5826 by directed evolution using error-prone PCR. The amino acid sequences of xylanase from the mutants with enhanced thermostability differed in 3 amino acids for mutant 2B7-6 and had single mutation for mutants 2B11-16 and 2B7-10. Only one amino acid substitution (D72G) and substitution (Y58F) of xylanase from mutant 2B11-16 and mutant 2B7-10 increased 2 and 2.5 times of half-life at 70^{o}C, respectively. Most amino acid substitution of the mutants, except mutant

2B7-10 occurred within the β-sheet of enzyme in which forms the hydrophobic region of the enzyme (Stephens *et al.*, 2007).

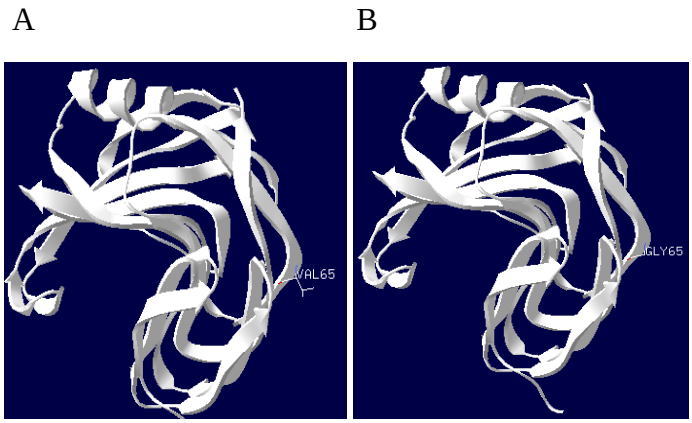


Figure 20 Three-dimension structures of xylanase produced by THKU-9 (A), THKU-49 (B) showing various amino acid.

The single amino substitution of THKU-49 xylanase (V96G) occurred on the outer surface of the β-sheet in which resulted in increasing hydrophilicity of enzyme resulted in increasing of stability. In contrast to this finding, glycine substitutions have been reported to unfavourably affecting the activity and stability of many enzymes (Betts *et al.*, 2003; Palackal *et al.*, 2004). Generally, one of aromatic bonds was formed by leucine in position 76 and tryptophan in position 39 of *T. lanuginosus* xylanases structure (Georis *et al.*, 2000).

In order to find relationship between xylanase sequences of thermophile, mesophile and thermophilic fungi, multiple-xylanase sequence alignment of our strains and other fungi such as *Aspergillus kawachi, Aspergillus niger, Bacillus stearothermophilus, Chaetomium thermophillum, Humicola grisea, Humicola insolens, Schizophyllum commune, Scytalidium thermophilum, Thermomyces lanuginosus, Trichoderma harzianum, Tricoderma viride* and *Trichoderma reesei*was made using the Genetyx5. Then, phylogenetic tree was constructed by the Neighbor-Joining using MEGA4 program as shown in Fig. 21. The total number of amino acid for comparison was 225 residues. The tree implied that xylanases of thermophile,

mesophile and thermophilic fungi appeared to be related to each other forming 3 groups. Xylanases from thermophilic fungi of *T. lanuginosus, S. thermophilum, H. inssolens* and *H. grisea var thermoidea* and *A. niger* seem to be related (group 1). Xylanases from mesophilic fungi of *T. reesei, T. harzianum* and *T. viride* and *C. thermophillum* also seem to be related (group 2). However, xylanase produced by strain of *T. reesei* was found to be distantly related to each other. Xylanases from two-different microorganisms of *A. kawachi, T. reesei* and *B. stearothermophilus* were also identical (group 3).

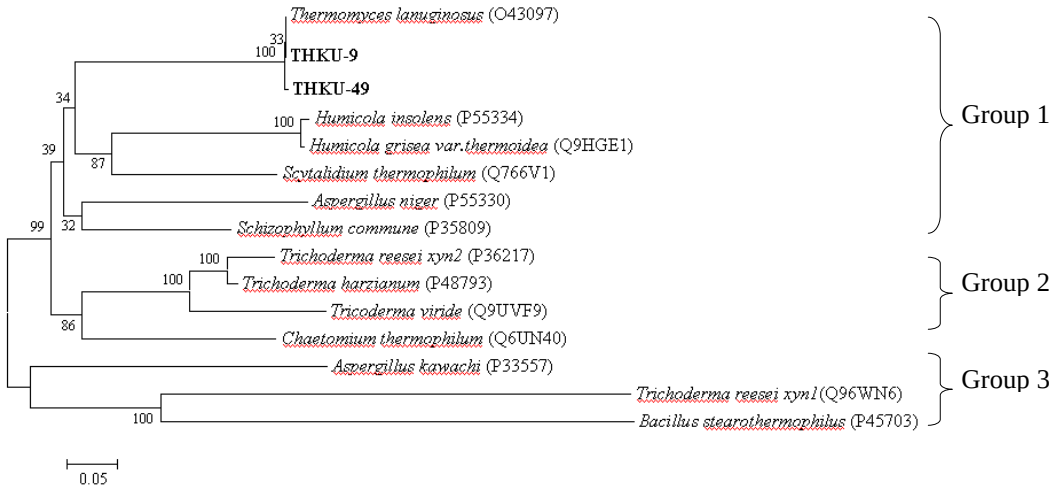


Figure 21 Phylogenetic tree of amino acid sequence analysis of xylanase of thermophile, mesophile and thermphilic fungi constructed by Neighbor-joining method from MEGA4 program.

The tree is rooted by the amino acid sequence of xylanase from *Bacillus stearothermophilus*. Scale bar shown distance values under the tree means 0.05 substitutions per amino acid position. Bootstrap analyses were performed with 1000 re-samplings and percent values are shown at the branching points. All xylanase sequences of our isolated in this study are bold letter.

3. Xylanase induction

Previous study, xylanase of *Humicola lanuginosa* (Griffon and Maublanc) Bunce in which was deposited at MIRCEN Bangkok culture collection, Thailand with name is *Thermomyces lanuginosus* TISTR 3645 was produced at high level when used xylan as a carbon source whereas very low activity was obtained when used xylose as a carbon source and its activity was similar level to that of glucose, galactose, cellobiose, and arabinose. (Koochareanpisan *et al.*, 2001). Hoq *et al.* (1994) also reported that low xylanase formation of *T. lanuginosus* RT9 was found when use xylose as a carbon source. In contrast to those, *T. lanuginosus* DSM5826 (Purkarthofer and Striner, 1995) and DSM 10635 (Xiong *et al.*, 2004) produced high levels xylanase when used xylose as a carbon source. It is still doubtful that this fungus may possess different induction-repression systems. In this study, the ability on xylanase production in the medium using xylan or xylose by different strains of *T. lanuginosus*, which were mostly-locally isolated, was studied. The effect of xylose on xylanase synthesis by the growing cell and resting cell of selected isolates of *T. lanuginosus* was investigated to explain induction-repression mechanism.

3.1 Growth experiments and xylanase production

3.1.1 Effects of xylan or xylose on xylanase production by different strains of *T. lanuginosus*

The xylanase production by 91 isolates of *T. lanuginosus* in the medium using either oat spelt xylan and xylose as an energy source and carbon source was diverse depended on the strains (Appendix Table A3). *T. lanuginosus* THKU-86 produced the highest xylanase activity of 134 units/ml in the xylan medium whereas *T. lanuginosus* THKU-11 produced the highest xylanase activity of 95.5 units/ml in the xylose medium as shown in Appendix Table A3. There was a group of isolates produced high xylanase either in the xylan or xylose medium. The ratio of xylanase activity from xylan medium to xylose medium was in a range of 1.1-1.5 (Table 15). The representative isolates of this group were THKU-11, THKU-25 and

ATCC 44008. In contrast to this group, there was another group of isolates that was able to produce high xylanase only in the xylan medium but less activity was detected in the xylose medium. The ratio of xylanase activity from xylan medium to xylose medium were higher than 3.0. The representative isolates were TISTR 3465, THKU-85 and ATCC 46882.

Table 15 Xylanase production by *T. lanuginosus* strains on the medium using either xylan or xylose as a carbon sources at 5th day cultivation.

Strain	Xylanase activity (U/ml)[a]		Ratio
	Xylan medium (A)	Xylose medium (B)	A/B
ATCC 44008	100	40.7	2.5
ATCC 46882	100	6.4	15.6
TISTR 3465	95	0.2	475.0
THKU-2	102	53	1.9
THKU-4	101	67.8	1.5
THKU-5	106	58.4	1.8
THKU-6	105	77.1	1.4
THKU-8	108	67.9	1.6
THKU-9	51	69.1	0.7
THKU-10	103	73.9	1.4
THKU-11	107	95.5	1.1
THKU-12	106	34.4	3.1
THKU-21	100	66.8	1.5
THKU-22	101	74.6	1.4
THKU-25	102	69.2	1.5
THKU-26	101	63	1.6
THKU-27	95	78	1.2
THKU-28	89	70.1	1.3
THKU-83	112	9.0	12.4
THKU-84	106	68	1.6
THKU-85	76	8.1	9.4
THKU-86	134	48.8	2.8
THKU-88	113	9.1	12.4

[a] mean value of duplication.

The representative isolates of each group were cultivated in the medium containing either oat spelt xylan or xylose as a carbon sources at concentration of 0.1%, 0.5% and 1%. It was found that xylanase production by

isolates of ATCC 44008, THKU-11, and THKU-25 were positively affected by xylan. The activities were increased when xylan concentration was increased (Table 16). Few xylanase activity was produced in xylose medium in regardless of xylose concentration.

Table 16 Xylanase production by *T. lanuginosus* strains on the medium using either xylan or xylose as a carbon sources at various concentrations at 5th day cultivation.

Strain	Xylanase activity (U/ml) [a]					
	Xylan concentration			Xylose concentration		
	0.1%	0.5%	1.0%	0.1%	0.5%	1.0%
ATCC 44008	43.4	68.9	100.2	0.0	26.7	40.7
THKU-11	42.4	72.4	107.4	0.0	24.9	95.0
THKU-25	44.5	61.8	102.0	0.0	11.9	69.0
ATCC 46882	58.5	86.7	99.9	0.0	5.1	6.4
TISTR 3465	34.4	53.8	94.7	0.0	0.0	0.2
THKU-85	43.4	68.9	76.0	0.0	2.7	8.1

[a] mean value of duplication.

3.1.2. Effect of xylose addition on xylanase production by the selected strains of *T. lanuginosus*

Xylanase production by the selected isolates of *T. lanuginosus* was rapidly increased within 4-day cultivation and then slightly constant. Addition of xylose or glucose to the 3rd day of xylan-grown culture of TISTR 3465, THKU-85 and ATCC 46882 decreased xylanase formation as shown in Figs.22 A, B, C.

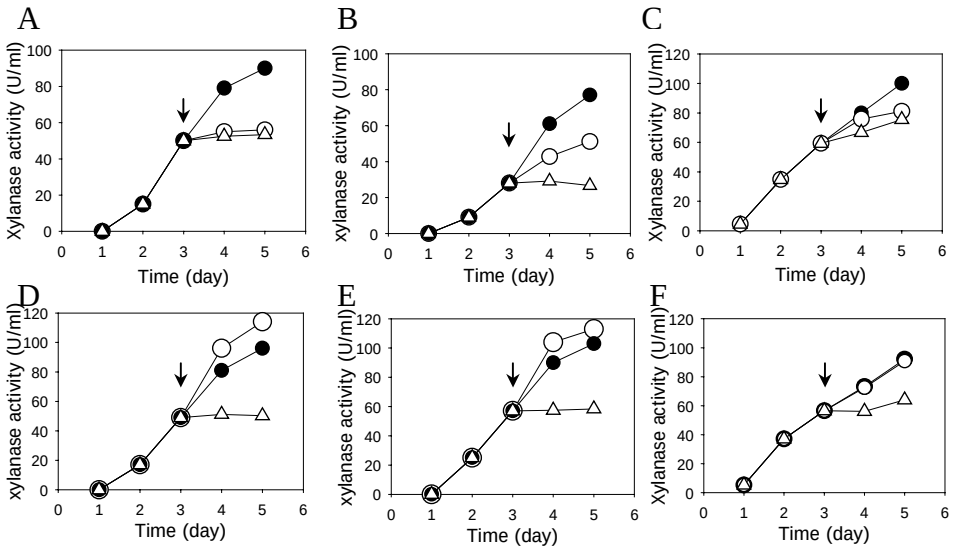


Figure 22 Effect of xylose or glucose addition to the 3rd day xylan-grown culture on the xylanase production by *T. lanuginosus* strains.
Xylan grown culture (-●-), 1% xylose added to xylan-grown culture (-○-), 1% glucose added to xylan-grown culture (-△-). Arrow indicated the addition of xylose or glucose

TISTR 3465 (A)	THKU-85 (B)	ATCC 46882 (C)
THKU-11 (D)	THKU-25 (E)	ATCC 44008 (F)

This finding was similar to the report of Hoq *et al.* (1994) in which found that low xylanase formation of *T. lanuginosus* RT9 in the xylose medium. The strain TISTR 3465 showed strong repression by xylose on xylanase formation. This was correlation very low xylanase activity on xylose medium. In contrast, addition of xylose to the xylan-grown cultures of THKU-11, THKU-25 and ATCC 44008 did not show any repression effect on xylanase formation whereas addition of glucose immediately decreased the activity of THKU-11, THKU-25 and slowly decreased the activity of ATCC 44008 (Figs. 22 D, E, F).

Addition of various concentrations at 0.1, 0.25, 0.5, 1.0 and 2% xylose in to xylan-grown cultures at the 3rd day of culture of TISTR 3465, THKU-85 and ATCC 46882 could effectively decreased β-xylanase formation when increasing

concentrations of xylose were added. This xylanase synthesis might be interpreted in terms of catabolite repression likewise described for other enzymes. Nevertheless, the strains of THKU-11, THKU-25 and ATCC 44008 gradually increased (Fig. 23). This result indicated that no catabolic repression cause by xylose did not cause of repression.

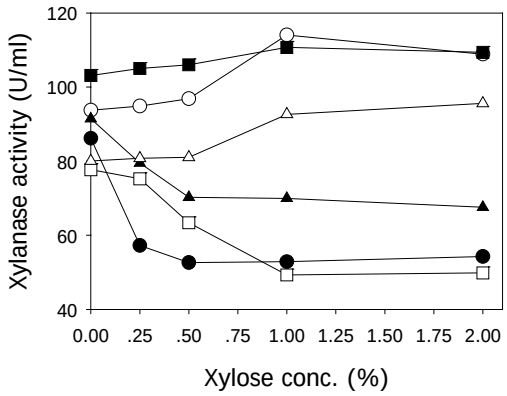


Figure 23 Effect of addition of xylose at various concentration to the 3rd day xylan-grown culture on the xylanase production by *T. lanuginosus* strains at 5-day cultivation.

TISTR 3465 (●) THKU-85 (□) ATCC 46882 (▲)
THKU-11 (○) THKU-25 (■) ATCC 44008 (△)

3.2 Induction experiments

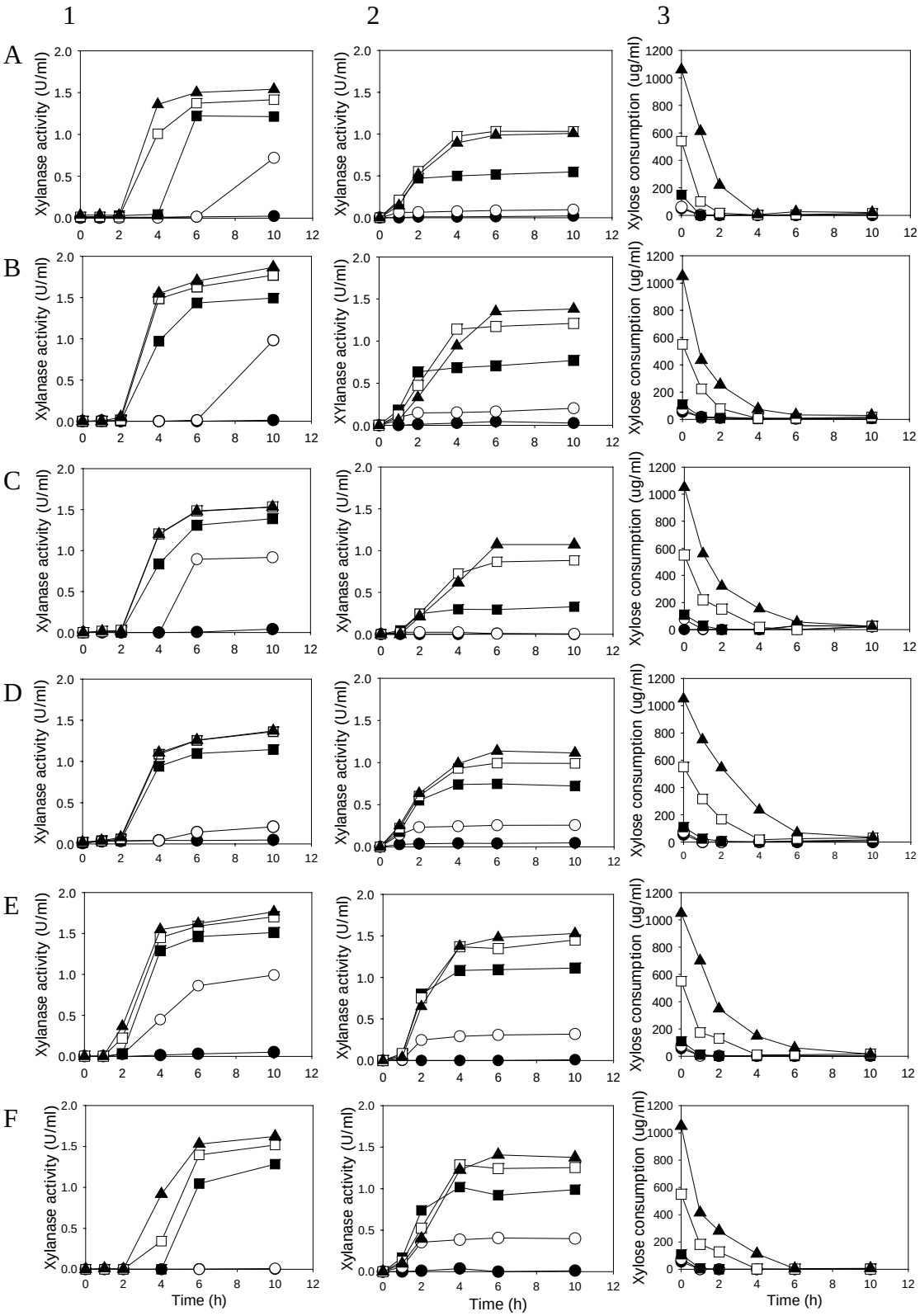
At various time intervals, the xylanase activity was determined in the incubated buffer. Xylan and xylose were able to induce xylanase formation in the strain THKU-11, THKU-25 and ATCC 44008 belonging to the group that xylose did not repress the enzyme synthesis. Unexpected, both xylan and xylose also induced the xylanase formation in the strains of TISTR 3465, THKU-85 and ATCC 46682 belonging to the group performed strong repression of xylanase by xylose. Xylanase activities were increased when the concentration of xylan or xylose was increased (Fig. 24). In the control of experiments showed very low xylanase activity because any xylanase activity formed constitutively. The xylanase formation during incubation

in presence of xylose was faster than xylan. However, xylanase formation using xylan as an inducer showed higher pronounced on induction on xylanase activity than using xylose (Fig. 24). It was possible to conclude that oat spelt xylan acted as the premier inducer of xylanase in strains of *T. lanuginosus*. However, since xylan was a large polymer in which could not penetrate the plasma membrane, it could not act as a direct inducer of xylanase. It could be anticipated that very low levels of extracellular xylanase in which was always produced and broke xylan into xylo-oligosaccharides and xylose. Then, they entered to the cells and mediate induction of xylanase. The result of xylan and xylose are inducer of xylanase synthesis was corresponded to Purkarthofer and Steiner (1995) who reported that xylanase production was induced in *T. lanuginosus* DSM 5826 with xylose in induction condition.

3.3 Random amplified polymorphic DNA (RAPD) analysis

Reproducible DNA polymorphisms were observed in the RAPD profiles of the *T. lanuginosus* strains for the used three primers. Both high and low intensity bands were produced when each primer were used. Minor bands were assumed to be non-specific amplification products that could have arisen by mismatching between primer and template DNA (Williams *et al.*, 1990). Generally, only the high intensity bands were considered in our assessments. When used recombinant Taq DNA polymerase as polymerizing enzyme and primer UBC235, UBC241 and UBC280 were used to amplify PCR fragments. The result also showed that recombinant Taq DNA polymerase gave smeary and unclear DNA bands which were difficult to distinguish the different between its bands. (Appendix Figure A6).

Figure 24 Time course of extracellular xylanase production by washed glutamic acid-grown mycelium of *T. lanuginosus* strains.
TISTR 3465 (A1,A2), ATCC 46882 (B1,B2), THKU-85 (C1,C2), THKU-11 (D1,D2), ATCC 44008 (E1,E2) and THKU-25 (F1,F2) during incubation of washed glutamic acid-grown mycelium in 50 mM phosphate buffer (pH 6.0) supplied with separate oat spelt xylan (A1-F1) and xylose (A2-F2) at concentrations of 0 mg/l (●), 10 mg/l (○), 100 mg/l (■), 500 mg/l (□), 1000 mg/l(▲). Concentrations of fungal mycelium are in range of 0.11-0.15 g dry weight/experiment. The consumption of xylose from xylose-supplemented condition was also shown (A3-F3).


1
2
3
A
B
C
D
E
F
Xylanase activity (U/ml)
XYlanase activity (U/ml)
Xylose consumption (ug/ml)
Time (h)
0.0
0.5
1.0
1.5
2.0
0
200
400
600
800
1000
1200
0
2
4
6
8
10
12

Whereas used Blend Taq Plus DNA polymerase as polymerizing enzyme, it gave the DNA band with clear sharp and easy to distinguish between DNA bands of this fungus. DNA profiles consisting of 2-4 fragments were obtained after amplification with primer UBC 235 with sizes ranging between 947 and 3530 bp (Appendix Figure A4). Using primer UBC 241, DNA profiles consisting of 5-9 fragments were obtained with sizes ranging between <564 and 3530 bp. Using primer UBC 280, DNA profiles consisting of 2-5 fragments were obtained with sizes ranging between 1375 and 3530 bp. These results showed that primer UBC 241 was the best primer for amplification.

The RAPD patterns received from Blend Taq plus DNA polymerase were distinguishable into four groups using primer UBC 241**.** Based on the presence or absence of bands, a dendrogram was generated which indicated the relationships among the strains into seven groups and two different clusters. The UPGMA tree from UBC241 was shown in Figure 25. Interestingly, the dendogram also distinguished between high level xylanase producing strains and low level xylanase producing strains using xylose as a carbon source (cluster A, B, C). For cluster A, *T. lanuginosus* strains THKU-56, ATCC 44008, THKU-30, THKU-33, THKU-49, THKU-86 and THKU-2 except THKU-77 that produced moderate amount of xylanase activity were apparently closely related. While cluster B, strains THKU-25, THKU-11 and THKU-9 that produced xylanase of 69.2, 95.5 and 69.1 U/ml, respectively of xylanase also showed a close relationship. Among this cluster, there was exception with strain TISTR 3465 that produced low level of xylanase. This indicated that RAPD analysis have ambiguous separation. In contrast to those, the dendogram could not distinguished between each cluster of strains producing high and low level of xylanase in the xylan medium (Fig. 25).

Singh *et al.* (2000b) also reported that RAPD analysis with primer UBC 241 (5’-GCCCGACGCG-3’) and *Taq* DNA polymerase (Life Technologies, Gibco, BRL, UK) resulted to distinguish between eight *T. lanuginosus* that produced high and low xylanase using corncob as a carbon source.

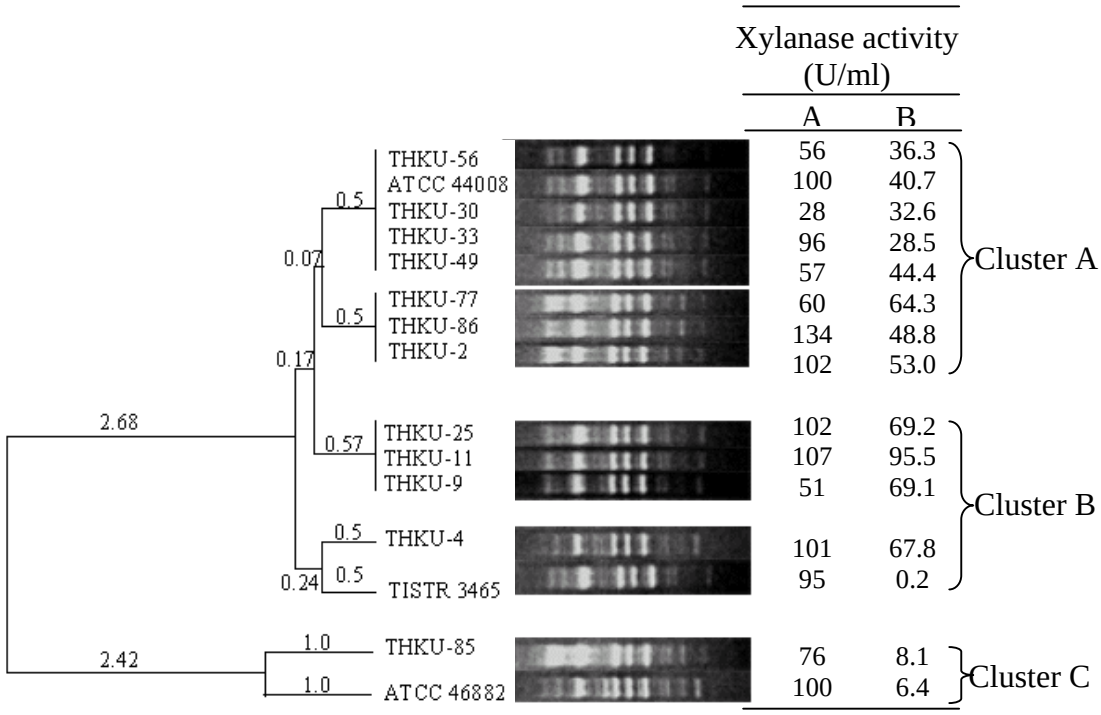


Figure 25 Dendogram indicating relationships of *T. lanuginosus* strains obtained with the primer UBC 241 of xylanase producing strains with xylanase activity obtained from 5-day cultivation using xylan (A) and xylose (B) as a carbon source.

The xylanase activities produced by each strain (Appendix Table A3) are shown in parentheses.

CONCLUSION AND RECOMMENDATIONS

Conclusion

In this study, 90-isolated strains of expected *T. lanuginosus* isolated from soil compost, and bark in Thailand, were identified using morphology as aleuriospores in which globose and irregularly sculptured criteria according to Cooney and Emerson (1964) and Domsch *et al.* (1993). Among those strains, 87-isolated strains were belonged to *Thermomyces lanuginosus.* Comparative taxonomy of the ITS rDNA sequences of 15-selected strains; THKU-2, THKU-3, THKU-9, THKU-11, THKU-16, THKU-17, THKU-25, THKU-30, THKU-33, THKU-43, THKU-49, THKU-55, THKU-79, THKU-80 and THKU-81 from those were studied with the sequences in the DDBJ database from DDBJ website. The ITS rDNA of selected strains were 99% homologous to that of *T. lanuginosus*. From morphological and ITS rDNA sequence of 15-selected strains as well as the comparative studies of the fungal characteristics, it was concluded that they were belonged to *T. lanuginosus*. It also could be concluded that the morphological identification was an effective method to identify this fungus to species level. Thus, other isolated strains should be approval as *T. lanuginosus.*

Crude enzymes obtained from 87 isolated strains, one strain of TISTR 3465 and two strains of ATCC 46882 and ATCC 44008 were screened based on thermostability at 70°C. Among these stains, 2 strains as THKU-9 and THKU-49 were selected depending on their low and high thermostable characters. The xylanases obtained from these 2 strains were purified by four steps; ammonium sulfate precipitation, DEAE-Sepharose fast flow column, hydroxylapatite column and Sephadex G-100 column. The enzymes recovered from these steps were proved to be purified as a single band of protein on SDS-PAGE. The specific activities of combined active fraction of xylanases of *T. lanuginosus* THKU-9 and THKU-49 were 306 and 552 unit/mg protein, respectively. The molecular mass of xylanases was found 24.9 kDa. Central composite design (CCD) was used to determine the

maximum activities of these xylanases. Their maximum xylanase activities of THKU-9 and THKU-49 were obtained at 66^{o}C (pH 6.3) corresponded to xylanase activities of 503 and 669 U/mg protein in confidential level of 95% whereas experimental activity, 510 and 712 U/mg protein were obtained, respectively. The CCD was also used to determine enzymatic thermostability at 70^{o}C in which reported as half-life. Long half-life means more stability. The longest thermostability of these enzymes obtained from equation model in range temperatures of 70-84^{o}C and pHs 5.6-8.4 of THKU-9 and THKU-49 were temperature of 70^{o}C (pH 7.5) and 70^{o}C (pH 7.3) having half-lives of 605 and 812 min, respectively. When buffer concentration increased, the half-life of xylanases from *T. lanuginosus* THKU-9 and THKU-49 were significantly decreased. Besides, half-life of their xylanases in phosphate buffer was longer than in citrate buffer and the longest when the enzymes were kept in buffer pH 7.0. The enzyme concentration was also effected of enzymatic thermostability. At highly enzymatic concentration, its half-life was longer than that of low concentration. When determine thermostability of enzymes at various temperatures in 50 mM phosphate buffer (pH 7.0), the enzymes of *T. lanuginosus* THKU-49 was fully stable to 1080 min up to 60^{o}C whereas the xylanase of *T. lanuginosus* THKU-9 was stable up to 50^{o}C and retained 87% at 60^{o}C. At 70^{o}C, residual activities of xylanase of *T. lanuginosus* THKU-9 and THKU-49 were 30% and 41%, respectively, after keeping for 1080 min. The results concluded that xylanases of THKU-49 were more stable than that of *T. lanuginosus* THKU-9.

When measured the xylanase activities of pure enzyme in presence of different metal ion, the considerably decreasing β-xylanase activity of THKU-49 was observed in the presence of Mn^{2+}, Sn^{2+} and EDTA at the concentration of 1 mM. The relative activities with Mn^{2+}, Sn^{2+} and EDTA were 85.5%, 83.2% and 74.6%, respectively. However, all added metal ions and chemicals had no effect on xylanase of THKU-9.

The substrate specificity of xylanases of *T. lanuginosus* THKU-9 and THKU-49 on soluble oat spelt xylan that was gotten the highest activities of 1.20 ± 0.010 U/ml (112.1% relative activity) and 1.06 ± 0.009 U/ml (112.9% relative activity) were observed from THKU-9 and THKU-49, respectively. The enzymes were able to

hydrolyse soluble substances better than in soluble substance. The K_m value of pure xylanase of *T. lanuginosus* THKU-49 towards oat spelt xylan, soluble oat spelt xylan and insoluble oat spelt xylan were 8.4 ± 1.300, 7.3 ± 0.236 and 60.2 ± 6.788 mg/ml, respectively. The K_m of pure xylanase of strain THKU-49 towards soluble oat spelt xylan and oat spelt xylan lower than K_m values of THKU-9. It could be concluded that xylanase of THKU-49 specified to soluble oat spelt xylan more than that of THKU-9.

Hydrolysis of soluble oat spelt xylan by both pure xylanase from *T. lanuginosus* resulted the main products as xylobiose, xylotriose and oligosaccharides. However, these enzymes could not hydrolyse xylobiose. This could be concluded that it was an endo-xylanase.

Substrate-binding analysis indicated that both pure enzymes bound to the insoluble oat spelt xylan and alkali treated corncob in differently relative adsorption values. But they were unable to bind carboxymethyl cellulose and cassava starch. Addition of NaCl did not increased absorption level between enzymes and insoluble-substrates.

Amino acid sequences transferred from nucleotides sequence of xylanase obtained from THKU-49 were different from that low thermostable xylanases of DSM 5826 and THKU-9 only one amino acid at hydrophobic region at position of V96G of the β-sheet (A5) at the outer surface of the enzyme structure, respectively.

β-Xylanase synthesis in *T. lanuginosus* was inducible enzymes. The isolated strains of *T. lanuginosus* could be separated to two groups using ability to produce xylanase when used xylose as a carbon source. The representative strains of THKU-11, THKU-25 and ATCC 44008 were selected from groups producing high xylanase. Addition of xylose to the 3-day xylan-grown cultures resulted in increase of xylanase formation to maximum level whereas addition of glucose deeply decreased xylanase formation. In contrast, the addition of separate xylose and glucose to the xylan grown culture of *T. lanuginosus* TISTR 3465, THKU-85 and ATCC 46882 in

which were selected from the groups producing low xylanases decreased xylanase formation.

The enzymes could be induced in washed glutamic acide-grown mycelium of THKU-11, THKU-25, ATCC 44008, TISTR 3465, THKU-85 and ATCC 46882 by xylan and xylose at various concentrations. The induction of xylanase by xylan lasted longer and the final total activities were significantly higher compared to the induction by xylose.

RAPD analysis proved to be a suitable method to detect genetic variability among strains of *T. lanuginosus*. Interestingly, primer UBC 241 yielded RAPD banding patterns that allowed us to distinguish between the high and low xylanase producing strains using xylose as a carbon source. This observation may prove useful in the further characterisation of *T. lanuginosus* strains that are hyperproducers of xylanase.

Recommendations

1. *T. lanuginosus* distribution should be determined in each season of Thailand.

2. To confirm the xylanase sequences should be determined amino acid sequence from pure enzyme.

3. To confirm the 3D structure of xylanase should be determined 3 D structure from crystallized protein.

4. Their stability should be confirmed by circular dichroism spectroscopy.

5. Cyclic AMP should be used to confirm catabolic repression of *T. lanuginosus* TISTR 3465, THKU-85 and ATCC 46882.

6. To confirm induction/repression system of xylanase gene expression should be determined.

7. *T. lanuginosus* THKU-11 and THKU-25 should be use to produce xylanase using xylose as a carbon source and then operate in large scale of fermentation.

8. Xylanase produced by *T. lanuginosus* THKU-49 should be application in biobleaching process because of its high thermostability.

LITERATURE CITED

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APPENDICES

Appendix A

Experimental results

Appendix Table A1 Comparison of number of thermophilic fungi and *T. lanuginosus* from samples collected at differently geographical location in Thailand.

Source	Sample no.	pH	Organic matter	Thermophilic fungi*		*T. lanuginosus*		Selected *T. lanuginosus*
				Colony/plate	cfu/g	Colony/plate	cfu/g	
A	1	6.0	10.0	100	11000	11	1210	1
	2	6.0	11.3	85	5100	9	540	0
	3	6.0	12.4	98	10780	0	0	0
	4	6.0	12.1	165	18150	15	1650	1
	5	6.0	6.6	115	3450	0	0	0
	6	6.0	7.0	125	7500	50	3000	3
	7	6.0	8.7	75	8250	0	0	0
	8	6.0	9.8	120	121200	23	23230	5
	9	6.0	6.5	320	35200	8	880	1
	10	6.0	11.8	120	13200	0	0	0
	11	6.0	12.0	170	18700	12	1320	1
	12	6.0	7.6	185	5550	15	450	0
	13	6.0	8.9	0	0	0	0	0
	14	6.0	8.3	0	0	0	0	0
	15	6.0	9.0	0	0	0	0	0
	16	6.0	12.1	0	0	0	0	0
	17	6.0	10.4	144	15840	30	3300	3
	18	6.0	10.0	140	15400	13	1430	2
	19	6.0	8.8	0	0	0	0	0
	20	6.0	7.0	132	14520	12	1320	1
	21	6.0	7.7	215	23650	0	0	0
	22	6.0	6.6	218	23980	20	2200	2
	23	6.0	7.1	0	0	0	0	0
	24	6.0	9.3	0	0	0	0	0
	25	6.0	6.6	0	0	0	0	0
	26	6.0	7.9	220	220000	6	6060	0
B	1	7.0	5.2	0	0	0	0	0
	2	7.0	4.1	0	0	0	0	0
	3	7.0	5.5	0	0	0	0	0
	4	7.0	5.7	0	0	0	0	0

Appendix Table A1 (Continued)

Sources	Sample no.	pH	Organic matter	Thermophilic fungi*		*T. lanuginosus*		Selected *T. lanuginosus*
				Colony/plate	cfu/g	Colony/plate	cfu/g	
C	1	6.0	0.9	23	690	0	0	0
	2	6.0	10.2	85	2550	6150	0	0
	3	6.0	10.0	120	3600	0	0	0
	4	6.0	13.5	90	2700	0	0	0
	5	6.0	0.8	50	1500	0	0	0
	6	6.0	13.7	125	3750	0	0	0
	7	6.0	11.5	69	2070	0	0	0
	8	6.0	13.7	98	2940	0	0	0
	9	6.0	13.5	150	4500	0	0	0
	10	6.0	0.7	205	6150	0	0	0
	11	6.0	0.6	156	4680	0	0	0
	12	6.0	0.9	69	2070	0	0	0
	13	6.0	10.9	103	3090	0	0	0
	14	6.0	9.9	203	6090	0	0	0
	15	6.0	13.2	95	2850	0	0	0
	16	6.0	12.8	36	1080	0	0	0
	17	6.0	10.6	87	2610	0	0	0
	18	6.0	0.7	96	2880	0	0	0
	19	6.0	0.9	140	4200	0	0	0
	20	6.0	11.1	0	0	0	0	0
D	1	6.0	13.0	0	0	0	0	0
	2	6.0	10.3	0	0	0	0	0
	3	5.0	3.4	0	0	0	0	0
	4	6.0	4.5	0	0	0	0	0
	5	5.0	13.7	0	0	0	0	0
	6	5.0	7.8	0	0	0	0	0
	7	6.0	3.8	0	0	0	0	0
	8	5.0	7.9	0	0	0	0	0
	9	6.0	9.1	0	0	0	0	0
	10	6.0	8.3	0	0	0	0	0
	11	6.0	13.7	0	0	0	0	0
	12	6.0	4.3	0	0	0	0	0

Appendix Table A1 (Continued)

Sources	Sample no.	pH	Organic matter	Thermophilic fungi*		*T. lanuginosus*		Selected *T. lanuginosus*
				Colony/plate	cfu/g	Colony/plate	cfu/g	
D	13	5.0	8.5	0	0	0	0	0
	14	5.0	9.7	0	0	0	0	0
	15	6.0	7.3	0	0	0	0	0
	16	6.0	7.1	0	0	0	0	0
	17	6.0	3.4	0	0	0	0	0
	18	6.0	5.4	0	0	0	0	0
	19	6.0	5.3	0	0	0	0	0
	20	6.0	7.9	0	0	0	0	0
	21	5.0	3.7	0	0	0	0	0
	22	5.0	10.4	0	0	0	0	0
	23	6.0	10.2	0	0	0	0	0
	24	5.0	12.8	0	0	0	0	0
	25	6.0	12.0	0	0	0	0	0
	26	5.0	8.9	0	0	0	0	0
	27	5.0	9.3	0	0	0	0	0
	28	5.0	5.9	0	0	0	0	0
	29	6.0	6.2	0	0	0	0	0
	30	6.0	9.4	0	0	0	0	0
	31	6.0	5.8	0	0	0	0	0
	32	6.0	6.2	0	0	0	0	0
	33	6.0	8.0	0	0	0	0	0
	34	6.0	5.9	0	0	0	0	0
	35	5.0	6.2	0	0	0	0	0
	36	5.0	7.9	0	0	0	0	0
	37	6.0	4.7	0	0	0	0	0
	38	5.0	12.9	0	0	0	0	0
E	1	6.0	3.4	0	0	0	0	0
	2	6.0	6.8	0	0	0	0	0
	3	5.0	3.2	0	0	0	0	0
	4	6.0	14.1	0	0	0	0	0
	5	6.0	11.4	0	0	0	0	0
	6	6.0	12.5	0	0	0	0	0

Appendix Table A1 (Continued)

Sources	Sample no.	pH	Organic matter	Thermophilic fungi*		*T. lanuginosus*		Selected *T. lanuginosus*
				Colony/plate	cfu/g	Colony/plate	cfu/g	
E	7	5.0	7.8	0	0	0	0	0
F	1	6.0	12.9	189	11340	9.5	570	0
	2	6.0	18.1	165	9900	21	1260	1
	3	6.0	10.4	145	8700	36	2160	4
	4	7.0	7.3	130	7800	10	600	2
	5	6.0	6.3	89	5340	18	1080	1
	6	6.0	8.6	24	1440	20	1200	2
	7	7.0	9.9	63	3780	14	840	0
	8	7.0	10.0	65	3900	5	300	0
	9	6.0	19.2	50	3000	6	360	0
	10	7.0	18.5	35	2100	8	480	0
G	1	7.0	34.3	0	0	0	0	0
	2	7.0	30.5	0	0	0	0	0
	3	6.0	20.3	106	6360	39	2340	0
	4	7.0	6.0	100	6000	20	1200	0
	5	6.0	15.8	86	5160	5	300	0
	6	6.0	5.9	120	7200	6	360	3
	7	7.0	8.8	65	3900	8	480	3
	8	6.0	7.3	63	3780	2	120	1
H	1	6.0	7.0	0	0	0	0	0
	2	6.0	6.9	0	0	0	0	0
	3	6.0	7.5	0	0	0	0	0
	4	6.0	7.3	0	0	0	0	0
	5	6.0	6.9	0	0	0	0	0
	6	6.0	7.4	0	0	0	0	0
	7	6.0	7.4	0	0	0	0	0
I	1	6.0	13.2	133	7980	17	1020	0
	2	6.0	14.1	189	11340	8	480	0
	3	6.0	10.5	200	12000	12	720	0
	4	6.0	7.6	208	12480	8	480	0
	5	6.0	8.9	121	7260	21	1260	1
	6	6.0	6.7	98	2940	0	0	0

Appendix Table A1 (Continued)

Sources	Sample no.	pH	Organic matter	Thermophilic fungi*		*T. lanuginosus*		Selected *T. lanuginosus*
				Colony/plate	cfu/g	Colony/plate	cfu/g	
F	7	6.0	7.2	165.5	4965	0	0	0
	8	6.0	6.7	0	0	0	0	0
G	9	6.0	14.0	125	3750	25	750	2
	10	6.0	12.1	141	4230	14	420	0
J	1	6.0	5.1	22	660	0	0	0
	2	6.0	15.5	10	300	0	0	0
	3	6.0	13.7	28	840	0	0	0
K	1	6.0	40.4	130	131300	11	11110	7
	2	6.0	41.2	140	15400	17	1870	7
	3	6.0	38.9	90	9900	6	660	4
	4	7.0	23.1	85.5	9405	10	1100	5
	5	6.0	30.5	134	14740	32	3520	15
	6	6.0	27.5	155	9300	17.3	1038	4
	7	7.0	30.7	140	15400	25	2750	8
L	1	6.0	12.4	32	32320	0	0	0
	2	6.0	13.0	120	13236	0	0	0
	3	6.0	13.7	121	13346	0	0	0
M	1	6.0	13.5	13	1430	7	210	1
	2	6.0	2.6	5	550	3	90	0
	3	6.0	10.2	12	720	5	150	0

* Triple plates of each diluted sample

A = Soils from orchard and plantation
B = Soils from cave
C = Soils from near seashore
D = Soils from road side
E = Soils from ancient remains
F = Soils from national park
G = Soils from botanical garden
H = Soils from hot spring
I = Soils from domestic area
J = Soils from embankment
K = Compost
L = Dung
M = Bark

Appendix Table A2 β-Xylanases production by different strains of *T. lanuginosus* and half-life of enzyme at 70°C.

Strain	β-xylanase (U/ml)	Half life (min)	Strain	β-xylanase (U/ml)	Half life (min)
TISTR 3465	95	21	THKU-26	101	20
THKU-2	102	10	THKU-27	95	28
THKU-3	99	17	THKU-28	89	28
THKU-4	101	140	THKU-29	91	16
THKU-5	106	20	THKU-30	28	3
THKU-6	105	15	THKU-31	93	19
THKU-7	93	142	THKU-32	95	20
THKU-8	108	52	THKU-33	96	12
THKU-9	51	20	THKU-34	91	80
THKU-10	103	41	THKU-35	88	40
THKU-11	107	70	THKU-39	89	140
THKU-12	106	42	THKU-40	91	19
THKU-13	90	32	THKU-41	55	30
THKU-14	79	29	THKU-42	52	61
THKU-15	73	20	THKU-43	53	179
THKU-16	78	76	THKU-44	55	59
THKU-17	90	18	THKU-45	47	43
THKU-18	42	20	THKU-46	57	254
THKU-19	88	16	THKU-47	47	80
THKU-20	91	81	THKU-48	35	231
THKU-21	100	95	THKU-49	57	266
THKU-22	101	65	THKU-50	42	31
THKU-23	94	20	THKU-51	64	68
THKU-24	98	70	THKU-52	58	127
THKU-25	102	69	THKU-53	59	26

Appendix Table A2 (Continued)

Strain	β-xylanase (U/ml)	Half life (min)	Strain	β-xylanase (U/ml)	Half life (min)
THKU-54	42	144	THKU-74	66	33
THKU-55	47	207	THKU-75	67	26
THKU-56	56	72	THKU-76	60	35
THKU-57	65	58	THKU-77	60	70
THKU-58	65	145	THKU-78	56	72
THKU-59	68	100	THKU-79	21	51
THKU-60	42	147	THKU-80	20	124
THKU-61	71	24	THKU-81	22	44
THKU-62	60	21	THKU-82	86	20
THKU-63	62	169	THKU-83	112	200
THKU-64	67	98	THKU-84	106	163
THKU-65	61	36	THKU-85	76	60
THKU-66	56	29	THKU-86	134	15
THKU-67	62	25	THKU-87	81	69
THKU-68	69	36	THKU-88	113	16
THKU-69	68	95	THKU-89	96	20
THKU-70	76	31	THKU-90	90	20
THKU-71	64	87	THKU-91	73	28
THKU-72	55	22	ATCC 44008	100	92
THKU-73	58	170	ATCC 46882	100	15

Appendix Table A3 Xylanase production by *T. lanuginosus* strains on the medium using either xylan or xylose as a carbon sources at 5th day cultivation.

Strain	Xylanase activity (U/ml)[a]		Ratio
	Xylan medium (A)	Xylose medium (B)	A/B
TISTR 3465	95	0.2	475.0
THKU-2	102	53	1.9
THKU-3	99	40.4	2.5
THKU-4	101	67.8	1.5
THKU-5	106	58.4	1.8
THKU-6	105	77.1	1.4
THKU-7	93	45.6	2.0
THKU-8	108	67.9	1.6
THKU-9	51	69.1	0.7
THKU-10	103	73.9	1.4
THKU-11	107	95.5	1.1
THKU-12	106	34.4	3.1
THKU-13	90	35.6	2.5
THKU-14	79	33	2.4
THKU-15	73	38.4	1.9
THKU-16	78	52.5	1.5
THKU-17	90	13.6	6.6
THKU-18	42	41.4	1.0
THKU-19	88	43.2	2.0
THKU-20	91	37.1	2.5
THKU-21	100	66.8	1.5
THKU-22	101	74.6	1.4
THKU-23	94	66	1.4
THKU-24	98	67.1	1.5
THKU-25	102	69.2	1.5

Appendix Table A3 (Continued)

Strain	Xylanase activity (U/ml)[a]		Ratio
	Xylan medium (A)	Xylose medium (B)	A/B
THKU-26	101	63	1.6
THKU-27	95	78	1.2
THKU-28	89	70.1	1.3
THKU-29	91	60	1.5
THKU-30	28	32.6	0.9
THKU-31	93	49.3	1.9
THKU-32	95	34	2.8
THKU-33	96	28.5	3.4
THKU-34	91	52.6	1.7
THKU-35	88	25.3	3.5
THKU-39	89	50.6	1.8
THKU-40	91	57.2	1.6
THKU-41	55	45.9	1.2
THKU-42	52	35	1.5
THKU-43	53	40	1.3
THKU-44	55	45.8	1.2
THKU-45	47	51.1	0.9
THKU-46	57	50.3	1.1
THKU-47	47	55.9	0.8
THKU-48	35	46.3	0.8
THKU-49	57	44.4	1.3
THKU-50	42	18	2.3
THKU-51	64	54.3	1.2
THKU-52	58	40.7	1.4
THKU-53	59	41	1.4
THKU-54	42	34.5	1.2
THKU-55	47	6.8	6.9

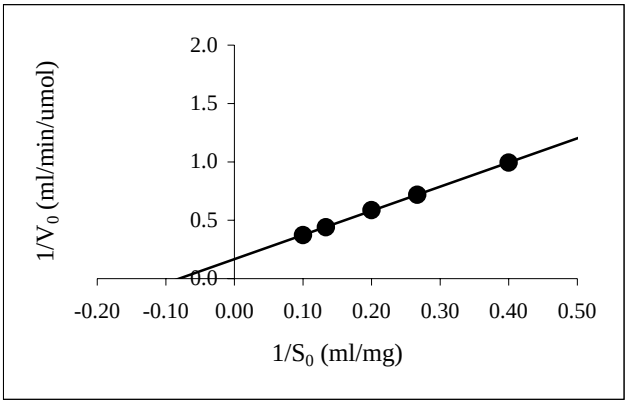
Appendix Table A3 (Continued))

Strain	Xylanase activity (U/ml)[a]		Ratio
	Xylan medium (A)	Xylose medium (B)	A/B
THKU-56	56	36.3	1.5
THKU-57	65	44.8	1.5
THKU-58	65	29.6	2.2
THKU-59	68	47.5	1.4
THKU-60	42	39.5	1.1
THKU-61	71	31.8	2.2
THKU-62	60	43.7	1.4
THKU-63	62	43.7	1.4
THKU-64	67	54.9	1.2
THKU-65	61	52.1	1.2
THKU-66	56	35.9	1.6
THKU-67	62	64.9	1.0
THKU-68	69	51.2	1.3
THKU-69	68	39.7	1.7
THKU-70	76	49.7	1.5
THKU-71	64	49.8	1.3
THKU-72	55	40.5	1.4
THKU-73	58	62.6	0.9
THKU-74	66	33.6	2.0
THKU-75	67	23.1	2.9
THKU-76	60	38.8	1.5
THKU-77	60	64.3	0.9
THKU-78	56	18.2	3.1
THKU-79	21	35.4	0.6
THKU-80	20	13.9	1.4
THKU-81	22	56.8	0.4
THKU-82	86	9.6	9.0

Appendix Table A3 (Continued)

Strain	Xylanase activity (U/ml)[a]		Ratio
	Xylan medium (A)	Xylose medium (B)	A/B
THKU-83	112	9	12.4
THKU-84	106	68	1.6
THKU-85	76	8.1	9.4
THKU-86	134	48.8	2.7
THKU-87	81	50.1	1.6
THKU-88	113	9.1	12.4
THKU-89	96	23.4	4.1
THKU-90	90	23.3	3.9
THKU-91	73	62.4	1.2
ATCC 44008	100	40.7	2.5
ATCC 46882	100	6.4	15.6

A



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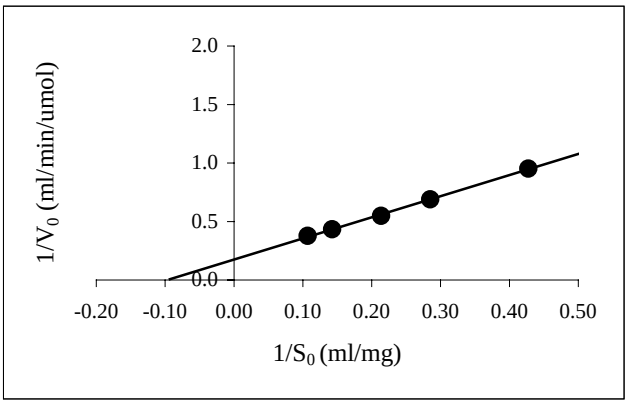

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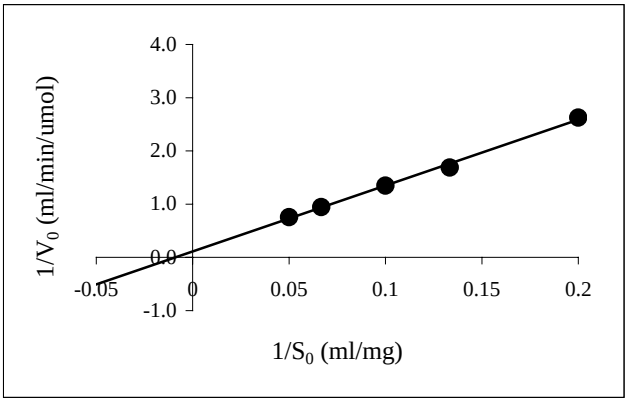
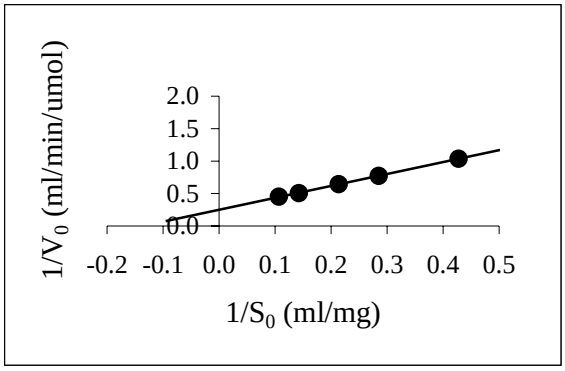


Appendix Figure A1 Lineweaver- Burk plot of xylanase from THKU-9 with soluble oat spelt xylan (A), oat spelt xylan (B) and insoluble oat spelt xylan (C).

A



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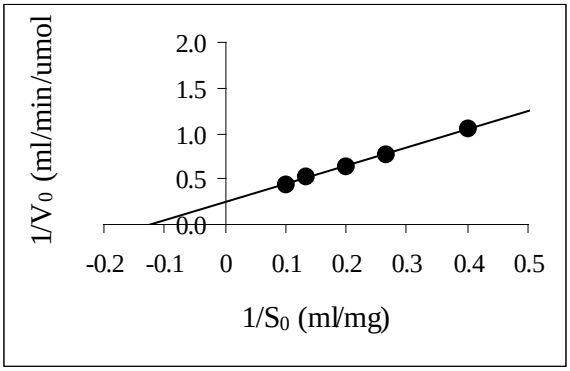

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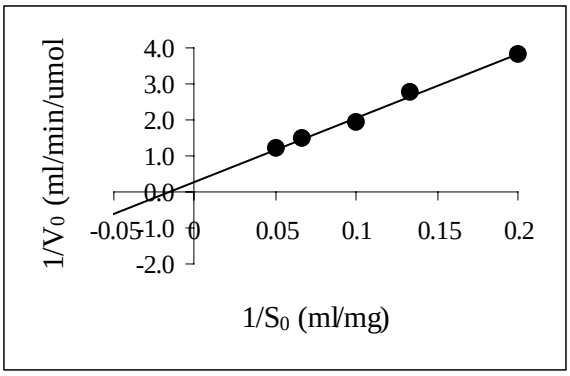
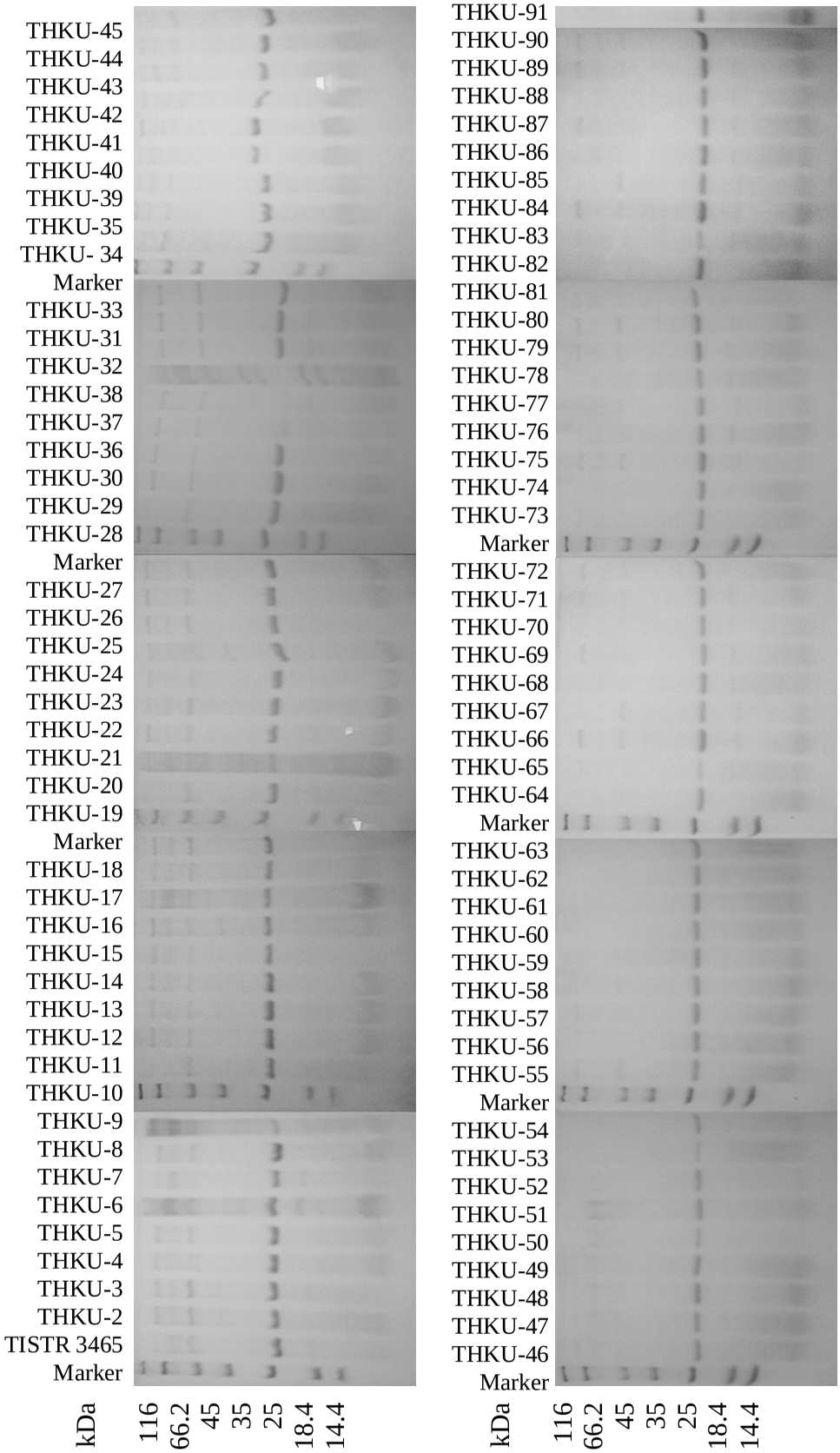
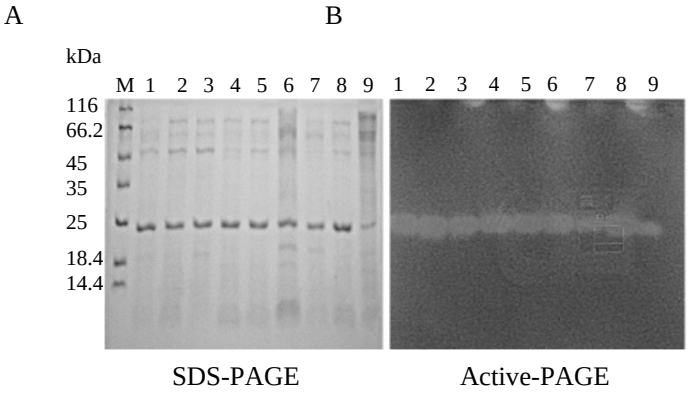


Appendix Figure A2 Lineweaver- Burk plot of xylanase from THKU-49 with soluble oat spelt xylan (A), oat spelt xylan (B) and insoluble oat spelt xylan (C).



Appendix Figure A3 SDS-PAGE profiles from crude enzyme produced by *T. lanuginosus* strains.



Appendix Figure A4 SDS-PAGE (A) and active-PAGE (B) of crude *T. lanuginosus* xylanases produced on culture medium.

Coomassie brilliant blue R-250 staining of gel. (A), Zymogram for detected xylanase activity (B).

Lane M, molecular weight calibration kit (SM0431 Fermentas INC, USA).

Lane 1, TISTR 3465

Lane 2, THKU-2

Lane 3, THKU-3

Lane 4, THKU-4

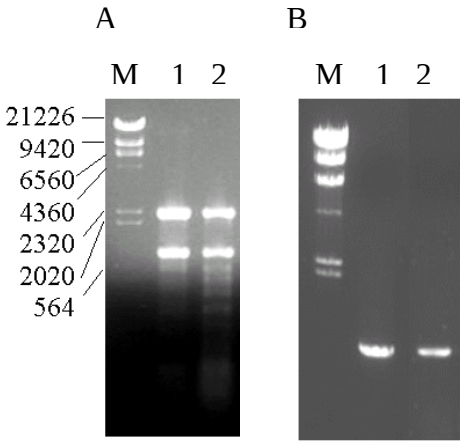
Lane 5, THKU-5

Lane 6, THKU-6

Lane 7, THKU-7

Lane 8, THKU-8

Lane 9, THKU-9



Appendix Figure A5 Formaldehyde agarose gel electrophoresis of total RNAs (A) and ethidium bromide-strained agarose gel containing RT-PCR products of xylanase genes (B) of *T. lanuginosus*.

M, lambda phage DNA digested with *Hind*III. Lanes 1-2, PCR product from THKU-9 and THKU-49, respectively. Total RNAs were separated in 1.5% agarose while RT-PCR products were separated in 1% agarose.

Appendix Figure A6 RAPD patterns of genomic DNA of selected strains of *T. lanuginosus* using Blend Taq Plus (A) and recombinant Taq (B) as DNA polymerase and using UBC 235, 241 and 280 as primers.

Lane 1, marker (lambda cut with *Hind*III&*Eco*RI)

Lane 2, *T. lanuginosus* THKU-56

Lane 3, *T. lanuginosus* TISTR 3465

Lane 4, *T. lanuginosus* ATCC 44008

Lane 5, *T. lanuginosus* THKU-30

Lane 6, *T. lanuginosus* THKU-33

Lane 7, *T. lanuginosus* THKU-49

Lane 8, *T. lanuginosus* THKU-77

Lane 9, *T. lanuginosus* THKU-86

Lane 10, *T. lanuginosus* THKU-85

Lane 11, *T. lanuginosus* ATCC 46882

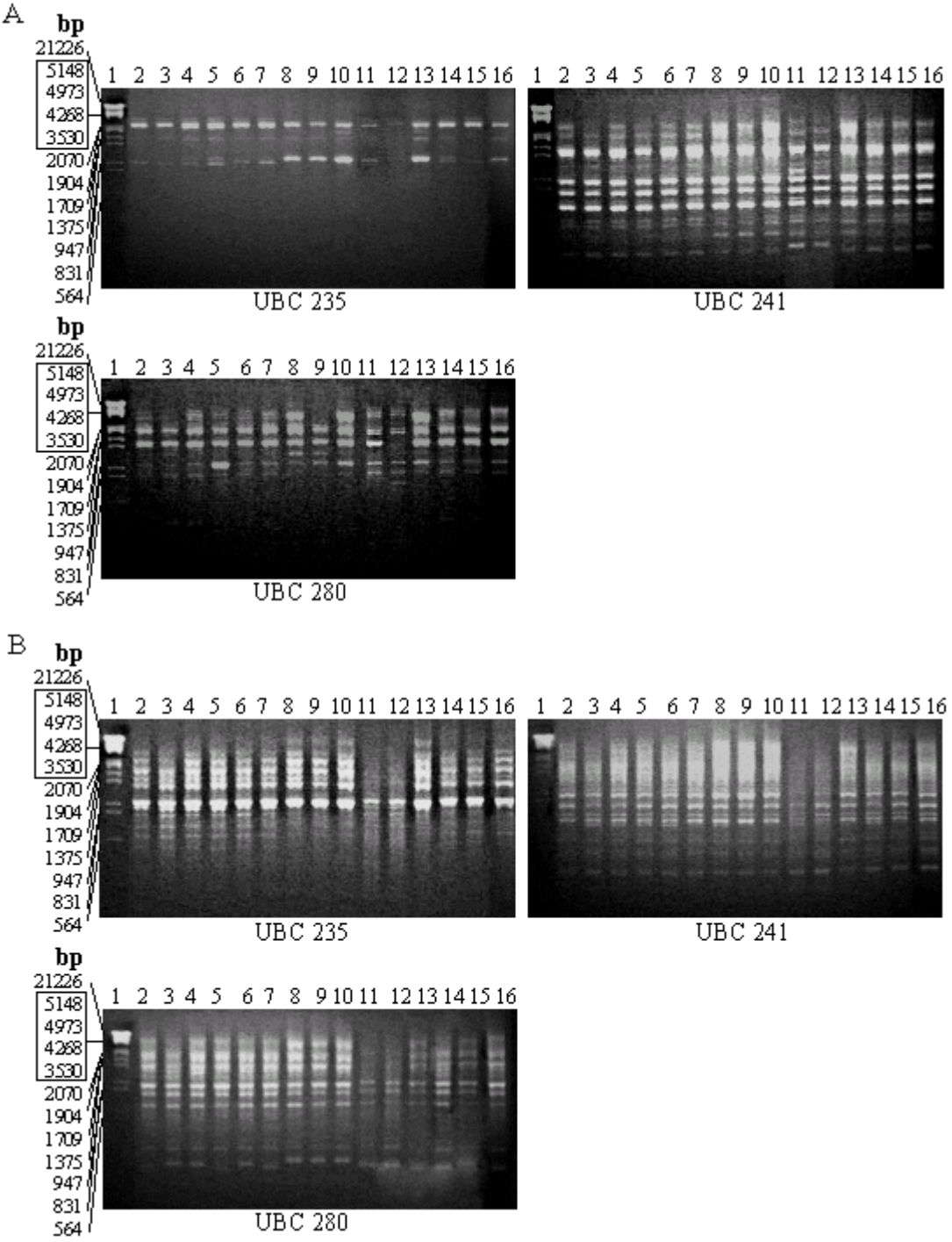
Lane 12, *T. lanuginosus* THKU-4

Lane 13, *T. lanuginosus* THKU-2

Lane 14, *T. lanuginosus* THKU-25

Lane 15, *T. lanuginosus* THKU-11

Lane 16, *T. lanuginosus* THKU-9


A
bp
21226
5148
4973
4268
3530
2070
1904
1709
1375
947
831
564
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
UBC 235
UBC 241
UBC 280
B
UBC 235
UBC 241
UBC 280

Appendix B

Reagents

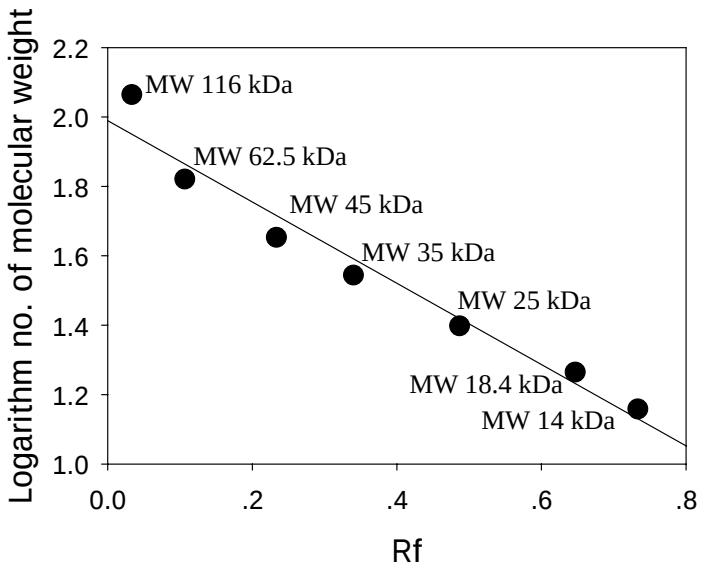
Composition of reagents of SDS-PAGE were as follows:

1. Acrylamide-bis Stock, 100 ml:	30% acrylamide
	0.8% N,N'-methylene bis acrylamide
2. 2x SDS-Running Buffer, 100 ml:	0.75 M Tris-HCl, pH 8.8
	0.2% SDS
3. 2x SDS-Stacking Buffer, 100 ml:	0.25 M Tris-HCl, pH 6.8
	0.2% SDS
4. 5x SDS-Electrode Buffer, 100 ml:	0.125 M Tris-HCl, pH 8.3
	0.96 M glycine
	0.5% SDS
5. TEMED	full strength
6. 2x SDS-SAB	0.125 M Tris-HCl, pH 6.8
	4% SDS
	20% glycerol
	0.002% Bromphenol blue
	10% mercaptoethanol
	(add just before use)

Coomassie brilliant blue staining for protein from PAGE

Compositions of coomassie brilliant blue stain and destain solution were as follows:

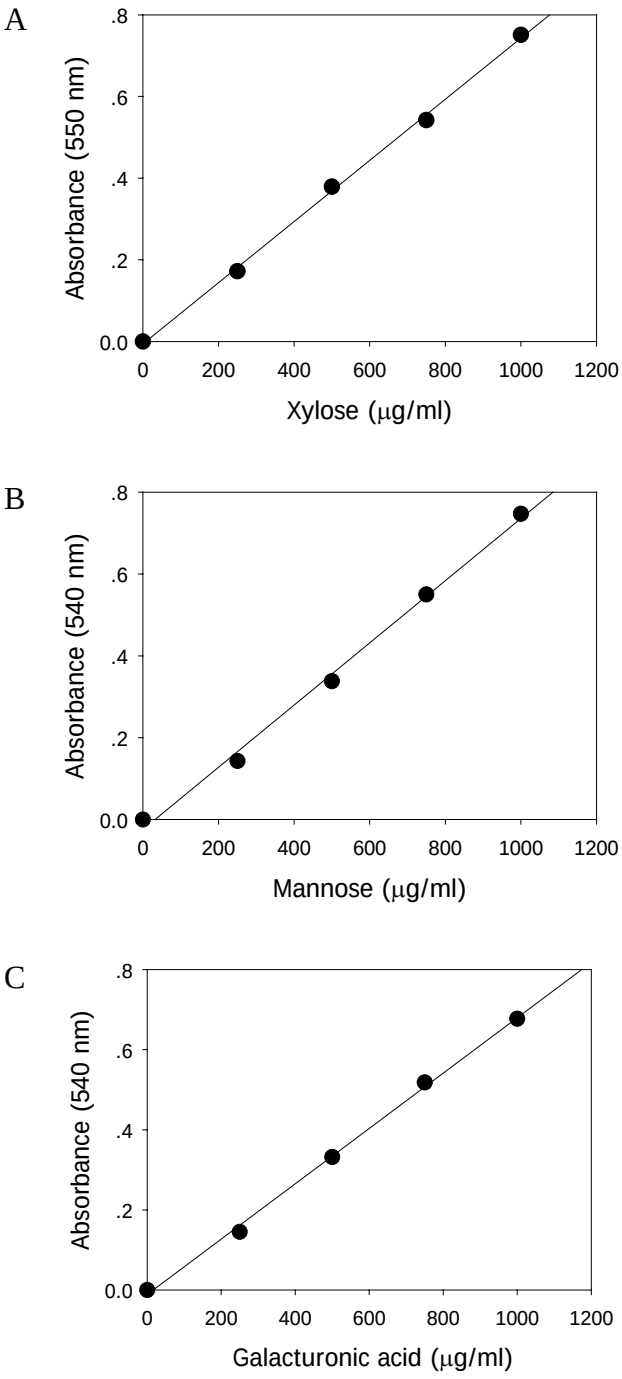
1. Stain:	450 ml water
	500 ml methanol
	75 ml acetic acid
	5 g Coomassie brilliant blue
2. Destain I:	1.0 litre water
	1.0 litre methanol
	200 ml acetic acid
3. Destain II:	150 ml methanol
	225 ml acetic acid
	bring to 1.0 litre with water



Appendix Figure B1 Standard curve of molecular weight standard protein from SDS-PAGE.

3,5-dinitrosalicylic acid reagents (DNS) solution

(1) Added 200 g potassium sodium tartrate to 500 ml of 2% NaOH solution
(2) Added 0.5 g sodium sulfite
(3) Added 10 g 3,5-dinitrosalicylic acid
(4) Added 2 g phenol
(5) Adjust volume of solution to 1 liter



Appendix Figure B2 Standard curve of xylose (A), mannose (B) and galacturonic acid (C) analysis by DNS.

Lowry-Folin protein assay reagents;

Solution A: 1% (w/v) $CuSO_4.5H_2O$

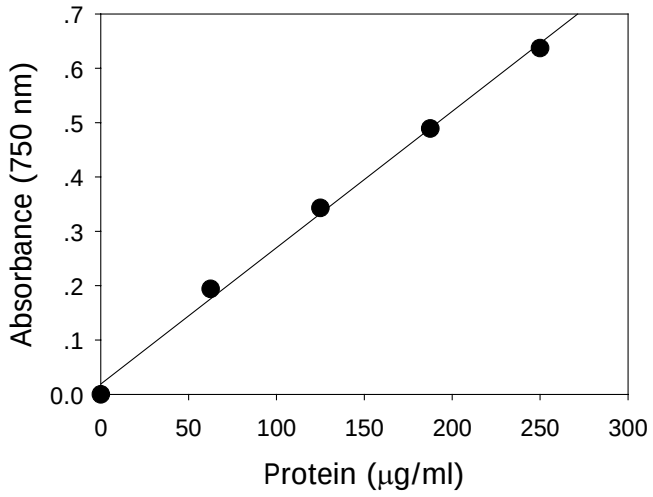
Solution B: 2% (w/v) Sodium potassium tartrate

Solution C: 0.2 M Sodium hydroxide

Solution D: 4% (w/v) Sodium carbonate

Solution E: Mixed 49 ml of Solution C with 49 ml of Solution D. Added 1 ml of Solution A and 1 ml of Solution B. (Freshly prepare before use)

Solution F: Diluted 1:1 (v/v) Folin-Ciocalteau reagent (Merck®) with distilled water



Appendix Figure B3 Standard curve of protein (bovine serum albumin) assay by Lowry-Folin method.

Reagent for Analysis of ITS rDNA

DNA extraction buffer

Cetyltrimethylammonium bromide (CTAB)	1 g
NaCl	4.1 g
1M Tris-HCl (pH 8.0)	5.0 ml
0.5 M EDTA (pH 8.0)	2 ml
Add distilled water to	1 liter
Sterilized by autoclaving at 121 °C 15 min	
Add 2-mercaptoethanol before used	1 ml

PCI (Phenol: Chloroform: Iso amyl alcohol)

TE saturated phenol	50 ml
CIA (Chloroform: Isoamyl alcohol)	50 ml

TE saturated phenol

Phenol	500 g
1M Tris-HCl (pH 8.0)	500 ml
8-hydroxyquinoline	0.5 g
Lower phase was collected.	

CIA (Chloroform: Isoamyl alcohol)

Chloroform	24 ml
Isoamyl alcohol	1 ml

1% agarose gel

Agarose	0.8 g

TBE buffer/TAE buffer	80 ml
10 mg/ml Ethidium bromide	4 μl

TE buffer

Tris-HCl	10.8 g
EDTA-2Na	0.83 g
pH was adjusted to 8.0 with HCl	
Add distilled water to	1,000 ml

TBE buffer (10X)

Tris-base	27 g
H_3BO_3 (Boric acid)	13.75 g
EDTA-2Na	2.32 g
Add distilled water to	250 ml

TAE buffer (10X)

Tris-base	12.1 g
100% acetic acid	2.9 ml
0.5 M EDTA (pH 8.0)	5 ml
Add distilled water to	250 ml

Loading dye

Bromophenol blue	1.25 g
Xylene cyanol FF	1.25 g
Glycerol	15 g
Add distilled water to	50 ml

CIRRICULUM VITAE

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SCHOLARSIP/AWARDS : In 2006, 2nd prize of poster section in 8th International Mycological Congress, Cairns, Australia

: In 2002, Royal Golden Jubilee Ph.D. program from Thailand Research Fund

: In 2001, Taguchi Prize award for outstanding master degree thesis

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