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β-xylanase from *Thermomyces lanuginosus* and its Biobleaching Application

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Abstract: *Thermomyces lanuginosus* is thermophilic fungus in which was isolated from widespread material. A high number of this fungus was found in composts especially mushroom composts. This fungus has been reported to produce a high level xylanase when cultivated in the medium containing xylan and corn cob as a carbon source. Various strains of *T. lanuginosus* produced a single xylanase with molecular masses in range of 22.0 to 29.0 kDa. Pure β-xylanase obtained from various strains of this fungus exhibited highly stability at high temperature and wide pH range. The optimal temperature and optimal pH of pure β-xylanase from various strains of *T. lanuginosus* have been reported in range of 60-75°C and pH 6.0-7.0, respectively. The great thermal stability was resulting from the present of hydrophilic amino acid on beta sheet of the surface of xylanase structure. Moreover, the relatedness between high and low xylanase producing strains can be distinguish by random amplification of polymorphic DNA (RAPD). Based on nucleotide sequences and *T. lanuginosus* xylanase gene has been classified to be a member of family 11 (formerly known as cellulase family G) glycosyl hydrolases. This enzyme was endo-type xylanase having main product are xylose and xylobiose. The expression of xylanase gene from *T. lanuginosus* was achieved in *Escherichia coli* and methylotrophic yeast *Pichia pastoris*. The ability of *T. lanuginosus* in which produced large amount of high thermos stable xylanase has made this fungus to be a source of xylanase production for biobleaching in pulp and paper process.

Key words: Xylanase, *Thermomyces lanuginosus*, biobleaching

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. 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). Hetero-β-1,4-D-xylans constitute the major portion of the hemicellulose in terrestrial plants (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). 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).

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*., 1994; Georis *et al*., 2000; Mesta *et al*., 2001; Turunen *et al*., 2001, 2002; Liu *et al*., 2002) 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). *Thermomyces lanuginosus* DSM 5826 produces a high level of cellulase-free, thermostable

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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.

CHARACTERISTICS OF *THERMOMYCES LANUGINOSUS*

Thermophilic fungus *Thermomyces lanuginosus* (formerly known as *Humicola lanuginosa*) was classified as a Deuteromycetes (imperfect fungus) that was unicellular or septate and reproduces asexually by forming aleurioconidia. The colonies of fungus grew rapidly at 45-50°C within 2 days. Initially, the colonies appeared white but soon turn grey or greenish-grey and mature colonies appeared dark brown to black. The colonies have a little various colour depend on isolated source (Fig. 1a) (Khucharoenphaisan and Kitpreechavanich, 2006). Immature conidial spore were colorless and smooth walled and turned dark brown and globe as mature (Fig. 1b). Mature aleuriospores showed reticulate sculpture with a diameter of 5.5-12 μm. The aleuriopores were

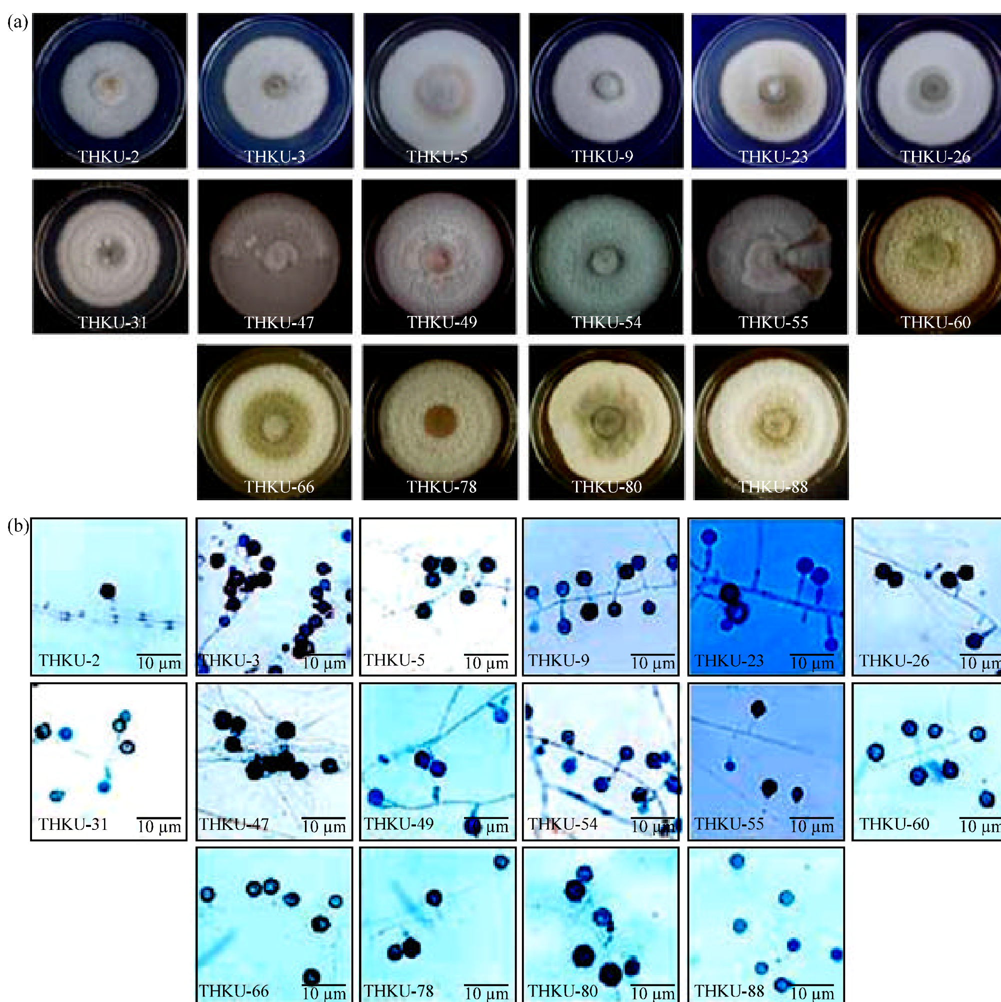

Fig. 1: Colony (a) and aleuriospores from light microscope (b) of some strains of *T. lanuginosus* (Khucharoenphaisan and Kitpreechavanich, 2006)

straight or curved, colorless or brown and smooth (Cooney and Emerson, 1964). *T. lanuginosus* was widely distributed (Emerson, 1968) and has been isolated in various location and ecology (Singh *et al.*, 2000a; Hoq and Deckwer, 1995; Hoq *et al.*, 1994). Large amount of this fungus was found in mushroom compost (Khucharoenphaisan and Kitpreechavanich, 2004). It grew at temperature of 30°C to 60°C with an optimum growth temperature of 50°C. The optimum pH for growth of most strains was 6.5.

The genetic diversity 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 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 appropriated for phylogenetic comparisons within this species. A homology search by BLAST (National Center for Biotechnology Information, USA) has shown that the ITS region and the 5.8 S rDNA sequences were highly conserved in others thermophilic fungi, suggesting a possible recent taxonomic divergence in this group of fungi.

The Deuteromyces genus *Thermomyces* was closely related to *Humicola* and has been combined with it by several authors. However, it could be distinguish 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, thick walled, with wrinkled surface, 6-10 µm. The genus of *Thermomyces* containing four species was *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* by a verrucose aleurioconidia 10-17 µm diameter. In case of *T. ibadanensis* has smooth walled aleurioconidia 4-8 µm diameter. *T. stellatus* has aleurioconidia in which was singly on the tip of the aleuriophore were dark brown and stellate with maturity, 5.3 µm diameter and 7.6 µm in length.

XYLANOLYTIC ENZYMES

Xylan was a complex structure of the hemicelluloses in wood. The two main enzymes using for de-structure of the xylan backbone were β-xylanase and β-xylosidase. β-xylanases hydrolyze randomly on the backbone of xylan to make shorter chain oligomers as xylooligosaccharides xylobiose and monosaccharide as xylose. β-xylosidases were essential for the complete breakdown backbone of xylan to xylose at the non-reducing end (Poutanen and Puls, 1988). To complete hydrolysis of xylan, debranching enzymes such as α-arabinofuranosidase, α-glucuronidase, acetylxylan esterase and hydroxycinnamic acid esterases that cleave side chain residues from the xylan backbone were required to release the substituents on the xylan backbone. A total hydrolysis of xylan to monosaccharide was achieved from this reaction (Fig. 2). All these enzymes act cooperatively to convert xylan to its constituents (Sunna and Antranikian, 1997).

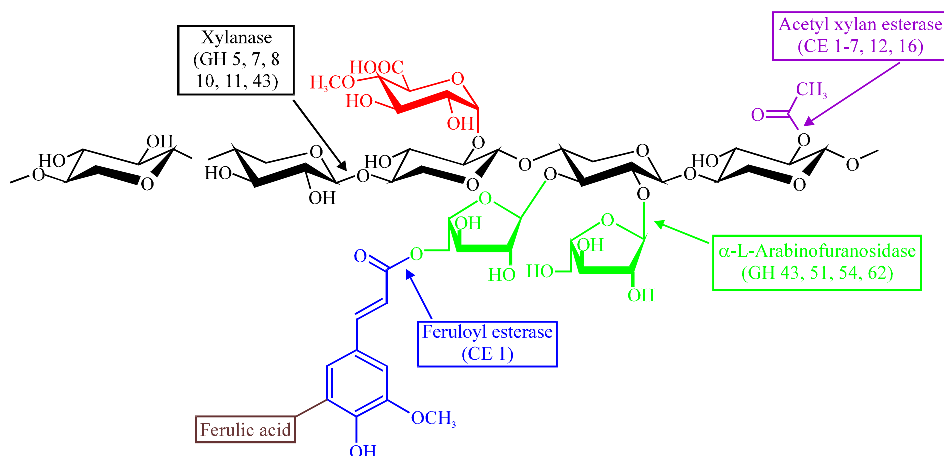

Fig. 2: Xylanolytic enzymes involved in the degradation of xylan (Dodd and Cann, 2009)

PRODUCTION OF β-XYLANASE BY *T. LANUGINOSUS*

T. lanuginosus strain SSBP has been reported to be the best producer of xylanase with an activity of 3575 U mL^{-1} than that of *T. lanuginosus* strains DSM 5826 and ATCC 46882 with xylanase activity of 2172 and 2726 U mL^{-1}, respectively 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 (Khucharoenphaisan *et al*., 2010a). Corncobs were found to be the most effective substrate for xylanase production among various lignocellulosic substrates such as corn leaf, wheat bran, wheat straw, barley husk and birchwood xylan (Singh *et al*., 2000a, c; Gomes *et al*., 1993a; Purkarthofer and Steiner, 1995; Bennett *et al*., 1998). The strain of THKU-86 produced high level of xylanase in the medium containing xylan as a carbon source whereas strain THKU-9 produced high level of xylanase in the medium containing xylose as a carbon source (Khucharoenphaisan *et al*., 2009). A shaking speed has most effect on xylanase production by this fungus. At 120 rpm was provided the optimal conditions for enzyme formation whereas decreased shaking speed to 100 rpm resulting in reduced dramatically enzyme production. At high shaking speeds of 150-250 rpm, the adversely affect was occurred on enzyme production due to greater hypha branching, mycelium fragmentation and early sporulation (Purkarthofer *et al*., 1993). The production of xylanase by *T. lanuginosus* THKU-49 was also studied in shaking cultivation at 45°C for 7 days using 1% oat spelt xylan as a carbon source. Xylanase production was rapidly increased during 4-day cultivation, yielded 45.7 U mL^{-1}. It then increased to some extent, 62.7 U mL^{-1} after 7 day cultivation (Khucharoenphaisan *et al*., 2010b).

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). This technique has been used for molecular genetic studies as it was a simple and rapid method for determining genetic diversity and similarity in various organisms. It also has the advantage that prior knowledge of the genome under research was not necessary (Yoon and Kim, 2001). Khucharoenphaisan *et al*. (2009) reported that this fungus could be difference into two groups based on their ability to produce xylanase using xylose as sole of carbon source. The phylogenetic analysis obtained from random amplified polymorphic DNA (RAPD) pattern using primer UBC 24 (5'-GCCCGACGCG-3') pointed to greater diversity of high (cluster B) medium (cluster A) and low (cluster C) xylanase producing strains using xylose as a carbon source as shown in Fig. 3. This result be in line with the formerly study of Singh *et al*. (2000b) whose examined the

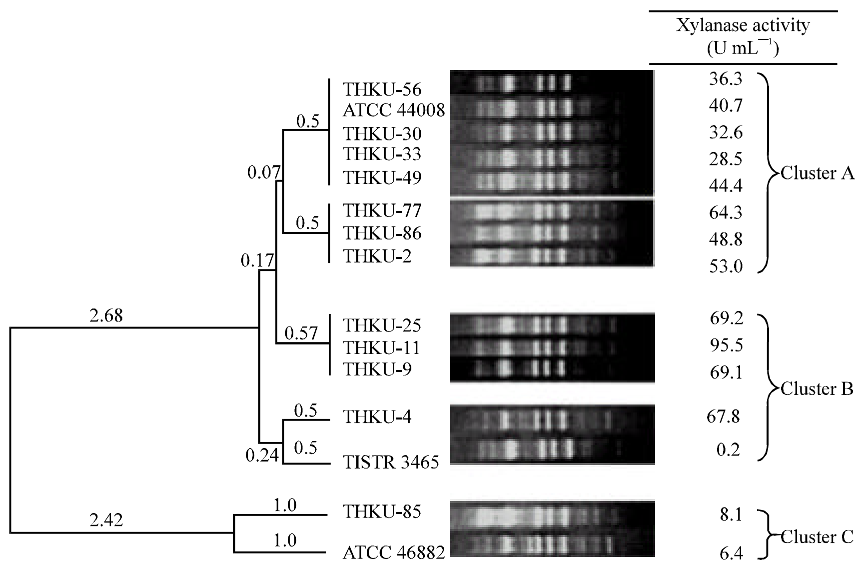

Fig. 3: Dendogram indicating relationships of *T. lanuginosus* strains obtained with the primer UBC 241 of xylanase producing strains with xylanase activity obtained from 5-day cultivation using xylose as a carbon source (Khucharoenphaisan *et al*., 2009)

phylogenetic properties of eight *T. lanuginosus* strains and found relationship between the RAPD pattern and levels of xylanase produced. It could be establish using certain primer UBC 241. Strains DSM 5826 and SSBP that produced xylanase of 32000 and 59600 nkat mL^{-1}, respectively, were apparently closely related while strains ATCC 28083 and ATCC 58160 that produced xylanase of 9000 and 6300 nkat mL^{-1}, respectively, also showed a close relationship. However, not all strains producing low levels of xylanase were 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.

REGULATION OF *T. LANUGINOSUS* XYLANASE SYNTHESIS

Xylanases has been shown to be inducible enzymes but rare constitutive xylanase expression has also been reported. Addition of inducer in the medium showed higher enzyme production than that of un-induced condition. In general, the xylanase induction was a complex phenomenon and the level of response to an individual inducer varied depend on the organisms. The substrate derivatives and the enzymatic end products might often play a key positive role on the induction of xylanases. However, they could also act as the end-product inhibitors, possibly at much high concentrations (Kulkarni *et al*., 1999).

Generally, xylanases were induced in most microorganisms during growth on substrates containing xylan (Purkarthofer and Steiner, 1995; Khucharoenphaisan *et al*., 2010a; Ahmed *et al*., 2003). Xylan having high molecular mass could not penetrate the cell wall. Thus, hydrolysates of xylan such as xylose, xylobiose, xylooligosaccharides and heterodisaccharides could play a key role in the regulation of xylanase biosynthesis (Kulkarni *et al*., 1999). Xylanase produced by *T. lanuginosus* was shown to correspond to an induction or repression mechanism with *Aspergillus sydowii* MG49 (Ghosh and Nanda, 1994). A low level of xylanase was constitutively formed without the presence of an inducing substance. Xylanase production of *T. lanuginosus* DSM 5826 was induced by D-xylose and having the strongest effect (1225 nkat mL^{-1}) indicating that D-xylose was the natural inducer. The highest xylanase activity (7100 nkat mL^{-1}) of *T. lanuginosus* DSM 5826 was found in xylan-grown culture whereas very low activity (3.5 nkat mL^{-1}) was found in glucose-grown culture (Purkarthofer and Steiner, 1995). This was differenced from the report of Khucharoenphaisan *et al*. (2010a) who reported that xylan was the best inducer for xylanase production in cell culture of *T. lanuginosus* TISTR 3465. This indicated that these two strains might have different induction mechanism. The induction mechanism of xylanase from *T. lanuginosus* TISTR 3465 by xylose and xylan in resting cell was also studied by Khucharoenphaisan *et al*. (2010a). With the sequential addition of xylan, xylanase formation was delayed but lasted longer. The 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). Hoq *et al*. (1994) reported that 10 g L^{-1} birch wood xylan and 30 g L^{-1} corncob induced xylanase synthesis in cultured growth of *T. lanuginosus* RT9 in which isolated in Bangladesh with activities of 8725 and 7110 nkat mL^{-1}, respectively. In contrast, Xylose (5 g L^{-1}) repressed xylanase synthesis of this fungus with activity of 19 nkat mL^{-1}. Moreover, the xylanase formation using 5 g L^{-1} glucose and non carbon source having 7 and 12 nkat mL^{-1}, respectively, were similar to xylanase level using xylose as a carbon source. Xiong *et al*. (2004) reported that 15 g L^{-1} of substrates such as xylan and xylose stimulated xylanase formation of *T. lanuginosus* DSM 10635 with activities of 497 and 83.2 U mL^{-1}, 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^{-1}, respectively.

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; Khucharoenphaisan *et al*., 2009; Khucharoenphaisan *et al*., 2010a). 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.

CHARACTERIZATION OF *T. LANUGINOSUS* XYLANASE

Xylanase of *T. lanuginosus* has been purified from a number of strains and used for characterization of enzyme. The molecular mass of the enzyme was found to be in the

Table 1: Biochemical properties of β-xylanase produced by *T. lanuginosus*

Strains	MW (kDa)	Optimal pH	Optimal temp. (°C)	Half-life at 70°C (min)	K_m (mg mL^{-1})	Reference
DSM 10635	25.5	6.5	70	40 (pH 6.5)	3.85	Xiong *et al.* (2004)
SSBP	24.7	6.5	70	232 (pH 6.5)	3.26	Singh *et al.* (2000a, b)
SSBP	23.6	6.5	70-75	-	-	Lin *et al.* (1999)
DSM 5826	25.5	7	60-70	201 (pH 6.5)	7.3	Cesar *et al.* (1996)
ATCC 46882	25.7	6-6.5	75	30 (pH 6.0)	-	Bennett *et al.* (1998)
THKU-49	24.9	6.3	66	336 (pH 6.0)	7.3	Khucharoenphaisan *et al.* (2008a)
THKU-9	24.9	6.2	66	186 (pH 6.0)	10.3	Khucharoenphaisan *et al.* (2008a)
(Griffon and Maublanc) Bunce	29	6	65	-	0.91	Anand *et al.* (1990)
195	22	-	-	-	-	Gaffney *et al.* (2009)

range of 22.0-29.0 kDa and pI value 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*., 1984; Bakalova *et al*., 2002; Xiong *et al*., 2004; Khucharoenphaisan *et al*., 2010a, b). The optimum temperature and pH of purified xylanase from various strains have been reported to be in the range of 60-75°C and 6.0-7.0, respectively (Table 1). These values were similar to those observed in crude extracts of xylanase (Lischnig *et al*., 1993; Singh *et al*., 2000a, c; Gomes *et al*., 1993a, b; Alam *et al*., 1994; Khucharoenphaisan *et al*., 2010b). The thermal stability was considered as an major characters xylanase from *T. lanuginosus*. The xylanase of *T. lanuginosus* strain SSBP was previously reported to be the most stable (half-life = 337 min at 70°C), whereas DSM 5826 strain and other strains showed lesser stability. The xylanase of *T. lanuginosus* strain SSBP retained its full activity at temperatures up to 65°C and 45% of its activity after 30 min at 100°C (Singh *et al*., 2000d). Up to date, xylanase produced by *T. lanuginosus* THKU-49 has the highest thermostability with half-life of 336 min. Moreover, this enzyme was more stable in phosphate buffer than that in citrate buffer. When the buffer concentration increased, the half-life of the enzyme decreased significantly. The high thermostability of this enzyme because of single substitution (V96G) with signal peptide was occurred at outer surface of the enzyme structure as shown in Fig. 4 (Khucharoenphaisan *et al*., 2008a).

The xylanase activity obtained from *T. lanuginosus* THKU-49 was inhibited by Mn^{2+}, Sn^{2+} and EDTA. The xylanase showed high activity towards soluble oat spelt xylan but it exhibited low activity towards insoluble oat spelt xylan. No activity was found to carboxymethylcellulose, avicel, filter paper, locust bean gum, cassava starch and *p*-nitrophenyl β-D-xylopyranoside. The apparent K_m value of the xylanase on soluble oat spelt xylan and insoluble oat spelt xylan was 7.3±0.236 and 60.2±6.788 mg mL^{-1}, respectively. 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

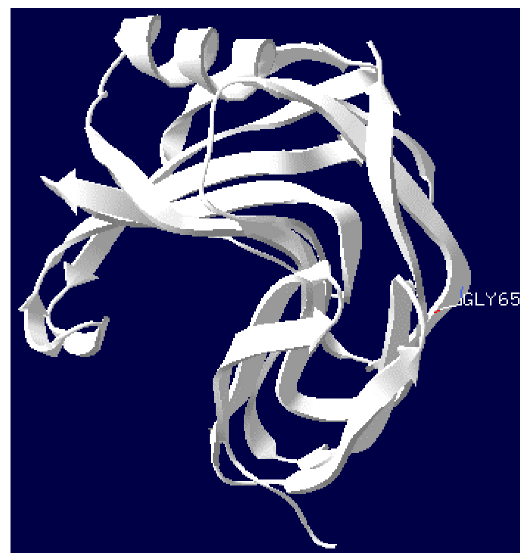

Fig. 4: Three-dimension structure of xylanase produced by *T. lanuginosus* THKU-49 without signal peptide (Khucharoenphaisan *et al*., 2008a)

xylose and xylobiose from beechwood 4-O-methyl-D-glucuronoxylan and in addition also released an acidic xylooligosaccharide from 4-O-methyl-D-glucuronoxylan. The hydrolysis of oat spelt xylan yield mainly xylobiose and xylose as end products. However, the xylanase could not release xylose from substrate as xylobiose. This suggested that it was an endo-xylanase (Khucharoenphaisan *et al*., 2010b).

The Central Composite Design (CCD) was a statistic method wildly used in many application including enzyme technology (Heck *et al*., 2006). The CCD had been used for optimization of culture condition (Couto *et al*., 2006) and also optimal temperature and optimal pH for maximum enzyme activity (Khucharoenphaisan *et al*., 2008b). The maximum xylanase activity of *T. lanuginosus* THKU-49 was obtained from CCD analysis was 66°C and pH 6.3 (Khucharoenphaisan *et al*., 2008b). The temperature stability of the purified xylanase from various strains differed somewhat depending on the experimental conditions (Table 1). 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 1).

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.

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 increasing of half-life for 2-time (from 89 min to 168 min at 70°C) and 2.5-time (from 89 min to 215 min at 70°C), 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, 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 (Stephens *et al*., 2007). Evolution in nature gives also rise to *T. lanuginosus* producing enzymes with different properties including their thermostability (Khucharoenphaisan *et al*., 2008a).

Molecular and structure of xylanase from *T. lanuginosus*. The complementary DNA (cDNA) of *T. lanuginosus* xylanase containing 989 bp and included open reading frame (ORF) 615 bp was firstly reporteded by Schlacher *et al*. (1996). An ATG codon (starting site) was identified on 36 bp downstream of the 5' of the cDNA. The ORF of xylanase gene encoding for 225 amino acids. 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 (Singh *et al*., 2003). The multiple alignment of amino acid sequence of xylanase gene obtained from highly thermostable producing strain (THKU-49) and low thermostable producing strain (THKU-9 and DSM 5826) showed some differentiation among these strains (Fig. 5). One amino acid differentiation was found at position 96. Valine (V) was found in both of low thermostable producing strain while valine was replaced by glycine (G) in highly thermostable producing strain. This may imply that they have some modification in this fungus (Khucharoenphaisan *et al*., 2008a).

The phylogenetic relationship between fungal xylanase was studied. Based on amino acid sequences,

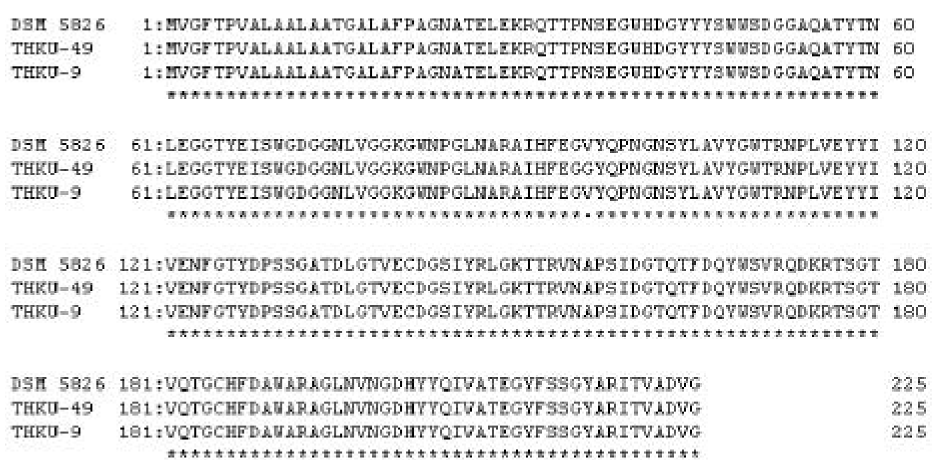

Fig. 5: Alignment of amino acid sequence of xylanase genes from different strains of *T. lanuginosus* strains. Alignment characters are indicated as follows: '*' indicates position with a conserved amino acid residue; '.' indicates position with a different amino acid residue (Khucharoenphaisan *et al*., 2008a)

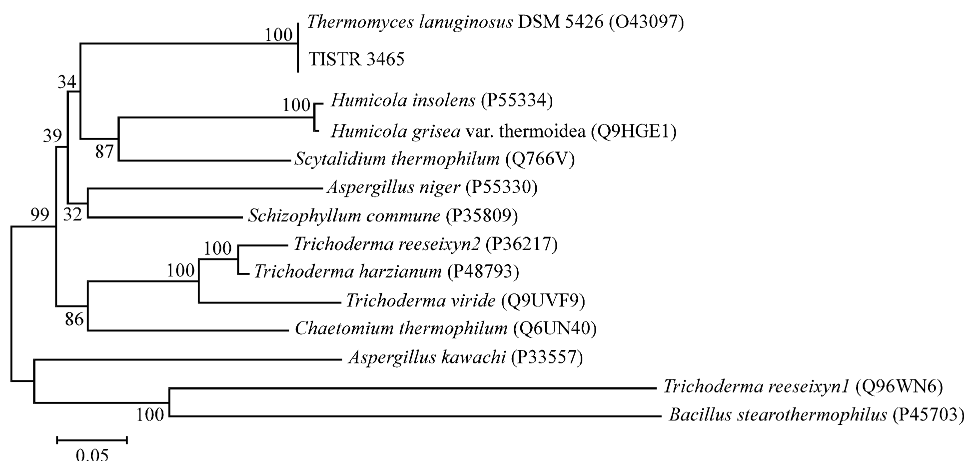

Fig. 6: Phylogenetic tree of amino acid sequence analysis of xylanase of thermophile, mesophile and thermophilic fungi constructed by Neighbor-joining method from MEGA 4 program. Scale bar shown distance values under the tree means 0.05 substitutions per amino acid position. Bootstrap analyses were performed with 1000 re-samplings and percent values are shown at the branching points (Khucharoenphaisan *et al*., 2010a)

xylanase from *T. lanuginosus* TISTR 3465 is identical to *T. lanuginosus* DSM 5826 and closely related to other thermophilic fungi especially *Humicola* sp. and *Scytalidium thermophilum* as shown in Fig. 6.

Xylanase produced by *T. lanuginosus* strain was folded into a single ellipsoidal domain. The overall structures of xylanase were similar and have been described as a partially closed right hand (Fig. 4). The active site was located at the concave side of the cleft (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).

Xylanase gene from *T. lanuginosus* has been expressed in *Escherichia coli* but the expression level was lower than that of original host. Most expressed xylanase was found as inclusion body in the cytoplasm of the cell. The absence of post translational modification such as glycosylation in *E. coli* and intracellular accumulation of recombinant xylanase have been suggested to be the key reason (Singh *et al*., 2003). The xylanase gene incorporating the secretion signal sequence of *T. lanuginosus* DSM 5826 was functionally expressed in *E. coli* but extracellular enzyme activity was not reported (Schlacher *et al*., 1996). Subsequently the recombinant *E. coli* was found to produce up to 240 U mL^{-1} of intracellular xylanase when induced by 0.1 mM isopropyl thiogalactoside (Singh *et al*., 2003). Xylanase gene (*xynA1*) including signal peptide from *T. lanuginosus* DSM 5826 was synthesized to construct the expression vector pHsh-*xynA1*. After optimization of mRNA secondary structure in the translation initiation, the expression level was increased from 1.3 to 13% of total cell protein. Maximum xylanase activity of 47.1 U mL^{-1} was obtained from cellular extract (Yin *et al*., 2008) The expression of xylanase gene from *T. lanuginosus* THKU-49 in *E. coli* has been compared between with and without 31 amino acid signal peptide from original but the expression level was not different. Recently, the using the methylotrophic yeast *Pichia pastoris* as a host is particular interesting in enzyme expression. A distinct advantage of eukaryotic expression host is capacity to facilitate the post translation modification of enzyme. Highly efficient expression of xylanase gene from *T. lanuginosus* IOC-4145 was achieved in *P. pastoris* under the control of the AOX1 promoter. The secretion level of recombinant XynA was in range of 90 to 126 U mL^{-1} after 90 h induction. The maximum expression of recombinant XynA was occurred after optimization by factorial design and showed the enzyme activity of 360 U mL^{-1} (Monica *et al*., 2003). The xylanase gene from *T. lanuginosus* 195 was also successfully expression in *P. pastoris*. However, maximum xylanase activity of 26.8 U mL^{-1} was obtained after 120 h induction of the recombinant culture without optimization of the condition (Gaffney *et al*., 2009). The enhancing of recombinant xylanase production in eukaryotic host may manipulation of codon usage. The signal sequence peptide also has been affected on the heterologous expression (Ghosalkar *et al*., 2008). It is anticipated that the xylanase expression level will be identical to original production in thermophilic fungus *T. lanuginosus*.

BIOLOGICAL APPLICATION OF XYLANASES IN PULPS

Among hemicellulytic enzymes obtained from *T. lanuginosus*, xylanase has widely use in biotechnological applications especially pulp and paper industry. The xylanase of *T. lanuginosus* belong to family 11 and found to be very efficient in biobleaching. Therefore, the high thermostable xylanase of *T. lanuginosus* was suitable for high temperature processes. The low molecular weight of this enzyme has assisted in penetrating the enzyme into the interior part of the fiber, resulting in removal of lignin compound from the pulp (Beg *et al*., 2001). There were two hypotheses about the role of xylanases in biobleaching process. In the first, the xylanases act on the xylan precipitated on the lignin (Viikari *et al*., 1994). It is able to remove xylan in which was precipitated at the end of the cooking stage. This action would leave lignin to the compounds employed in the bleaching of cellulose pulp. The second hypothesis was inhibited lignin to form complexes with polysaccharides such as xylan during the kraft process (Buchert *et al*., 1992). The xylanases acted also by cleaving the interaction between the lignin and xylan resulting on open the structure of the cellulose pulp. Thus low amount of chemical could increase brightness of pulp (Paice *et al*., 1992).

Xylanase was applied as a bleaching agent to reduce amount of chlorine required for increasing brightness of kraft and sulfite pulp which was produced from sugar cane bagasse, eucalyptus and beech (Manimaran *et al*., 2009). A diagrammatic flowchart involving xylanase in bleaching technology was represented in Fig. 7. It showed excellent results by enhance the extractability of lignin in pulp bleaching process (Viikari *et al*., 1994). Several studies have been reported about the application of xylanase in biobleaching of softwood and hardwood (Khucharoenphaisan *et al*., 2001; Oakley *et al*., 2003). However, studies on biobleaching of non-woody plant pulps such as wheat straw and bagasse pulp also has been reported (Li *et al*., 2005; Manimaran *et al*., 2009; Christopher *et al*., 2005). 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). The brightness of bagass pulp was improved by two units with 50 U gram pulp^{-1} of crude xylanase obtained from *T. lanuginosus* SSBP (Manimaran *et al*., 2009) as compared to xylanase obtained from *T. lanuginosus* ATCC 46882 and ATCC 36350 (Christopher *et al*., 2005). The brightness of the wheat straw pretreated with xylanase of *T. lanuginosus* CBS 288.54 showed 7.8 points increase (Jiang *et al*., 2006). Xylanase from *T. lanuginosus* TISTR 3465 (*Humicola lanuginosa*) exhibited promising result when applied as prebleaching agent to paper mulberry pulp and eucalyptus pulp. Each pulp was prebleached at 50°C for 3 h with *T. lanuginosus* TISTR 3465 β-xylanase obtained from solid state culture. In case of eucalyptus pulp, the enzyme treatment resulted in 1.4 unit reduction in kappa number and increase in brightness value of 5.3 points. The combination of enzyme treatment and peroxidase extraction resulted to reduce the kappa numbers by 5.7 unit and have brightness value of 17.4 points. In contrast, the enzymes could not increase the reduction of kappa no. and brightness of paper mulberry pulp with or without peroxide extraction. Therefore, the effectiveness of *T. lanuginosus* TISTR 3465 β-xylanase for biobleaching may be depended on the nature and quality of pulps (Khucharoenphaisan *et al*., 2001). It is possible that the different on brightness improvement from each *T. lanuginosus* xylanase on pulps could be due to different amount of xylan present in each type of pulp.

Scanning electron microscope was used to determine to morphological of pulp after pretreated with xylanase. The control pulps such as bagasse and wheat straw were smooth surface and uniform whereas xylanase pretreated pulp showed irregular on the peeled fibers on the surface as shown in Fig. 8a and b (Manimaran *et al*., 2009).

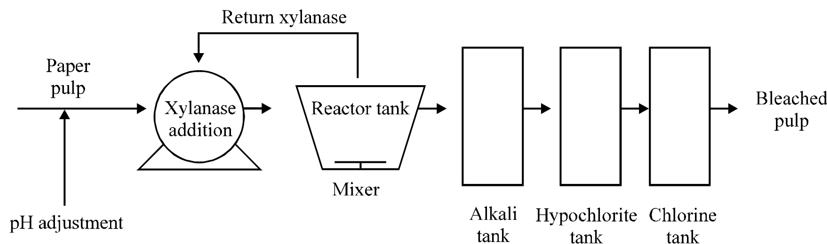

Fig. 7: Xylanase application processes flowchart

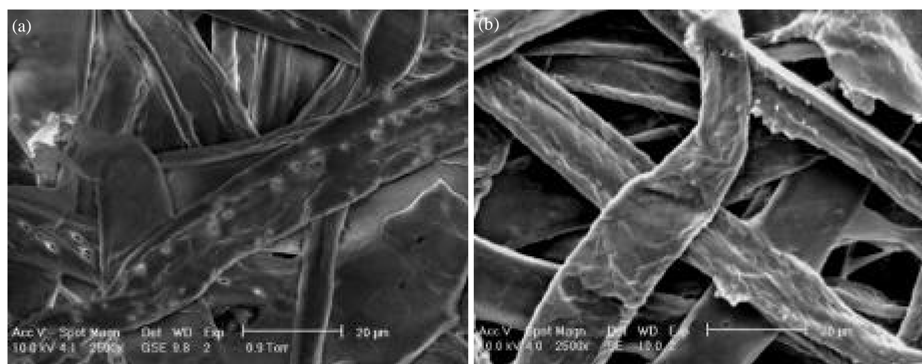

Fig. 8: (a) Scanning electron micrographs of control bagasse pulp and (b) treated with crude xylanase at 50°C for 3 h (Manimaran *et al*., 2009)

PERSPECTIVE

T. lanuginosus showed significant vary in xylanase productivity using corn cob and xylose as substrate in submerge cultivation. This apparent contradiction was found in RAPD analysis. The xylanase of *T. lanuginosus* has considerable for its biotechnological potential in biobleaching of pulp. The properties of xylanase are suitable for biobleaching of pulp due to a highly stable on high temperature and alkaline pH. However, attempt to improve the stability especially more pH stability is needed because strong alkaline pH was occurred in pulping process. To remove the effect of unknown extracellular component contributed the thermostability, the pure enzyme should be used as sample and amount of enzyme should be reported because enzyme concentration also effected on its thermostability. It is not clear why xylanase obtained from *T. lanuginosus* TISTR 3465 more induced by xylan than xylose, xylobiose and xylooligosaccharide at various concentrations. This reports differed from *T. lanuginosus* DSM 5826. This may imply that there is another factor importance for xylanase induction in this fungus which interesting to future research.

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